Université de Bourgogne
UFR des Sciences de la Vie

UMR CNRS 5561
Biogéosciences
Equipe "Différenciation et Espèces"

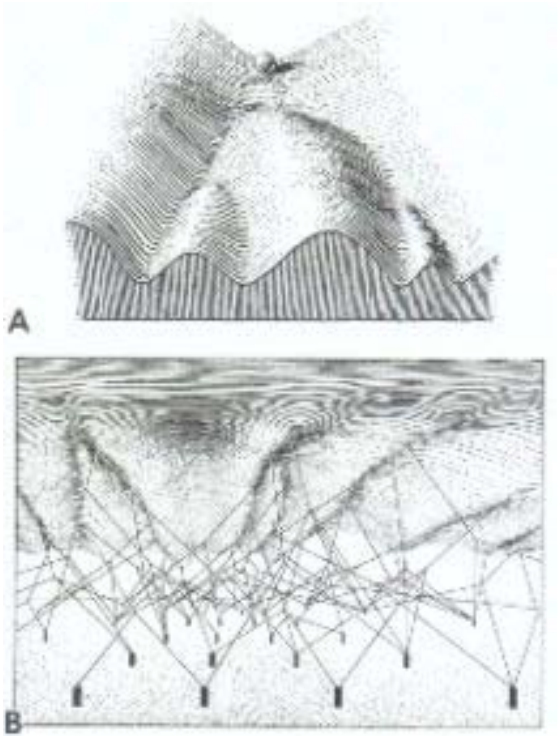
Ecole doctorale
Buffon (IMON)

Habilitation à Diriger des Recherches

De la microévolution à la macroévolution : exemples d'apports de la morphométrie

par

Paul Alibert



Novembre 2006

Composition du jury :

Rapporteurs :
Christiane Denys, Professeur, Muséum National d'Histoire Naturelle, Paris
Christian Peter Klingenberg, Senior Lecturer, University of Manchester, UK
Christophe Thébaud, Professeur, Université P. Sabatier, Toulouse

Examinateurs :
Jean-Christophe Auffray, DR CNRS, Université Montpellier II
Frank Cézilly, Professeur, Université de Bourgogne
Bruno David, DR CNRS, Université de Bourgogne

TABLE DES MATIERES

Curriculum Vitae

Né le 16 novembre 1968
Marié, 3 enfants
Nationalité : Française

Adresse professionnelle :
Equipe "Différenciation et Espèces" UMR CNRS 5561 Biogéosciences
Université de Bourgogne, Bât. Gabriel, 6, boulevard Gabriel, 21 000 DIJON
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SITUATION ACTUELLE

Maître de Conférences (67ème section CNU) à l'Université de Bourgogne
- rattaché à l'UFR des Sciences de la Vie
- rattaché à l'UMR CNRS 5561 Biogéosciences (sections 18 et 29 CNRS)

Domaine de recherche : Etude de la différenciation des populations et de la spéciation par le biais d'approches morphométriques

PARCOURS PROFESSIONNEL

- ***depuis janv. 2004*** Directeur du GDR CNRS 2474 "Morphométrie et Evolution des Formes".
- ***depuis janv. 2003*** Responsable de l'équipe "Différenciation et Espèces" du laboratoire Biogéosciences.
- ***depuis sept. 1997*** MAITRE DE CONFERENCES**,** Université de Bourgogne, UMR CNRS 5561 Biogéosciences.
- ***1995-1996*** ATER Université Montpellier II, Institut des Sciences de l'Evolution, UMR CNRS 5554.
- ***1992- 1995*** THESE DE DOCTORAT (spécialité Biologie des Populations et Ecologie). Soutenue le 20 décembre 1995 à Montpellier.
  Lieu : Institut des Sciences de l'Evolution, UMR CNRS 5554
  Sujet : "*Coadaptation génomique et spéciation chez la souris domestique : approche morphométrique de la divergence et stabilité du développement*". Directeur : Pr. L. Thaler. Jury composé de Pr. L. Thaler (Pdt), Pr. J. H. Graham (Rap.), Pr. B. Laurin (Rap.), Dr. J.-C. Auffray, Dr. F. Bonhomme, Dr. F. Renaud. Mention très honorable avec félicitations du jury.
- ***1992*** DEA Biologie de l'Evolution Université Montpellier II

BOURSES ET PRIMES

- ***depuis 2002 :*** Titulaire de la Prime d'Encadrement Doctoral et de Recherche
- ***1997*** **:** Obtention bourse Postdoctorat 12 mois "Marie Curie" (EU)
  Laboratoire d'accueil : Norwich University, UK, *Collaborateur :* Prof. G.M. Hewitt
  Sujet : "Hybridisation and speciation : a morphological approach using geometric morphometrics and developmental stability on the grasshopper model".
- ***1997 :*** Obtention bourse Postdoctorat 12 mois "Alexander von Humboldt" (bourse Allemande)
  Laboratoire d'accueil : J. Gutenberg-Universität, Mainz, Allemagne, *Collaborateur :* Prof. N. Schmidt-Kittler
  Sujet : "Comparative analysis of the evolutionary trends in dental morphology among fossils and living herbivorous lineages"

Ces deux offres ont été déclinées en raison de mon recrutement comme maître de conférences à l'Université de Bourgogne à cette même période.

- ***1996 :*** Obtention d'une bourse "*European Science Fundation*" (EU) pour participer au Workshop ESF : "Developmental stability, individual performance and population biology", Gilleleje, DANEMARK, Sept. 1996
- ***1992-1995 :*** Allocation de Recherche MNERT (+ allocation de monitorat)

EXPERIENCE D'ENSEIGNEMENT

• ***Université de Bourgogne*** (depuis 1997)

☞ **DEUG B 2ème année/ L2 VTES**

- TD et TP de Zoologie des invertébrés (54 à 72 heures éq. TD/an) jusqu'en 2001
- Cours de Biologie Evolutive/Ecologie (env. 30 heures éq. TD/an)

☞ **Maîtrise BPE/ Master 1 VTES mention BOP**

- Cours, TD et TP de Biologie Evolutive (jusqu'à 60 heures éq. TD/an)
- Cours, TD et TP de Biologie de la Conservation (jusqu'à 60 heures éq. TD/an)
- TP Dynamique des pop. et relations interspécifiques (jusqu'à 25 heures éq. TD/an)

☞ **Troisième cycle**

- Cours DESS (puis M2 pro.) Espace Rural et Environnement (3-6 heures éq. TD/an)
- Cours de Biologie Evolutive et Morphométrie DEA (puis M2 recherche) Géosystèmes, Evolution, Environnement (env. 10 heures éq. TD/an)
- Préparation au CAPES et à l'Agrégation (10-20 heures éq. TD/an, CM, TD, leçons)

Répartition globale des enseignements : **25% Cours Magistraux, 40% TD et 35% TP.**

• ***Université Montpellier II*** (1992-1996, Monitorat, ½ poste ATER)

☞ **DEUG B 1ere année**

- TD de Génétique Mendélienne (jusqu'à 20 heures éq. TD/an)
- TD et TP de Biologie Cellulaire (jusqu'à 20 heures éq. TD/an)
- TP Embryologie (Aide spécifique)

☞ **DEUG B 2ème année**

- TD et TP de Zoologie des vertébrés (jusqu'à 40 heures éq. TD/an)

• ***Autres***

☞ Mai 2005 **: Chargé de cours Master Biologie, Evolution, Conservation, Université de Lausanne (Suisse)** – De la macroévolution à la microévolution, Morphométrie (11 heures éq. TD).

☞ Intervenant invité dans le cadre d'actions de formation permanente "Morphométrie" destiné aux chercheurs CNRS à Montpellier, UMR 5554 (1995) et à Lyon, UMR 5565 (1997).

RESPONSABILITES ADMINISTRATIVES (HORS RECHERCHE)

• ***Actuelles***

- Membre des commissions de spécialistes de l'Université de Bourgogne (élu, 67ième section CNU) et de l'Université de Franche-Comté (nommé, 66-69ième section CNU).

- Membre nommé de la commission "Relations Internationales" de l'UFR Sciences-Vie de l'Université de Bourgogne.

- Membre nommé du comité scientifique de la SEIVA, Structure d'Echange et d'Information sur Valduc (site CEA Installation nucléaire de base secrète, Côte d'Or).

• ***Passées***

- Directeur adjoint du Département "Biologie Animale" de l'Université de Bourgogne (2004-2005).

- Co-responsable de la Maîtrise "Biologie des Populations et des Ecosystèmes" de l'Université de Bourgogne (2000-2003).

- Secrétaire du Pôle Evolution du Vivant de l'Université de Bourgogne. Organisation de séminaires hebdomadaires, organisation d'une exposition "R'évolution, l'Odyssée du vivant" pour les Muséums d'Histoire Naturelle de Bourgogne (1997-2000).

Animation de la recherche

THEMATIQUES DE RECHERCHE

Mes activités de recherche se concentrent autour de l'étude de la différenciation des populations et de la spéciation par le biais d'approches morphométriques. Elles s'articulent autour de **trois axes principaux complémentaires** :

- **Axe 1**- quantification morphométrique de la différenciation et sa mise en relation avec les patrons de différenciation génétique et l'histoire évolutive des taxons considérés.
- **Axe 2**- évaluation de l'impact de la différenciation morphologique sur la spéciation par l'étude de l'instabilité de développement des hybrides entre taxons en voie de différenciation
- **Axe 3**- étude des mécanismes sous-jacents à l'instabilité de développement.

Les principaux modèles biologiques d'étude sont les insectes (carabes, drosophiles…), les oursins (réguliers et irréguliers) et les rongeurs.

MANAGEMENT -ADMINISTRATION

Gestion d'équipes de recherche

- Directeur du GDR CNRS 2474 "Morphométrie et Evolution des Formes".
- Responsable de l'équipe "Différenciation et Espèces" du laboratoire Biogéosciences, UMR CNRS 5561.

Gestion des structures de recherche

- Membre élu du conseil de l'UMR 5561 CNRS Biogéosciences.
- Membre nommé de la commission" Recherche" de l'UFR Sciences-Vie de l'Université de Bourgogne.

Participation à des programmes de recherche et contrats

- Obtention (mai 1997) de deux financements (une bourse Marie-Curie et une bourse "Alexander von Humboldt") pour deux stages post-doctoraux (12 mois chacun) mais offres déclinées en raison de mon recrutement à l'université de Bourgogne.
- Obtention d'une bourse post-doctorale de la région Bourgogne sur un projet d'étude de l'ontogénie de l'asymétrie fluctuante chez les oursins : accueil d'avril 2004 à avril 2005 du Dr L. C. Stige (de nationalité Norvégienne).
- Obtention d'un financement Accueil Jeunes Chercheurs Région Bourgogne (1998).
- Participation à des programmes financés dans le cadre d'appels d'offre nationaux : GIP Ecofor (1998-2000), Institut Français de la Biodiversité (2001-2002, 2 projets), ANR ECCO (2006-2007).

EXPERTISES
JURYS

- Relecteur pour les revues internationales : *Evolution, Functional Ecology, Archives of Environmental Contamination and Toxicology, Heredity, Parasite*, *Mammalia, Sarsia.*
- Expert extérieur pour le "Natural Environment Research Council (NERC)"
- Examinateur dans les jurys de thèses de N. Cadée (soutenue le 27/01/2000, Univ. Paris 7, Dir. thèse : A. P. Møller) et de V. Debat (soutenue le 22/12/2000, Univ. Montpellier II, Dir. thèse : J.-C. Auffray).
- Membre des comités de thèses de V. Debat (Université Montpellier II, 1997-2000), C. Teplisky (Université Lyon I, 1999-2003), E. Gazave (Université Montpellier II, 2000-2003), E. Renvoisé (Université de Bourgogne, 2005-2007).
- Expertises régulières de mémoires de DEA GEE (master 2 CEPS) – Université de Bourgogne, master GSA – Université de Bourgogne, DEA Pal & Sed, Université Lyon I, DESS (master Pro) ERE – Université de Bourgogne.

FORMATION PAR LA RECHERCHE
ENCADREMENT 3EME CYCLES

Doctorats

- Angeline BERTIN (1999-2002)

Sujet : "Etude morphologique et comportementale des patrons d'appariement chez l'aselle *Asellus aquaticus*". Co-direction avec F. Cézilly (Biogéosciences-Dijon).

Parcours post-thèse : Stage post-doctoral (18 mois) University of Rochester, USA , puis ATER Université Montpellier II. Actuellement "Research assistant" à la Southwest Foundation for Biomedical Research, San-Antonio (USA).

Publication co-signée :
Bertin, A., David, B., Cézilly, F. & **P. Alibert** (2002) Quantification of sexual dimorphism in *Asellus aquaticus* (Crustacea: Isopoda) using outline approaches. *Biological Journal of the Linnean Society* 77: 523-533.

- Stéphane GARNIER (1999-2003)

Sujet : "Rôle de l'hybridation naturelle dans la diversification des carabes forestiers (Coléoptères *Chrysocarabus*)". Co-direction avec J.-Y. Rasplus (INRA, Montpellier).

Parcours post-thèse : ATER Université de Bourgogne, puis stage post-doctoral (9 mois) University of Edinburgh (Ecosse). Actuellement Maître de Conférences au Laboratoire Biogéosciences de l'Université de Bourgogne.

Publications co-signées :
Garnier, S., **Alibert, P**., Audiot, P., Prieur, B. & J.-Y. Rasplus (2004) Isolation by distance and sharp discontinuities in gene frequencies: implications for the phylogeography of an alpine insect species, *Carabus solieri*. *Molecular Ecology* 13: 1883-1897.

Garnier, S., Magniez-Jannin, F., J.-Y. Rasplus & **P. Alibert** (2005) When morphometry meets genetics: inferring the phylogeography of *Carabus solieri* using Fourier analyses of pronotum and male genitalia. *Journal of Evolutionary Biology* 18: 269-280.

Garnier, S., Gidaszewski, N., Charlot, M., Rasplus, J.-Y. & **P. Alibert** (2006) Hybridization, developmental stability and functional significance of morphological traits in the carabid beetle *Chrysocarabus solieri* (Coleoptera, Carabidae). *Biological Journal of the Linnean Society* 89: 151-158.

DEA-Master 2 recherche

- Benoit Moureau (1997-1998)

Sujet : "Etude de la différenciation morphologique chez un carabe forestier (*C. auronitens*) : approche par les méthodes de morphométrie géométrique". DEA Analyse et Modélisation des Systèmes Biologiques, Université de Lyon. Co-encadrement avec J.-L. Dommergues (Biogéosciences-Dijon).

Parcours post-DEA : Thèse à Lausanne (Suisse), actuellement en stage post-doctoral en Angleterre

Publication co-signée :
Alibert, P., B. Moureau, J.-L. Dommergues & B. David. (2001) Differentiation at a microgeographical scale within two species of ground beetles, *Carabus auronitens* and *C. nemoralis* (Coleoptera, Carabidae): a geometrical morphometric approach. *Zoologica Scripta* 30 (4): 299-311.

• Djamel Bousbassi (1999-2000)
Sujet : "Relation différenciation morphologique-différenciation génétique dans le complexe d'espèce *C. solieri* (Carabidae)". DEA Géosystèmes, Evolution, Environnement, Université de Bourgogne.
Parcours post-DEA : Vie active (hors recherche).

• Hélène Marchand (1999-2000)
Sujet : "Stabilité de développement, stress et habitats fragmentés chez *Clethrionomys glareolus* (Arvicolinae, Rodentia)". DEA Géosystèmes, Evolution, Environnement, Université de Bourgogne. Co-encadrement avec S. Montuire (Biogéosciences-Dijon).
Parcours post-DEA : Thèse au MNHN, Paris.

• Ludovic Journaux (2000-2001)
Sujet : "Etude de la variabilité morphologique (par les méthodes de morphométrie géométrique) chez trois espèces d'Hyménoptères haplo-diploides (genre *Nasonia*) et de leurs hybrides". DEA Analyse et Modélisation des Systèmes Biologiques, Université de Lyon. Co-encadrement avec M.-J. Perrot-Minnot (Biogéosciences-Dijon).
Parcours post-DEA : Thèse au laboratoire LE2I, Université de Bourgogne.

• Nelly Gidaszewski (2001-2002)
Sujet : "Etude des patrons de variabilité morphologique chez plusieurs espèces de campagnols : tentative d'estimation de la stabilité de développement sur du matériel fossile". DEA Géosystèmes, Evolution, Environnement, Université de Bourgogne. Co-encadrement avec S. Montuire (Biogéosciences-Dijon).
Parcours post-DEA : Thèse en Angleterre (Manchester).
Publication co-signée : Garnier, S., N. Gidaszewski, M. Charlot, J.-Y. Rasplus and **P. Alibert**. 2006. Hybridization, developmental stability and functional significance of morphological traits in the carabid beetle *Chrysocarabus solieri* (Coleoptera, Carabidae). *Biological Journal of the Linnean Society* 89: 151-158

• Elodie Colin (2001-2002)
Sujet : "Quantification des convergences morphologiques chez les oursins de type dollars des sables". DEA Géosystèmes, Evolution, Environnement, Université de Bourgogne. Co-encadrement avec B. David (Biogéosciences-Dijon).
Parcours post-DEA : Vie active (hors recherche).

• Jessica Meredith (2002-2003)
Sujet : "Différenciation et couleurs chez le carabe forestier, *Chrysocarabus solieri* (Coleoptera, Carabidae)". DEA Géosystèmes, Evolution, Environnement, Université de Bourgogne. Co-encadrement avec S. Garnier (Biogéosciences-Dijon).
Parcours post-DEA : Vie active (Retour aux USA, domaine de la biologie de la conservation).

• Paul-Eric Bourgeon (2004-2005)
Sujet : "Relation asymétrie fluctuante-fitness chez *Drosophila melanogaster*". Master 2 Gènes, Sélection, Adaptation, Université de Bourgogne. Co-encadrement avec L. C. Stige (Biogéosciences-Dijon).
Parcours post-DEA : Stage DSER, recherche de financement de thèse
Publication co-signée : Stige, L.C., Bourgeon, P.-E., Flotterer, F.-X. & **Alibert, P**. Relationship between fluctuating asymmetry and fitness in a selection experiment on *Drosophila melanogaster*. (en préparation)

• Yoland Savriama (2004-2005)
Sujet : "Etude de l'instabilité de développement chez l'oursin régulier *Paracentrotus lividus*. Master 2 Climatologie, Environnement, Paléontologie, Sédimentologie, Université de Bourgogne. Co-encadrement avec L. C. Stige & B. David (Biogéosciences-Dijon).
Parcours post-DEA : Thèse en Angleterre (Manchester)
Publication co-signée : Savriama, Y., Stige, L. C., **Alibert, P**., Perez T. & David, B. Pentaradial fluctuating asymmetry: the case of two species of sea urchins in contrasted environments in the mediterranean sea. (en préparation)

• Remy Laffont (2005-2006)
Sujet : "Variabilité morphologique et modularité chez le campagnol". Master 2 Climatologie, Environnement, Paléontologie, Sédimentologie, Université de Bourgogne. Co-encadrement avec S. Montuire (Biogéosciences-Dijon).

Autres encadrements

• Encadrement de 3 stages annexes de DEA (BEE Montpellier, AMSB Lyon) en 1999, 2000 et 2002.

• Depuis 1997, encadrement de 10 stages de recherche de Maîtrise-Master (2ème cycle)

FORMATION PAR LA RECHERCHE
ENSEIGNEMENTS 3EME CYCLES

Responsabilité de modules de DEA (-Master 2)

• Co-responsable (avec P. Neige, UFR Sciences de la Terre) du module *Patterns et Processus de l'Evolution* du Master 2 recherche CEPS, Université de Bourgogne (depuis 2000).

• Responsable du module *Mécanismes de l'Evolution* (1999-2000) du DEA GEE, Université de Bourgogne.

Enseignements 3ème cycles

• Enseignements en DEA GEE devenu Master 2 recherche CEPS, Université de Bourgogne : biologie évolutive, morphométrie.

• Enseignements en DESS ERE devenu Master 2 professionnel, Université de Bourgogne : biologie évolutive, biologie de la conservation.

• Chargé de cours Master BEC, Université de Lausanne (avril 2005) : Evolution, Morphométrie.

Séminaires

• Intervenant invité dans le cadre d'actions de formation permanente "Morphométrie" destiné aux chercheurs CNRS à Montpellier, UMR 5554 (1995) et à Lyon, UMR 5565 (1997).

• Intervenant invité dans le module de morphométrie de l'école doctorale du MNHN, Paris (avril 2004).

• Invitation à divers séminaires invités par des laboratoires : laboratoire Ecologie des Hydrosystèmes fluviaux, Université de Lyon I (mars 2000), laboratoire d'Ecologie, Université Paris VI (avril 2001), séminaires STIC, Université de Bourgogne (avril 2002), Laboratoire PEPS, Université de Lyon I (décembre 2003).

DIFFUSION DE LA RECHERCHE
VERS LE GRAND PUBLIC

• Co-organisateur d'une exposition "*R'évolution, l'Odyssée du vivant*" pour les Muséums d'Histoire Naturelle de Bourgogne (2001).

• Porteur d'un projet (en collaboration avec B. Faivre et D. Raichvarg) de site internet "*De mémoire d'oiseau : base de données scientifiques et culturelles interactive sur les oiseaux nicheurs de Bourgogne*" dont un des objectifs est d'expliciter aux utilisateurs du site (étudiants scientifiques, associations, grand public) le lien, quand il existe, entre les espèces et leur utilisation comme modèle de recherche (depuis 2002).

• Participation à fête de la science en octobre 2006 (Thème : La biodiversité) Participation à la réalisation de posters (exposés à l'université et en ville) et conférence à destination du grand public au Lycée de Cluny.

Liste des travaux

PUBLICATIONS

Seules les publications publiées ou sous presse sont reportées ici.
Celles indiquées par "*" sont directement issues de ma thèse de doctorat.
Le facteur d'impact (IF) des revues est indiqué entre parenthèse à la suite de la référence (source JCR 2004).

Articles de rang A

17- STIGE, L. C., DAVID, B. & **P. ALIBERT** (2006) On hidden heterogeneity in directional asymmetry - can systematic bias be avoided? ***Journal of Evolutionary Biology*** 19: 492-499 (IF = 2,893)

16- GARNIER, S., GIDASZEWSKI, N., CHARLOT, M., RASPLUS, J.-Y. & **P. ALIBERT** (2006) Hybridization, developmental stability and functional significance of morphological traits in the carabid beetle *Chrysocarabus solieri* (Coleoptera, Carabidae**). *Biological Journal of the Linnean Society*** 89: 151-158 (IF = 1,935)

15- SAUCEDE, T., **ALIBERT, P**., LAURIN, B. & B. DAVID (2006) Environmental and ontogenetic constraints on developmental stability in the spatangoid sea urchin *Echinocardium* (Echinoidea). ***Biological Journal of the Linnean Society*** 88: 165-177 (IF = 1,935)

14*- BRITTON-DAVIDIAN, J., FEL-CLAIR, F., LOPEZ, J., **ALIBERT, P.,** & P. BOURSOT (2005) Postzygotic isolation between the two European subspecies of the house mouse: estimates from fertility patterns in wild and laboratory-bred hybrids. ***Biological Journal of the Linnean Society*** 84: 379-393. (IF = 1,935)

13- GARNIER, S., MAGNIEZ-JANNIN, F., J.-Y. RASPLUS & **P. ALIBERT** (2005) When morphometry meets genetics: inferring the phylogeography of *Carabus solieri* using Fourier analyses of pronotum and male genitalia. ***Journal of Evolutionary Biology*** 18: 269-280. (IF = 2,893)

12- GARNIER, S., **ALIBERT, P**., AUDIOT, P., PRIEUR, B. & J.-Y. RASPLUS (2004) Isolation by distance and sharp discontinuities in gene frequencies: implications for the phylogeography of an alpine insect species, *Carabus solieri*. ***Molecular Ecology*** 13: 1883-1897. (IF = 4,375)

11- TERRAL, J.-F., ALONSO, N., BUXO I CAPDEVILLA, R., CHATTI, N., FABRE, L., FIORENTINO, G. MARINVAL, P., PEREZ-JORDA, G., PRADAT, D., ROVIRA, N. & **P. ALIBERT**. (2004) Historical biogeography of olive domestication (*Olea europea* L.) as revealed by geometrical morphometry applied to biological and archaeological material. ***Journal of biogeography***, 31(1): 63-77. (IF = 2,329)

10- BERTIN, A., DAVID, B., CÉZILLY, F. & **P. ALIBERT** (2002) Quantification of sexual dimorphism in *Asellus aquaticus* (Crustacea: Isopoda) using outline approaches. ***Biological Journal of the Linnean Society*** 77: 523-533. (IF = 1,935)

9- **ALIBERT, P.**, L. BOLLACHE, D. CORBERANT, V. GUESDON & F. CÉZILLY (2002). Parasitic infection and developmental stability: fluctuating asymmetry in *Gammarus pulex* infected with two acanthocephalan species. ***Journal of Parasitology*** 88(1): 47-54. (IF = 1,439)

8- **ALIBERT, P**., B. MOUREAU, J.-L. DOMMERGUES & B. DAVID. (2001) Differentiation at a microgeographical scale within two species of ground beetles, *Carabus auronitens* and *C. nemoralis* (Coleoptera, Carabidae): a geometrical morphometric approach. ***Zoologica Scripta*** 30 (4): 299-311. (IF = 2,411)

7*- DEBAT, V., **ALIBERT P.** , DAVID, P., PARADIS; E. & J.-C. AUFFRAY (2000). Independence between developmental stability and canalization in the skull of the house mouse. ***Proceeding of the Royal Society of London. B***, 267: 423-430. (IF = 3,653)

6*- AUFFRAY, J.-C., RENAUD, S., **ALIBERT, P**., & E. NEVO (1999) Developmental stability and adaptative radiation in *Spalax ehrenbergi* superspecies in the middle East. ***Journal of Evolutionary Biology*** 12: 207-221. (IF = 2,893)

5*- **ALIBERT, P**., FEL-CLAIR, F., MANOLAKOU, K., BRITTON-DAVIDIAN, J. & J.-C. AUFFRAY (1997) Developmental stability, fitness and trait size in laboratory hybrids between European subspecies of the house mouse. ***Evolution*** 51(4): 1284-1295. (IF = 3,719)

4*- LENORMAND, T., FEL-CLAIR, F., MANOLAKOU, K., **ALIBERT, P**., & J. BRITTON-DAVIDIAN (1997) Centromeric transmission bias in laboratory hybrids between wild strains of the two European subspecies of house mice. ***Genetics*** 147(3), 1279-1287. (IF = 4,138)

3*- GANEM, G., **ALIBERT, P**. & J.B. SEARLE (1996) An ecological comparison between standard and chromosomally divergent house mice in northern Scotland. ***Zeitschrift für Saugetierkunde*** 61, 176-188. (IF = 0,302)

2*- AUFFRAY, J.-C., **ALIBERT, P**., LATIEULE, C. & B. DOD (1996) Relative warp analysis of skull shape changes across the house mouse (*Mus musculus*) hybrid zone in Europe. ***Journal of Zoology, London*** 240: 441-445. (IF = 0,889)

1*- **ALIBERT, P**., RENAUD, S., DOD, B., BONHOMME, F. & J.-C. AUFFRAY (1994) Fluctuating asymmetry in the *Mus musculus* hybrid zone: a heterotic effect in disrupted co-adapted genomes. ***Proceeding of the Royal Society of London. B***, 258: 53-59 (IF = 3,653)

Chapitres d'ouvrages expertisés

1- **ALIBERT, P**. & J.-C. AUFFRAY (2003).Genomic coadaptation, outbreeding depression and developmental instability. In *Developmental instability: Causes and Consequences*, Ed M. Polak, New York: Oxford University Press, 116-134.

2- AUFFRAY, J.-C., DEBAT, V. & **P**. **ALIBERT** (1999). Shape asymmetry and developmental instability. In *On Growth and Form: Spatio-temporal Patterning in Biology*, Eds. M. Chaplain *et al*., London: John Wiley and Sons. pp. 309-324.

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PARTICIPATION A DES COLLOQUES

Auteur ou co-auteur de 14 communications en congrès internationaux (9 communications orales et 5 posters) et 14 communications en congrès nationaux (9 communications orales et 5 posters), dont participation à un workshop invité par la "European Science Foundation". Pour les communications orales, le nom souligné correspond à l'auteur de la communication.

4ème Symposium de Morphométrie, Paris, FRANCE, Décembre 2005 (2 communications orales)

- BOURGEON P.-E., STIGE L.C., FLOTTERER F.-X. & **P**. **ALIBERT** "Étude de la relation asymétrie fluctuante/fitness chez *Drosophila melanogaster* : impact de la sélection directionnelle."
- SAVRIAMA Y., STIGE L.C., **ALIBERT P**. & B. DAVID "Asymétrie fluctuante et instabilité de développement chez des organismes pentaradiés dans des environnements contrastés de Méditerranée Occidentale."

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MEMOIRE

CADRE GENERAL

Comprendre comment les divergences entre groupes d'organismes peuvent aboutir à l'apparition d'espèces nouvelles est un problème majeur en Evolution. Qu'ils se concentrent sur la notion même d'espèce, sur les modalités de mise en place de la différenciation des populations ou encore sur les facteurs intrinsèques responsables de celle-ci, les travaux relatifs la différenciation et à la spéciation sont particulièrement nombreux (Barton 2001; Coyne & Orr 2004). Il semble même que cet intérêt ce soit accru ces dernières années. La mise en place de nouvelles approches de génétique (en particulier dans le domaine de la génétique quantitative), l'accumulation de données biologiques et paléontologiques, mais aussi l'émergence de questions centrées sur le problème de la notion d'espèce et de l'hybridation inter-spécifique dans le domaine de la biologie de la conservation en sont probablement partiellement responsables.

L'étude de la spéciation est un enjeu majeur en biologie évolutive

The study of speciation is a major topic in evolutionary biology

Force est de constater cependant qu'en dépit de la portée essentielle de la notion d'espèce et de spéciation en Evolution et de l'effort de recherche considérable dans ce domaine, il demeure aujourd'hui une absence de consensus sur le sujet. Une multitude de définitions et de concepts de l'espèce ont été proposés (concepts biologique, typologique, phylogénétique, évolutif...) et l'importance relative accordée aux différents modèles de spéciation (allopatrique, sympatrique...) varie considérablement selon les auteurs (King 1993; Hey 2001; Turelli *et al.* 2001; Via 2001). Les causes de cette absence de vision consensuelle sont nombreuses. Citons par exemple la difficulté de concilier nos méthodes de classement (plus ou moins intuitives) en catégories taxonomiques très délimitées, avec la réalité du processus complexe et graduel que constitue la différenciation (et donc à terme la spéciation). D'autres causes sont à rechercher dans la variété des objectifs des scientifiques travaillant dans ce domaine (par exemple, dans le cadre du débat qui a opposé les partisans du modèle d'évolution graduelle aux partisans du modèle d'évolution ponctuée, les modes de spéciation impliqués sont résolument différents). D'autres raisons encore peuvent être liées aux modèles biologiques étudiés. Quoi qu'il en soit, le sujet reste largement ouvert et, comme le soulignent Turelli *et al.* (2001), les besoins se situent aujourd'hui

Des questions nombreuses et largement ouvertes…

Numerous questions and far to be solved…

beaucoup plus dans l'étude de nouveaux exemples et l'obtention de nouvelles données plutôt que dans l'élaboration de nouvelles théories.

Trois axes de recherche

Three research axes

Le travail que j'ai initié durant ma thèse et que j'ai par la suite largement développé dans le cadre de mon recrutement à l'Université de Bourgogne s'inscrit dans ce cadre général et ambitieux. Ma – modeste – contribution à cette thématique combine des approches descriptives centrées sur la quantification de la divergence entre taxons en voie de différenciation et des approches plus tournées vers les conséquences de la différenciation en termes de perturbation du développement. Pour cela, mes recherches s'articulent autour de trois axes majeurs. Le premier s'attache à quantifier la différenciation par le biais d'approches morphométriques et à la mettre en relation avec les patrons de différenciation génétique et avec l'histoire évolutive des taxons considérés. Le second se concentre sur l'étude de la relation entre divergence de taxons en voie de différenciation et instabilité de développement de leurs hybrides. Enfin, le troisième vise à mieux comprendre les mécanismes sous-jacents à l'instabilité de développement..

Le présent mémoire, après une présentation rapide des principaux modèles biologiques étudiés et de l'approche générale utilisée, est organisé en quatre grandes parties. Les trois premières sont consacrées à la présentation de chacun des trois axes majeurs de recherche évoqués ci-dessus. Pour chacune de ces parties les travaux les plus représentatifs (parce qu'ils ont été publiés et/ou parce qu'ils sont le résultat d'encadrement d'étudiants) sont brièvement résumés. J'ai tenté autant que possible de reporter le minimum d'informations méthodologiques (celles strictement nécessaires à la compréhension de l'étude ou de ses résultats) et de n'évoquer que les résultats principaux, l'objectif étant d'éviter autant que possible une traduction inutile des articles déjà publiés. Pour tous les travaux évoqués qui ont fait l'objet d'une publication le lecteur est invité à se reporter à la fin du mémoire où il trouvera l'article en version intégrale (les travaux publiés se situant à la fin du mémoire sont signalés par une astérisque lors de leur première citation dans le texte). La quatrième partie présente, après avoir rappelé l'intérêt de l'étude du contrôle de la variation phénotypique, quelques exemples de projets de recherche que je souhaiterais développer dans les années à venir.

Les modèles biologiques

Des modèles biologiques variés…

Studied biological models are various…

Mes recherches portent sur des modèles biologiques très variés. Le groupe des carabes forestiers est celui sur lequel j'ai débuté lors de mon arrivée à Dijon. Ce choix pourrait paraître pour le moins curieux puisque d'une part j'ai réalisé ma thèse sur les souris domestiques et d'autre part le modèle de prédilection de mon équipe d'accueil Dijonnaise était les oiseaux. Il était cependant justifié sur deux points au moins, l'un purement scientifique, l'autre que l'on pourrait qualifier de "scientifico-politique". L'argument scientifique est que les espèces retenues dans un premier temps, les carabes forestiers *Chrysocarabus auronitens* et *C. nemoralis* sont des espèces

répandues dans les forêts françaises et notamment Bourguignonnes. Il était donc aisé d'avoir un matériel d'étude conséquent rapidement et qui permettait donc de démarrer dès mon arrivée un programme de recherche avant même de solliciter des fonds. C'est ce qui a été fait quelques semaines après mon arrivée avec le démarrage du travail de DEA de Benoît Moureau (1997-1998) qui portait sur la différenciation à l'échelle micro-géographique de populations de *C. auronitens* et de *C. nemoralis* (voir plus loin pour un résumé des principaux résultats). L'argument scientifico-politique (ou scientifico-stratégique, c'est selon…) était qu'un certain nombre de paléontologues de l'UMR 5561 (B. David, J.-L. Dommergues et F. Magniez notamment) avaient débuté quelques années auparavant des études sur la morphométrie des carabes. La morphométrie (notamment géométrique) constituait un point commun avec mes recherches, les carabes allaient en devenir un autre. Les points communs entre l'équipe d'Ecologie et l'UMR 5561 étaient très précieux et encouragés puisque c'est à cette époque que ces deux laboratoires ont proposé au CNRS de fusionner pour former l'UMR Biogéosciences. Nous verrons par la suite que mes recherches ont également porté sur le carabe de Solier *Chrysocarabus solieri* dans le cadre du travail de thèse de Stéphane Garnier. Les carabes ont par conséquent été des modèles essentiels pour aborder les questions relatives aux deux premiers axes de recherche cités plus haut.

Les carabes: un choix scientifique et stratégique

The carabid beetles: a scientific and strategic choice

Un deuxième modèle biologique important dans mes recherches est celui des oursins. Ce groupe constitue un modèle phare de l'UMR 5561 depuis de nombreuses années (notamment par le biais des programmes de recherche menés par B. David). Mon travail sur ce modèle a débuté par une collaboration avec B. David dans le cadre du travail de DEA de Thomas Saucède (1997-1998). C'était un premier pas prudent vers le monde des structures pentaradiées. Prudent car il s'agissait d'étudier les niveaux d'asymétrie fluctuante sur des oursins irréguliers et par conséquent se concentrer, comme l'immense majorité des études, sur des structures bilatérales. Ce premier travail nous a permis d'entrevoir le potentiel de ce modèle biologique mais aussi l'intérêt d'étudier la stabilité de développement sur des structures homologues autres que bilatérales. C'est par le biais d'un financement de stage post-doctoral attribué par la région Bourgogne (pour la période avril 2004-mars 2005) qu'a ensuite été initié par Leif C. Stige un travail directement orienté sur l'étude de l'ontogénie de la stabilité de développement chez les oursins. Le modèle biologique des oursins est remarquablement adapté à l'étude de l'ontogénie de la stabilité de développement car les plaques constituant leur test calcaire témoignent, sur l'individu adulte, des différentes étapes de son développement. Parmi les oursins, les spatangues incubant sont tout particulièrement indiqués, car ils se développent sans passer par une phase larvaire et toutes les étapes de leur ontogenèse sont accessibles. La deuxième particularité du modèle réside dans la façon d'appréhender la stabilité de développement. Les oursins peuvent en effet présenter deux types de plan de symétrie. La symétrie pentaradiée des échinides réguliers peut être comprise comme un équilibre achevé entre les 5 zones de croissance. La symétrie bilatérale, secondairement acquise par les échinides irréguliers, correspond au contraire à un déséquilibre entre zones de croissance, notamment entre les trois zones antérieures et les deux postérieures. Ainsi, l'étude de l'instabilité de développement se fera, selon l'espèce considérée, de façon classique en estimant la variation entre

Des oursins pour l'ontogénie et la symétrie pentaradiée

Sea urchins for ontogeny and pentaradial symmetry

structures bilatérales ou, de façon plus originale, entre les cinq parties homologues. Ce dernier type d'approche est particulièrement intéressant car il permet d'estimer une variabilité entre structures homologues, non pas à partir de deux valeurs (droite et gauche) comme c'est classiquement le cas, mais à partir de cinq. Le gain de puissance dans la détection d'un signal traduisant de l'instabilité de développement est donc en théorie non négligeable.

Les drosophiles permettent l'approche expérimentale

The drosophila model allow the experimental approach

Les carabes comme les oursins ont cependant, en tant que modèles biologiques d'études, une limite importante qui est celle des très fortes contraintes liées à l'élevage (difficultés techniques de maintien en conditions contrôlées, reproduction, durée importante des périodes larvaires…) qui rendent difficile toute approche expérimentale. Nos études, et en particulier celles liées aux mécanismes de contrôle de l'instabilité de développement, ne peuvent cependant faire l'économie de l'approche expérimentale. C'est essentiellement pour cette raison que nous nous sommes récemment tourné vers le modèle biologique des drosophiles. Les grandes facilités d'élevage, le temps de génération très court ou encore les nombreuses études déjà effectuées sur ce modèle biologique constituaient autant d'éléments en faveur du choix de la drosophile pour conduire une étude sur les relations entre niveau d'asymétrie fluctuante et valeur sélective (travail de M2 recherche de Paul-Eric Bourgeon, 2004-2005).

Des crustacés pour des questions ciblées

Crustacean for specific questions

Certains de mes travaux ont également porté sur les crustacés isopodes (aselles) et amphipodes (gammares). De la même manière que les carabes et les oursins ont constitué un point d'accroche pour les collaborations avec les membres d'autres équipes au sein de l'UMR, les crustacés ont été le moyen de collaborer avec des membres de l'équipe d'Ecologie (équipe à laquelle j'ai appartenu jusqu'en 2002). Les aselles m'ont notamment fourni l'opportunité, à l'occasion du co-encadrement du travail de thèse d'Angéline Bertin (avec F. Cézilly), de tester l'utilité et la puissance des méthodes de morphométrie géométrique dans le cadre d'études de sélection sexuelle. L'étude sur les gammares a quant à elle permis d'étudier pour la première fois la relation entre les niveaux d'asymétrie fluctuante et le parasitisme chez un hôte intermédiaire et d'aborder certains aspect du déterminisme de l'instabilité de développement. Nous verrons plus loin que les résultats très intéressants obtenus sur ces deux modèles biologiques suffisent à démontrer l'intérêt qu'il y a eu à étendre un peu le spectre de nos modèles d'études.

Des campagnols pour l'avenir?

Voles for the future?

Enfin, le lecteur pourra constater que les campagnols viendront certainement prendre une place plus affirmée dans l'avenir. L'orientation d'une partie de mes recherches vers l'étude conjointe des patrons de covariation morphologique (notions d'intégration morphologique et de modularité) et d'instabilité de développement m'amène à accentuer les collaborations déjà engagées avec deux autres membres de mon équipe, S. Montuire et C. Tougard. Les justifications du choix du modèle rongeur seront développées dans la partie de ce mémoire consacrée aux perspectives.

La figure 1 situe les différents axes de recherche et les modèles biologiques utilisés par rapport au schéma classique de différenciation spatiale et temporelle des populations et de spéciation. La partie supérieure de la figure montre clairement la prise en compte successive de différents niveaux de différenciation et concerne les études plus descriptives, la partie basse regroupe les approches plus mécanistiques.

Figure 1 : Situation des différents travaux de recherche

… mais des modèles centrés sur les mêmes questions

…but models which allow to address the same questions

Dispersion néfaste ou diversité enrichissante ? Le lecteur en jugera. Résumons cependant deux éléments (présents en filigrane dans la présentation du choix des modèles biologiques ci-dessus) qui me semblent essentiels à cette réflexion. Le premier est d'ordre purement scientifique. Il correspond à ce que je répète inlassablement aux étudiants qui viennent me demander des conseils d'orientation parce qu'ils souhaitent travailler sur les baleines, les félins, les primates ou je ne sais quel autre groupe emblématique (souvent doté de grands yeux noirs émouvants) : ce qui est important en Science, et particulièrement en biologie, c'est la question posée et non le modèle d'étude. Ce dernier doit être choisi de manière à permettre de répondre du mieux possible à la question retenue. Il est évident que dans le cas présent un modèle biologique unique permettrait très difficilement d'aborder l'ensemble des questions posées. L'étude conjointe des patrons de différenciation de taxons en voie de différenciation, d'impact de cette différenciation sur la rupture de coadaptation des systèmes de gènes impliqués dans la stabilité de développement mais aussi l'étude des mécanismes sous-jacents à l'instabilité de développement pose à chaque fois des questions relativement spécifiques. Le deuxième élément combine l'effet de la contingence (si chère aux paléontologistes Dijonnais) et celui de la

nécessité (si chère aux écologistes Dijonnais). Même si le modèle biologique idéal pour répondre à l'ensemble des questions posées dans un programme de recherche existait, il resterait à savoir quelles seraient les conditions de la mise en place d'un tel programme. Le démarrage d'un projet de recherche dans un laboratoire différent de celui où la thèse a été effectuée nécessite de s'adapter aux problématiques de la structure d'accueil (problématiques pas trop éloignées des siennes si le recrutement est cohérent…) et assez souvent de changer de modèle biologique d'étude. Comme expliqué plus haut, le choix de mon premier modèle d'étude relève justement à la fois de la contingence (dans le sens où certains paléontologues travaillaient sur les carabes et que mon recrutement à Dijon était complètement étranger à ce fait) et de la nécessité (les carabes offraient la possibilité de démarrer très vite un programme de recherche permettant le rapprochement avec ces mêmes paléontologues). Ces deux facteurs, hasard et nécessité, sont encore intervenus plusieurs fois dans le choix des modèles biologiques : le jeu des attributions de bourses en 1999 (par le biais d'un chassé-croisé sur un candidat et un sujet entre Dijon et Montpellier) a eu par exemple pour conséquence de définir un sujet de thèse, initialement non prévu, combinant des approches morphométrique et comportementale chez l'aselle (thèse d'Angéline Bertin). La participation à un programme comme celui du GIP Ecofor sur l'impact des types de traitement forestier sur la biodiversité fournit un autre exemple.

J'admets qu'un risque que peut courir un chercheur ayant exploité autant de modèles biologiques différents, qui plus est par le biais d'une approche unique (morphométrique en l'occurrence), est d'être assimilé à un "prestataire de service". Je fonde néanmoins l'espoir que ce mémoire démontre le contraire. Les liens unissant les questions abordées par les différents travaux présentés ici me semblent forts et ils devraient être le gage de la cohérence de mon bilan et de mon projet.

L'approche morphométrique

L'étude de la morphologie a toujours joué un rôle central en Evolution. Il serait vain de tenter de proposer ici une liste exhaustive des différents apports des approches morphométriques dans le cadre de recherches liées à l'identification ou la quantification des similitudes morphologiques, la confrontation actuel–passé, l'étude de la sélection et des contraintes ou d'une manière plus générale la liaison entre l'évolution des génomes et l'évolution des phénotypes. La forme des objets biologiques est un élément d'appréciation majeur des phénotypes et un arsenal technique performant est aujourd'hui disponible pour l'étude de ces formes. L'avènement des nouvelles méthodes de morphométrie (en particulier la morphométrie géométrique) permet aujourd'hui de quantifier la variabilité morphologique selon tous ses aspects (notamment par l'étude indépendante de la taille et de la forme) et de traiter l'information contenue dans la morphologie comme n'importe quelles données quantitatives (Rohlf & Marcus 1993; Adams *et al.* 2004). Ces nouvelles techniques d'investigation du vivant ont en commun de ne plus se fonder sur des mesures euclidiennes (les mensurations) mais sur les

Des développements récents qui étendent considérablement le champ d'investigation des approches morphométriques

Recent developments which considerably increase the field of morphometry

coordonnées de points de repères ou de points sur le contour numérisés par analyse d'image sur les structures morphologiques étudiées. La quantification de la divergence morphologique couplée avec des méthodes de représentation qui en facilitent l'interprétation – par exemple les grilles de déformation ou *Thin Plate Spline* (Bookstein 1991) ou les transformées de Fourier (Kuhl & Giardina 1982; Rohlf & Archie 1984) – constituent un outil d'une très grande efficacité pour l'approche des taux, des rythmes et des modalités de l'évolution morphologique. Les différences de forme entre individus ou échantillons sont alors analysées par le biais des méthodes statistiques multivariées classiques réalisées sur les paramètres obtenus à partir des fonctions d'ajustement (voir David *et al.* (2004) pour une présentation des espaces morphologiques). Le développement de la morphométrie géométrique doit beaucoup aux travaux de F. L. Bookstein et de F. J. Rohlf (cf. Rohlf & Marcus 1993). Grâce aux diverses méthodes d'acquisition (analyse d'image) et de morphométrie géométrique, la très classique morphologie – dont la contribution à l'étude de l'évolution des espèces est, depuis Darwin, considérable – s'est incontestablement dotée des qualités d'une science moderne reposant sur une technologie performante et en constante amélioration.

PARTIE 1

Quantification morphométrique de la différenciation
(et mise en relation avec les patrons de différenciation génétique et avec l'histoire évolutive des taxons considérés).

La différenciation précède la spéciation

Differentiation preceed speciation

La majorité des modèles de spéciation (notamment allopatrique et parapatrique dans le cadre du très répandu concept biologique de l'espèce) présuppose une phase de différenciation durant laquelle des mécanismes d'isolement reproducteur vont se mettre en place (Dobzhanski 1970; Mayr 1970; Coyne & Orr 2004). Sous la vision néodarwinienne classique, la différenciation[1] entre populations de la même espèce est la conséquence de l'effet prépondérant des forces évolutives responsables de l'apparition et du maintien des différences dans les pools géniques des populations (mutations, sélection, dérive génétique), par rapport à l'effet du flux génique (qui tend à homogénéiser ces mêmes pools géniques). Si les conditions évolutives semblent bien identifiées, le problème de la spéciation est cependant loin d'être résolu. Parmi les questions essentielles qui demeurent on peut citer les suivantes: quel est le temps nécessaire pour que la différenciation mène à l'isolement reproducteur ? La différenciation concerne-t-elle, de la même manière, l'ensemble du génome ? Est-ce que certains caractères ou certaines fonctions ont un rôle prépondérant dans l'isolement reproducteur ?

L'étude de cas présentant différents niveaux de différenciation est une approche possible

The study of cases presenting different levels of differentiation is one possible approaches

Il est aisé d'imaginer que la réponse à ces questions ne peut venir de l'étude unique du produit de la spéciation à savoir les espèces elles-mêmes. L'examen, au sein de l'espèce, de la différenciation entre les populations plus ou moins différenciées est une approche incontournable. A cette échelle plusieurs niveaux d'étude sont possibles. Un premier niveau peut se situer à l'échelle de populations géographiquement plus ou moins isolées mais ne présentant pas forcément d'éléments laissant supposer un processus de spéciation avancé (absence de caractères diagnostiques par exemple). Le travail que nous avons mené sur les carabes *C. auronitens* et *C. nemoralis* s'inscrit dans cette démarche. Il nous a, entre autre, permis d'appréhender quels pouvaient être les niveaux de différenciation morphologique en relation avec l'isolement géographique des populations mais aussi en relation avec la biologie des espèces. Il a également été un moyen de tester la puissance des méthodes de morphométrie géométrique (voir paragraphe 1 ci-dessous). Un deuxième niveau d'étude concerne les situations où la différenciation inter-

[1] Que l'on définira ici simplement comme la résultante de l'accumulation des différences.

populationnelle est plus avancée et en particulier les cas où des entités différenciées sont reconnues (on identifie par exemple des sous-espèces). Un champ d'investigation plus large s'ouvre alors et il devient particulièrement informatif d'inclure l'étude des individus hybrides. C'est à cette échelle que s'inscrivent les travaux conduits sur le carabe de Solier (voir paragraphe 2 ci-dessous)

1- Etude de la différenciation morphologique à une échelle micro-géographique : les cas de *C. auronitens* et *C. nemoralis*

Résultats publiés sous la référence:
Alibert, P., B. Moureau, J.-L. Dommergues, and B. David (2001) Differentiation at a microgeographical scale within two species of ground beetles, *Carabus auronitens* and *C. nemoralis* (Coleoptera, Carabidae): a geometrical morphometric approach. *Zoologica Scripta* **30**:299-311. *

Des modèles d'étude de l'impact de la fragmentation des habitats sur la différenciation morphologique

Study models of the impact of fragmentation of habitats on morphological differentiation

La fragmentation et la destruction des habitats sont considérées comme les causes majeures de déclin de la biodiversité. Cependant, si beaucoup d'études se concentrent sur les facteurs de disparition des espèces liés à cette altération des habitats, peu s'intéressent à une conséquence moins négative: la différenciation des populations (et donc à terme éventuellement l'apparition de nouvelles espèces) dans les fragments restants. Dans le cadre du travail de recherche de DEA de Benoît Moureau (1997-1998), nous avons voulu mesurer l'impact de la fragmentation de la forêt sur la différenciation et la variabilité morphologique de deux espèces de carabes forestiers du genre *Chrysocarabus*. A partir d'échantillons prélevés sur le terrain (dans la région de Dijon), nous avons apprécié les niveaux de différenciation morphologique (de taille et de forme) entre populations géographiquement proches (de l'ordre du kilomètre) et populations plus éloignées (de l'ordre de la quarantaine de kilomètres). L'influence de divers facteurs écologiques tels que la présence de barrières naturelles (routes, cours d'eau, surfaces exploitées) a également été considérée.

Une différenciation morphologique pour la population la plus éloignée

A morphological differentiation for the more distant population

Nous avons pu mettre en évidence une différenciation morphologique (de taille comme de forme) significative entre les populations les plus éloignées (distantes d'une quarantaine de kilomètres) pour l'espèce la plus inféodée aux habitats forestiers (*C. auronitens*). A l'inverse, entre les populations géographiquement proches (de l'ordre du kilomètre) aucun rôle significatif des barrières physiques à la dispersion n'a été noté. Dans leur ensemble ces résultats indiqueraient que (1) la distance géographique a un rôle plus important que celui joué par les barrières physiques pour ce type d'organisme (rappelons ici que les carabes ont perdu la fonction du vol), et que (2) les méthodes de morphométrie utilisées s'avèrent très bien adaptées à ce type d'étude à l'échelle intra-spécifique. L'absence de différenciation significative entre les populations de *C. nemoralis* pourrait être liée au caractère plus sténotopique de cette espèce: moins strictement inféodée aux habitats forestiers les populations doivent certainement être plus interconnectées.

Cette étude a apporté des enseignements précieux en terme de connaissance du matériel biologique, d'adaptation des différentes approches méthodologiques ou encore du choix des caractères à étudier. Néanmoins la morphométrie n'est qu'un outil et son utilisation prend tout son sens dans la

confrontation, quand cela est possible, avec d'autres approches et notamment les approches de génétique. Dans le cas des modèles *C. auronitens* et *C. nemoralis* aucune donnée de structuration génétique des populations n'était disponible et pour diverses raisons le choix n'a pas été fait d'entamer des recherches dans cette direction. En particulier à cette même période (1998-1999) des opportunités de collaborations avec J.-Y. Rasplus (INRA-Centre de Biologie et de Génétique des Populations de Montpellier) se sont présentées pour travailler sur une autre espèce de chrysocarabes, le carabe de Solier *C. solieri*. Ce modèle, tout en restant dans le cadre des chrysocarabes, présentait deux atouts majeurs: (1) il permettait de travailler à une échelle plus "avancée" de différenciation (au niveau de la sous-espèce) et (2) il bénéficiait d'un corpus de données de génétique des populations et de phylogénie moléculaire non négligeable.

2- Dynamique de la différenciation au sein d'un complexe de sous-espèces : le cas de *C. solieri*

Une systématique infra-spécifique est complexe et mal définie

A *complex and unsolved infraspecific systematics*

C. solieri est une espèce de carabe forestier protégée, dont l'aire de répartition s'étend du massif de l'Esterel aux Alpes Liguriennes. La caractéristique certainement la plus remarquable de cette espèce est son très grand niveau de variabilité intra-spécifique en dépit d'une aire de répartition somme toute très limitée. De nombreuses variations de forme, de taille, de couleur mais aussi génétiques ont été décrites (Bonadona 1967; Darnaud *et al.* 1978; Rasplus *et al.* 2001). En conséquence, la systématique infra-spécifique de cette espèce demeure imprécise, le nombre de sous-espèces variant de trois à six selon les auteurs. Les évènements d'hybridation entre les différentes entités semblent par ailleurs fréquents. L'ensemble de ces éléments souligne l'intérêt du modèle *C. solieri* dans le cadre de nos thématiques générales de recherche puisque cette espèce semble être le siège d'une dynamique active de spéciation. Trois raisons au moins pourraient expliquer ces niveaux élevés de différenciation : (1) les capacités de dispersion limitées de l'espèce (les ailes membraneuses sont dégénérées et ne subsistent plus qu'à l'état vestigial), (2) le relatif isolement des populations en raison de la fragmentation de l'habitat forestier et (3) les oscillations climatiques pléistocène avec pour conséquence une succession de phases de rétraction et d'extension de l'aire de répartition (Garnier 2003). La figure 2 présente l'aire de répartition de *C. solieri* et mentionne les limites géographiques de 6 groupes de populations distinguables par la couleur et la localisation géographique (notons ici que ces groupes n'ont pas de valeur taxonomique mais permettent une description s'affranchissant de la confusion régnant autour de la systématique).

Six entités sont définies

Six entities are defined

Dans ce contexte notre étude avait pour objectif général de mettre en relation les patrons de différenciation et d'introgression de différents marqueurs génotypiques et phénotypiques. Ce projet a constitué le cadre directeur de la thèse de Stéphane Garnier (thèse soutenue en décembre 2003) et a également été à l'origine de deux mémoires de DEA (Djamel Bousbassi, 2000 et Jessica Mérédith, 2003).

La structuration génétique

Résultats publiés sous la référence:
Garnier, S., P. Alibert, P. Audiot, B. Prieur, and J.-Y. Rasplus (2004) Isolation by distance and sharp discontinuities in gene frequencies: implications for the phylogeography of an alpine insect species, *Carabus solieri*. *Molecular Ecology* **13**:1883-1887. *

Quatre groupes génétiques ont été identifiés.

Four genetical groups have been identifed.

Une première étape du travail de Stéphane Garnier a été d'augmenter très significativement les données sur la structuration génétique des populations de *C. solieri*. Ce travail, réalisé au CBGP à Montpellier s'est notamment basé sur l'étude de 10 marqueurs microsatellites. Une analyse d'isolement par la distance couplée à une analyse de partition a été réalisée sur un échantillon de plus de 1000 individus répartis sur 41 localités (voir Garnier et al. 2004 pour un détail de la méthodologie employée). Quatre groupes génétiques principaux ont ainsi pu être identifiés (Rasplus *et al.* 2001; Garnier *et al.* 2002; Garnier *et al.* 2004). Un premier groupe correspond aux populations de Bonnetianus (plus certaines populations de Curtii), un deuxième rassemble les populations de Solieri-C, de Clairi et les populations restantes de Curtii, un troisième groupe comprend l'ensemble des populations de Solieri I mais se subdivise en deux en séparant les populations de l'Est de celles de l'Ouest.

C'est dans ce contexte génétique clarifié qu'il devenait particulièrement intéressant de voir si il existait une concordance dans les patrons de structuration génétique et morphologique.

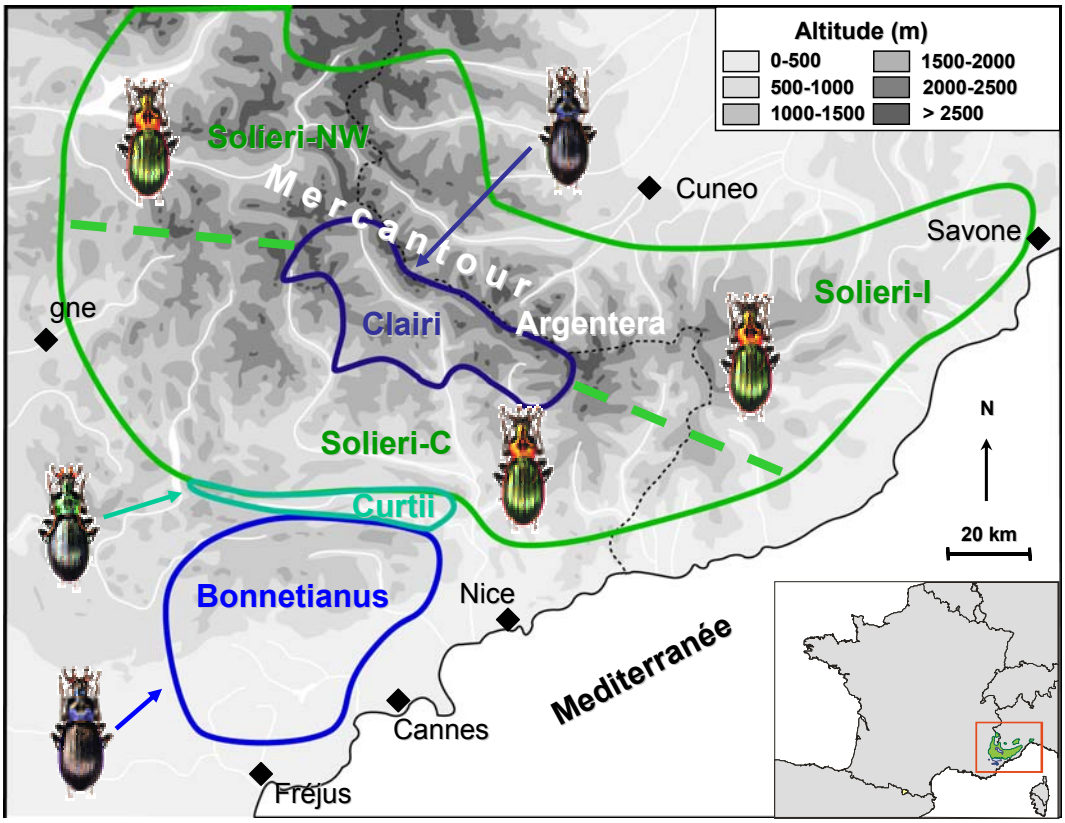


Figure 2 : Aire de répartiion de l'espèce *C. solieri*. Les limites des aires de répartition des 6 groupes définis sur la base de la couleur et de la géographie sont indiquées.

La structuration morphologique

Résultats publiés sous la référence:
Garnier, S., F. Magniez-Jannin, J.-Y. Rasplus, and P. Alibert (2005) When morphometry meets genetics: inferring the phylogeography of *Carabus solieri* using Fourier analyses of pronotum and male genitalia. *Journal of Evolutionary Biology* **18**:269-280. *

Une étude morphométrique de la forme du pronotum et de l'édéage.

A morphometric study of the shape of pronotum and aedeagus.

L'étude morphométrique a porté sur la forme du pronotum et des édéages (génitalia externes) mâles. Ces deux structures ont été retenues en raison de leur utilisation fréquente dans les études de systématique des carabes. Ce sont par ailleurs des structures sclérifiées très rigides et par conséquent certainement peu sujettes aux déformations liées à la conservation des spécimens dans l'alcool. Les pronotums comme les édéages ne présentant que très peu de points de repères facilement identifiables, nous avons choisi de réaliser des analyses de contour par le biais des transformées de Fourier. Le principe de ces analyses est que l'échantillonnage des points le long du contour fournit un signal qui est décomposé en une somme de fonctions trigonométriques de longueurs d'onde décroissantes (appelées harmoniques). Chaque harmonique est caractérisée par une paire de coefficients, les coefficients ou descripteurs de Fourier (il s'agit d'une paire de valeurs correspondant à la phase et à l'amplitude, ou de deux coefficients a et b reliés mathématiquement à la phase et à l'amplitude). Le signal périodique issu du contour a dans notre cas été obtenu par échantillonnage des variations des coordonnées cartésiennes (x,y) en fonction de l'abscisse curviligne de 128 points équidistants. Une approche [analyse de Fourier discrète ou *Dual Axis Fourier Shape Analysis* en anglais (Moellering & Rayner 1981, 1982; Bertin *et al.* 2002)] utilisant une notation complexe des coordonnées (Z=X+iY) a ensuite permis le traitement simultané des valeurs des abscisses et des ordonnées (ces deux paramètres sont traités séparément dans le cas de la plus classique transformée de Fourier elliptique). Des analyses préliminaires ont montré qu'en combinant l'information issue des calculs d'erreur de mesure à celle des qualités de reconstruction des contours *via* les transformées de Fourier inverses il était opportun de retenir 15 harmoniques dans le cas du pronotum et 12 dans celui de l'édéage. Au total 1094 individus provenant de 41 localités ont été étudiés pour le pronotum et 310 individus provenant de 24 localités pour l'édéage.

Une corrélation négative significative entre taille et altitude

A significant negative correlation between size and elevation

Concernant la taille, les ANOVA réalisées sur les valeurs des racines carrées de la surface des structures étudiées ont révélé des différences significatives de taille entre les échantillons pour les deux structures morphologiques (pronotum: $F_{40,1053}=36.55$, $p<0.0001$ et édéage: $F_{23,286}=36.38$ et $p<0.0001$). Il existe une corrélation significative et négative entre taille et altitude plus marquée pour le pronotum ($R^2=0.49$, $F_{1,39}=36.89$, $p<0.001$) que pour l'édéage ($R^2=0.18$, $F_{1,22}=4.75$, $p<0.001$). En revanche, il n'existe pas de patron de variation de la taille en liaison avec les différents groupes de populations (Bonnetianus, Curtii, Clairi, Solieri).

Des différences de forme significatives entre les populations

Significant shapes differences among populations.

Concernant la forme, les MANOVA réalisées sur les variables de forme (les coefficients de Fourier) indiquent que la forme du pronotum varie de façon significative entre les populations (MANOVA, Wilk's lambda=$5.4\ 10^{-5}$, $F_{2400,\ 33514.18}=4.80$, $p<0.0001$). La projection des scores moyens des populations sur les deux premiers axes factoriels de l'analyse discriminante est

représentée sur la figure 3. Trois groupes de populations s'individualisent (ils sont distingués sur la figure par des contours en traits pleins et en pointillés). Le premier correspond à la quasi-totalité des populations du groupe Bonnetianus, le deuxième correspond aux populations du groupe Solieri-NW et le troisième est constitué des autres populations. Notons également que sur le premier axe factoriel, les populations sont approximativement ordonnées selon leur latitude. Les contours reconstruits indiquent que ce sont essentiellement les bords latéraux des pronotums qui sont concernés par les changements de forme.

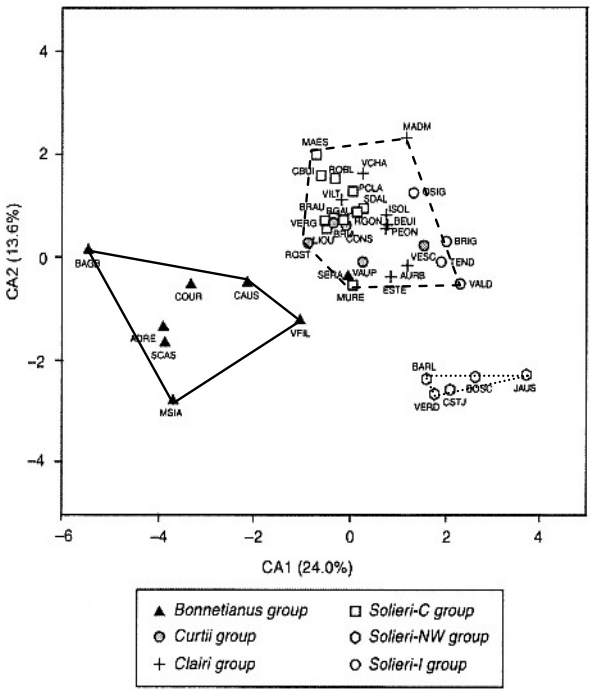


Figure 3 : Espace morphologique défini par les deux premiers axes canoniques et construit à partir de l'analyse des contours des pronotums. Seuls les centres de gravité des populations sont représentés sur la figure. Les symboles désignent les groupes définis à partir des analyses génétiques et de la couleur (d'après Garnier *et al.*, 2005*).

De la même manière il existe une variation de forme significative sur les paramètres de forme décrivant l'édéage (MANOVA: Wilk's lambda=3.2 10^{-4}, $F_{552,\ 4468.34}$=5.03, p<0.0001). La projection des scores moyens des populations sur les deux premiers axes factoriels (représentant un peu plus de 50% de la variation) ne permet pas une individualisation de groupes de populations aussi distincte que dans le cas du pronotum mais les positions relatives des populations respectent une certaine logique, le groupe Bonnetianus reste excentré et le groupe Solieri-I se situe à l'autre extrémité de l'espace morphologique (figure 4). La distinction entre les groupes Solieri-I et Solieri-NW est moins nette pour la forme de l'édéage que pour celle du pronotum. Les reconstructions des différents contours moyens indiquent que les variations de forme de l'édéage demeurent très subtiles.

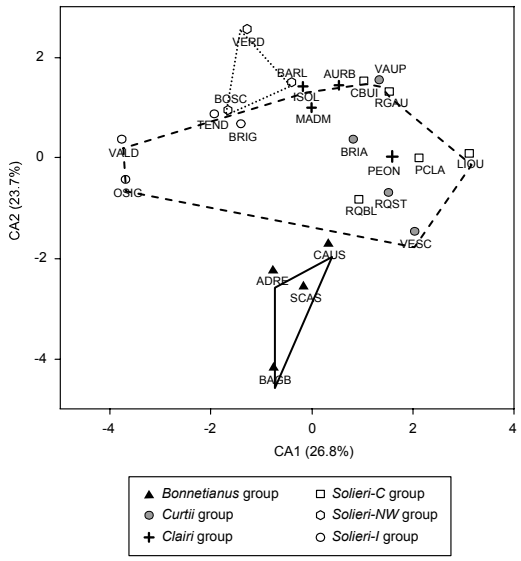


Figure 4 : Espace morphologique défini par les deux premiers axes canoniques et construit à partir de l'analyse des contours des édéages. Seul les centres de gravité des populations sont représentés sur la figure. Les symboles désignent les groupes définis à partir des analyses génétiques et de la couleur (d'après Garnier *et al.*, 2005).

Les distances morphologiques sont corrélées aux distances génétiques

Morphological distances are correlated to genetic distances

Les tests de Mantel indiquent que les distances morphologiques entre populations (distances de Mahalanobis dans les espaces discriminants précédemment obtenus) sont significativement corrélées aux distances génétiques d'une part et aux distances géographiques d'autre part (tableau I). Cependant, les tests de corrélations partielles révèlent que seules les corrélations entre distances morphologiques et génétiques demeurent significatives lorsque l'on tient compte de leur éloignement géographique.

Tableau I : Résultats des tests de corrélations de Mantel simples et partielles entre distances morphologiques, géographiques et génétiques. Les corrélations significatives sont indiquées en gras.

	Pronotum		Edéage	
Tests de Mantel simples	r	*p*	r	*p*
Morpho – Géo	**0.46**	**0.0001**	**0.40**	**0.0150**
Morpho – Génét	**0.69**	**<0.0001**	**0.59**	**<0.0001**
Tests de Mantel partiels	r'	*p*	r'	*p*
Morpho – Géo – Génét	0.09	0.1800	0.12	0.1700
Morpho – Génét - Géo	**0.58**	**<0.0001**	**0.48**	**<0.0001**

Des résultats qui contrastent avec ceux d'études antérieures.

Results differ from those reported by previous studies.

Ce travail a montré qu'il existait au sein de l'espèce *C. solieri* une différenciation morphologique importante. Un élément témoignant en faveur de la solidité de nos résultats et que les patrons de variabilité obtenus à partir des deux structures morphologiques considérées sont globalement congruents. Notons cependant que ces derniers contrastent avec des études antérieures basées sur des approches de morphométrie traditionnelle (portant, entre autre, sur le pronotum) menées par Bonadona (Bonadona 1967, 1973). Cet auteur conclut notamment à une séparation de certaines populations au sein du groupe Bonnetianus et à une distinction entre les groupes Clairi et Solieri-C. Nos résultats n'appuient aucun de ces deux éléments. La faiblesse de l'échantillonnage, la valeur peu informative des caractères mesurés pour retranscrire les variations de forme du pronotum, l'erreur de mesure potentielle ou encore la non indépendance des éléments permettant la définition des entités (morphométrie et couleur) sont autant

d'éléments qui nous ont amené à discuter de la validité des résultats de Bonadona (Garnier *et al.* 2005).

Les approches génétique et morphométrique sont congruentes.

Genetical and morphometrical approaches are congruent.

Un des intérêts de notre travail sur *C. solieri* résidait dans la confrontation des résultats entre approche génétique et approche morphométrique. La congruence des résultats issus des deux études démontre, si besoin en était, tout l'intérêt de cette double approche. Plus spécifiquement ils montrent également que les méthodes de morphométrie géométrique permettent de détecter des différences morphologiques à la fois subtiles et complexes. Ils sont une illustration supplémentaire que ces méthodes peuvent être des outils puissants même à de faibles niveaux taxonomiques (Baylac & Daufresne 1996; Adams & Funk 1997; Alibert *et al.* 2001; Baylac *et al.* 2003).

A l'issue de l'étude de structuration génétique des populations nous avions avancé trois hypothèses pour tenter de préciser la phylogéographie de l'espèce. L'étude de morphométrie nous a permis d'en favoriser une, celle envisageant que les groupes Solieri-C et Clairi qui forment une entité génétique propre proviennent d'une large introgression entre les deux entités représentées aujourd'hui par les groupes Bonnetianus et Solieri-I (voir Garnier et al., 2005 pour une discussion détaillée).

L'étude quantitative de la couleur

L'étude d'un caractère original mais primordial (car il est un élément important de la systématique du groupe), la couleur, a été réalisée par le biais d'une collaboration avec le laboratoire "Electronique, Informatique et Image" de l'université de Bourgogne. Les couleurs des élytres et des pronotums des carabes ont été quantifiées (mesure des niveaux de RVB) et fournissent une mesure objective qui peut être utilisée comme un marqueur au même titre que les marqueurs génétiques ou morphométriques. Cette quantification a été réalisée dans le cadre du travail de DEA de Jessica Mérédith (2003) et constitue une partie du travail de thèse de Stéphane Garnier. L'étude comparative des profils d'introgression des différents marqueurs (génétique, morphologique, couleur) n'a, faute de temps, pour l'instant pas encore été conduite. Elle devrait permettre, au sein des zones d'échanges génétiques, une étude plus fine des forces sélectives impliquées dans la différenciation.

3- Quantification morphométrique de la différenciation intra-spécifique : exemple du dimorphisme sexuel chez l'aselle *Asellus aquaticus*.

Résultats publiés sous la référence:
Bertin, A., B. David, F. Cézilly, and P. Alibert (2002) Quantification of sexual dimorphism in *Asellus aquaticus* (Crustacea: Isopoda) using outline approaches. *Biological Journal of the Linnean Society* **77**:523-534. *

Ce travail a été réalisé dans le cadre de la thèse d'Angéline Bertin que j'ai co-encadrée avec F. Cézilly et qui portait sur la relation entre variabilité morphologique et sélection intra-sexuelle chez le crustacé isopode *Asellus aquaticus*. L'étude du dimorphisme sexuel dans un chapitre consacré à la quantification morphométrique de la différenciation peut paraître

surprenante. En effet, il ne s'agit pas ici d'une démarche visant à identifier et quantifier des différences révélatrices, voire participantes, d'une divergence pouvant conduire à la spéciation, mais plutôt d'étudier les forces sélectives impliquées dans certains mécanismes de sélection sexuelle. Si la question de fond est différente, la démarche méthodologique est néanmoins similaire puisque notre objectif était d'identifier des variations intra-spécifiques, parfois subtiles, mais aussi de les quantifier de manière à pouvoir comparer différents groupes d'intérêt.

Quelle est la relation entre le comportement de gardiennage précopulatoire et la forme de certaines structures morphologiques?

What is the relationship between mate guarding behavior and shape of some morphological structures?

Chez l'aselle, la copulation est précédée d'une période appelée gardiennage pré-copulatoire durant laquelle le mâle reste agrippé à la femelle (et le reste jusqu'à ce que l'insémination soit possible). Ce comportement est responsable d'un dimorphisme sexuel, en particulier au niveau de la première et de la quatrième paire de péréiopodes (pattes ambulatoires) qui présentent respectivement des apophyses et sont réduits et courbés chez les mâles. L'objectif de l'étude était double puisqu'il il s'agissait (1) de tester si le dimorphisme sexuel était quantifiable et (2) de voir si le dimorphisme sexuel de forme était exclusivement relié au comportement sexuel pré-copulatoire. La réponse à ce deuxième objectif passait par l'étude de caractères morphologiques non impliqués dans ce comportement sexuel.

Au total 5 caractères morphologiques ont été considérés: le protopodite du péréiopode 1, les carpopodites des péréiopodes 4 et 5, la tête et le pléotelson (voir figure 1 dans Bertin *et al.*, 2002*). Les deux premiers caractères étaient connus pour être dimorphiques, les trois autres non. La taille et la forme ont été analysés par le biais des transformées de Fourier discrètes appliquées sur les contours de ces caractères. Une analyse préliminaire de l'erreur de mesure a montré que la tête et le péréiopode 1 étaient mesurés avec trop d'imprécision puisque la variabilité entre séries de mesures sur les mêmes individus pouvait être supérieure à la variabilité inter-individuelle. Ces deux caractères ont par conséquent été exclus des analyses. Le dimorphisme sexuel a été analysé qualitativement par inspection des espaces des formes obtenus *via* des ACP sur les coefficients de Fourier. Un dimorphisme sexuel de taille existant chez l'aselle, des MANCOVA ont été réalisées de manière à tester l'effet potentiel de relations allométriques (variables dépendantes: amplitudes standardisées, effet: sexe, covariable: taille).

Un dimorphisme de forme entre mâles et femelles. Les mâles non appariés pourraient avoir une forme intermédiaire

A shape dimorphism beteen males and females .Unpaired males should have a n intermediate shape

Les deux résultats majeurs de cette étude sont les suivants. Le premier est que le dimorphisme sexuel de forme est important et présent sur les trois caractères finalement retenus. Rappelons ici que deux (péréiopode 5 et pléotelson) n'étaient pas identifiés jusqu'alors comme dimorphiques. La nette séparation des individus mâles et femelles est visible sur la figure 5 (même si elle est moins marquée pour le pléotelson). Le deuxième résultat est la différence de forme significative notée entre les mâles appariés et les mâles non appariés pour le péréiopode 4 et le pléotelson. Ce qui est remarquable dans ce résultat c'est que morphologiquement les mâles non appariés semblent se situer entre les mâles appariés et les femelles (visible également sur la figure 5). L'utilisation de transformées de Fourier inverses permet de constater que cette différence entre mâles appariés et non appariés concerne les mêmes parties des structures étudiées que celles impliquées dans le dimorphisme sexuel.

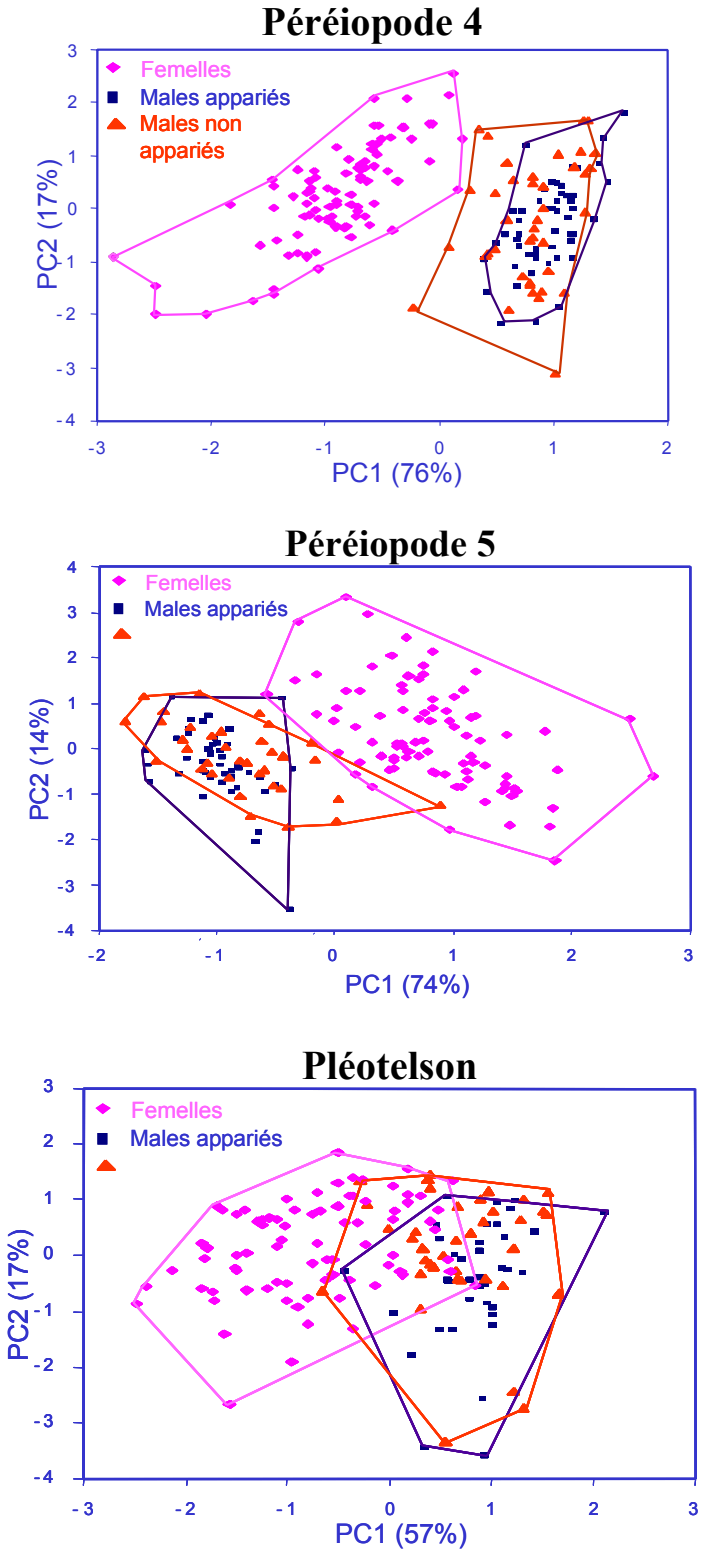


Figure 5 : Espaces morphologiques définis par les deux premières composantes principales et construits à partir des analyses des contours des trois structures étudiées. Les femelles, les mâles appariés et les males non appariés sont distingués (d'après Bertin *et al.*, 2002).

Nos résultats vont dans le sens de l'idée proposant que la compétition entre mâles est un déterminant majeur de l'évolution du dimorphisme sexuel chez l'Aselle (Vandel 1926; Balesdent 1964). Le gardiennage pré-copulatoire semble avoir une influence sur la morphologie des aselles puisque les

différences de forme mises en évidence entre mâles appariés et non appariés suggèrent que c'est la sélection sexuelle, au travers des capacités d'appariement des mâles, qui pourrait être en partie responsable du dimorphisme sexuel entre ces deux caractères. Ce sont d'ailleurs les mêmes régions des caractères étudiés qui sont concernées par les différences entre mâles et femelles, et entre mâles appariés et non appariés. Notons tout de même qu'un dimorphisme sexuel a également été noté pour le péréiopode 5, caractère pour lequel il n'y a pas de différences en fonction du statut d'appariement. Cet élément suggérerait que le gardiennage pré-copulatoire n'est pas le seul facteur responsable de l'évolution de la forme des caractères. Un paramètre biologique tel que le comportement de recherche du partenaire (développé chez les mâles, absent chez les femelles) peut fournir une explication alternative.

Ce travail combinant les approches morphométrique et comportementale apparaît comme assez innovant et prometteur. Il illustre, une fois encore, la capacité des méthodes d'analyse de forme à mettre en évidence des différences morphologiques pouvant être subtiles et par là même démontre leur potentiel pour les études de microévolution. Les études centrées sur la sélection sexuelle sont très demandeuses d'approches objectives et quantifiées des phénotypes. Notre étude montre que les méthodes de morphométrie modernes (en particulier la morphométrie géométrique) peuvent élargir considérablement le champ d'investigation de l'écologie comportementale.

PARTIE 2

Etude de la relation entre divergence de taxons en voie de différenciation et instabilité de développement de leurs hybrides

Un moyen d'estimer la divergence entre certains sytèmes de gènes

A *way to estimate the divergence between some gene systems*

Dans le cadre de la problématique générale de la dynamique de la différenciation, et quand on est en mesure d'identifier des entités (génétiques et/ou morphologiques, comme dans le cas de *C. solieri*), une question essentielle se pose quant aux conséquences de cette différenciation : les entités vont-elles continuer à se différencier et devenir à terme des espèces différentes ? ou, à l'inverse, les flux géniques vont-ils mener à leur mélange et leur homogénéisation ? Si la question est simple, apporter une réponse est toujours complexe. Dans le cas précis de *C. solieri*, et à ce stade de l'étude, aucun argument ne permet par exemple de favoriser le scénario de la spéciation par rapport à celui du mélange secondaire. Le phénomène de spéciation n'est pas observable à une échelle humaine et les seuls éléments dont nous disposons sont des indications indirectes, en général issues de l'étude des hybrides. Ces derniers offrent en effet l'opportunité d'étudier les systèmes impliqués dans des incompatibilités entre les entités parentales et par conséquent dans la spéciation. Plusieurs approches sont possibles, elles peuvent notamment concerner l'étude comparative des clines de fréquences de différents marqueurs dans les zones d'hybridation et/ou plus généralement, l'estimation de différents paramètres de la valeur sélective des hybrides, *in natura* ou dans des croisements expérimentaux. L'étude de l'instabilité de développement morphologique, parce qu'elle permet d'évaluer l'étendue de la divergence des systèmes de gènes impliqués dans cette fonction particulière chez les différentes entités parentales, s'inscrit dans ce cadre (Graham 1992; Auffray *et al.* 1996; Alibert & Auffray 2003).

L'asymétrie fluctuante comme mesure de l'instabilité de développement

Fluctuating asymmetry as a measure of developmental instability

L'instabilité de développement se définit comme le résultat des processus qui, dans un environnement donné, perturbent le développement le long d'une trajectoire développementale (Palmer 1994)[2]. Ce "bruit de fond" développemental, non héritable, est lié à des évènements indépendants qui perturbent le déroulement normal du développement et qui entraînent une variation d'origine stochastique dans la croissance (Polak 2003). Pour mesurer la part de variabilité morphologique liée à ce processus il est indispensable de s'affranchir des autres sources de variabilité morphologique que sont la variabilité génétique et la variabilité environnementale. Ainsi, le moyen le plus

[2] *A contrario*, la stabilité de développement se définit comme le résultat des processus qui résistent à ces perturbations (voir la partie 3 pour une présentation plus précise et plus exhaustive des définitions des différents patrons et processus liés au contrôle du développement).

courant de quantification de l'instabilité de développement est la mesure de la variabilité morphologique intra-individuelle entre structures homologues répétées. Cette approche repose sur le postulat que des caractères homologues (appelés aussi caractères répétés) au sein d'un individus sont contrôlés par les mêmes gènes et sont soumis au même environnement au cours de leur développement. Toute variation entre ces caractères traduira donc de l'instabilité de développement. Les structures répétées les plus fréquentes dans la nature sont les structures bilatérales. La mesure de l'asymétrie fluctuante (Van Valen 1962) est par conséquent l'approche quantitative la plus largement utilisée dans ce contexte (Palmer 1994). Elle consiste à mesurer les différences survenant entre les côtés droit et gauche de caractères morphologiques bilatéraux normalement symétriques ; toute déviation à la symétrie étant interprétée comme une diminution de la stabilité du développement.

L'asymétrie fluctuante est une mesure simple mais qui possède une profonde signification biologique. La stabilité de développement peut être en principe affectée par des facteurs environnementaux ou génétiques. Par exemple, il a été montré une augmentation des niveaux d'asymétrie fluctuante chez des individus issus de populations soumises à des stress environnementaux tels que l'appauvrissement ou la pollution du milieu (Pankakoski 1985; Parsons 1990; Pankakoski *et al.* 1992; Graham *et al.* 1993). Par ailleurs, l'hétérozygotie, la coadaptation génomique ou l'effet de gènes particuliers sont les conditions génétiques identifiées comme susceptibles de modifier la stabilité de développement (Clarke 1993; Markow 1995; Alibert & Auffray 2003). De fait, l'asymétrie fluctuante est considérée comme un indicateur de condition génotypique et phénotypique et a trouvé beaucoup d'applications dans le contexte des études de biologie évolutive (sélection sexuelle par exemple), comme dans celui des études plus appliquées telle que le biomonitoring (Møller & Swaddle 1997).

Des débats nourris…

A rather debated topic…

Ces deux dernières décennies, l'engouement autour de cette approche morphométrique a été extrêmement important. Plusieurs centaines d'articles utilisant l'asymétrie fluctuante ont été publiés. Les débats ont été (et restent) vifs, notamment autour de la généralisation des différentes hypothèses liées à l'utilisation de l'asymétrie fluctuante et en particulier comme marqueur de valeur sélective (Leung & Forbes 1997; Møller 1997; Palmer & Strobeck 1997; Clarke 1998a; Møller 1999; Clarke 2003; Tomkins & Simmons 2003; Tracy *et al.* 2003). Une partie des critiques a également été d'ordre méthodologique (Swain 1987; Palmer 1994; Swaddle *et al.* 1995; Van Dongen *et al.* 1999; Palmer & Strobeck 2003). Notre objet n'est cependant pas ici de présenter et de discuter l'ensemble de ces éléments. Soulignons simplement que le contexte est en effet particulièrement propice aux débats, les études d'asymétrie fluctuante présentant tous les ingrédients nécessaires pour nourrir les controverses : les méthodologies adoptées sont hétérogènes, les résultats parfois contradictoires (voir par exemple les méta-analyses de Møller & Cuervo (2003) et de Tomkins & Simmons (2003) qui n'interprètent pas de la même façon les résultats hétérogènes), et les effets, quand ils sont significatifs, sont faibles.

Ces controverses ont eu entre autres conséquences positives d'inciter les auteurs à une plus grande rigueur dans les expérimentations, à plus de prudence dans certaines interprétations et ont par voie de conséquence entraîné une augmentation du nombre d'études et de données plus fiables

(Polak 2003). Aujourd'hui, même si la majorité des critiques est justifiée et toujours valable, le bien fondé et l'utilité des approches utilisant l'asymétrie fluctuante ont été démontrés dans la plupart des domaines. L'appréciation de l'étendue des divergences par l'étude de l'impact de l'hybridation entre entités en voie de différenciation sur la stabilité de développement en est un.

1- Instabilité de développement et hybridation : à quoi doit-on s'attendre?

Une discussion générale de ce thème a été publiée sous la référence:
Alibert, P. and J.-C. Auffray (2003) Genomic coadaptation, outbreeding depression and developmental instability. In *Developmental instability: Causes and Consequences*, Ed M. Polak, New York: Oxford University Press, 116-134. *

Coadaptation génomique et hétérozygotie : des rôles antagonistes

Genomic coadaptation and heterozygosity: the antagonistic roles

La coadaptation génomique[3] et l'hétérozygotie sont les deux conditions génétiques principales identifiées comme ayant une action sur les niveaux d'instabilité de développement d'un organisme (les effets de certains gènes sont également documentés mais de façon beaucoup plus marginale). Les interactions génétiques jouent donc un rôle prépondérant (tableau II). Dans ce contexte, le cas des hybrides s'avère particulièrement intéressant. Lors d'un évènement d'hybridation entre entités différenciées, les niveaux de coadaptation génomique mais également d'hétérozygotie sont modifiés et les effets de ces modifications devraient être opposés. D'un côté l'instabilité de développement devrait augmenter en raison de la rupture de la coadaptation génomique (liée au mélange de deux génomes ayant évolué partiellement indépendamment), et d'un autre elle devrait diminuer en raison de l'augmentation du taux d'hétérozygotie attendu chez les hybrides. L'image d'une balance entre les effets opposés de ces deux conditions génétiques est généralement utilisée, l'équilibre de celle-ci dépendant de l'éloignement génétique entre les entités impliquées dans l'hybridation (Vrijenhoek & Lerman 1982; Graham 1992).

Tableau II : Résumé des interactions et des mécanismes liés aux différentes conditions génétiques agissant sur l'instabilité de développement

[3] La coadaptation génomique pourrait se définir comme le résultat de la sélection ayant favorisé, au cours de l'histoire évolutive d'une population donnée, l'accumulation et le maintien de gènes fonctionnant de façon hamonieuse. En termes de génétique quantitative la coadaptation génomique correspond aux effets non additifs sélectionnés, entre (épistasie) et au sein (dominance et super dominance) des loci (Alibert & Auffray, 2003).

Les prédictions sont difficiles à établir

Predictions are difficult to make

Nous avons cependant pu montrer dans une travail de synthèse portant sur les relations entre coadaptation génomique et instabilité de développement que même si il existait une tendance globale dans le sens prédit par le modèle de Vrijenhoek & Lerman (1982), les nombreuses exceptions interdisent toute généralisation (Alibert & Auffray, 2003). Le résultat du recensement des études présenté dans le tableau III montre par exemple qu'il existe un nombre non négligeable d'hybridations inter-subspécifiques (voire spécifiques) qui se traduisent par une diminution des niveaux d'instabilité de développement. On peut constater également que les croisements entre populations, races ou lignées génèrent de façon quasi équilibrée les trois types de résultats possibles.

Tableau III : Nombre d'études indiquant une augmentation, pas de différences ou une diminution des niveaux d'instabilité de développement chez des groupes hybrides en comparaison de leurs groupes parentaux (d'après Alibert & Auffray 2003).

Niveau de croisement	Augmentation ID	ID stable	Diminution ID	*Total*
Genres ou espèces	17	6	1	*24*
Sous-espèces	3	0	6	*9*
Populations différenciées, lignées ou races	5	5	4	*14*
Total	*25*	*11*	*11*	*47*

Une grande hétérogénéité entre les études

A high heterogeneity among studies

Cette constatation démontre que les liens unissant divergence des protéines (=distance génétique[4]) et divergence des gènes de régulation (estimée ici par le niveau d'instabilité de développement) ne sont pas toujours linéaires. Ce résultat n'a rien de surprenant dans la mesure où les études sont finalement très hétérogènes. Tout d'abord les organismes considérés sont très différents et l'on compare ici des résultats obtenus sur des plantes à ceux obtenus sur des vertébrés ou des invertébrés. Même si le phénomène de coadaptation génomique concerne tous les organismes et peut être considéré comme un mécanisme général, il est fort probable que les processus de contrôle de la stabilité de développement diffèrent entre les organismes et soit le fruit d'histoires évolutives différentes au sein des grandes lignées. Par ailleurs, il existe également une hétérogénéité au niveau des méthodes statistiques de traitement de l'asymétrie fluctuante (qu'il s'agisse des tests préliminaires ou de la comparaison des indices d'asymétrie) qui peuvent rendre les comparaisons difficiles. Dans le cas particulier de l'hybridation se pose également le problème de l'homogénéité des échantillons d'hybrides. Dans un certain nombre d'études les hybrides sont réunis dans un seul et même échantillon. Le mélange (éventuellement déséquilibré) de génotypes potentiellement caractérisés par des niveaux d'instabilité de développement différents entraîne nécessairement un problème d'interprétation mais également un problème statistique (Graham 1992; Palmer & Strobeck 1992; Arnold & Hodges 1995; Alibert & Auffray 2003).

[4] Distance soit effectivement mesurée soit simplement supposée, la taxonomie ne se basant pas toujours sur des études de génétique.

2- Instabilité de développement chez les hybrides au sein du complexe *C. solieri*.

Résultats publiés sous la référence:
Garnier, S., N. Gidaszewski, M. Charlot, J.-Y. Rasplus and P. Alibert (2006) Hybridization, developmental stability and functional significance of morphological traits in the carabid beetle *Chrysocarabus solieri* (Coleoptera, Carabidae). *Biological Journal of the Linnean Society* 89: 151-158 *

Deux évènements d'hybridation successifs

Two successives hybridisation events

L'étude comparée des niveaux d'instabilité de développement morphologique de groupes parentaux et hybrides a donc été un des axes de recherche du travail de thèse de Stéphane Garnier sur la dynamique de l'hybridation au sein du complexe *C. solieri*. Cette approche était motivée par deux éléments principaux. Premièrement, ce modèle présente des évènements d'hybridation à des échelles spatiales et temporelles différentes. Le premier évènement, qui a eu lieu entre les deux entités ancestrales, peut être considéré comme relativement ancien, et en tous cas comme un évènement passé car il n'y a actuellement pas d'échanges génétiques entre les groupes Bonnetianus et Solieri NW (les groupes dérivés des deux entités ancestrales). Le deuxième évènement d'hybridation, qui est celui qui serait à l'origine du groupe Curtii, est plus récent et probablement encore effectif. Deuxièmement, *C. solieri*, comme tous les Chrysocarabes, est brachyptère ce qui signifie que ses élytres sont soudées et que ses ailes membraneuses sont atrophiées et peuvent être par conséquent qualifiées de vestigiales. Parmi les explications possibles de l'hétérogénéité des résultats évoqués ci-dessus, la nature des caractères étudiés est souvent évoquée. L'hypothèse est que plus les caractères sont soumis à la sélection naturelle, plus leur développement devrait être contrôlé et soustrait au bruit développemental et aux influences génétiques et développementales (Debat & David 2001). Cette hypothèse demeure néanmoins difficile à tester car il n'est pas si évident de définir *a priori* l'intensité de la sélection affectant un caractère donné. La comparaison entre caractères fonctionnels et caractères vestigiaux en fournit l'opportunité.

Un total de 678 individus appartenant à 27 populations situées le long d'un transect Sud-Ouest – Nord-Est (voir figure 1 *in* Garnier *et al.*, 2006*) ont été analysés. Quatre caractères bilatéraux ont été considérés: longueur et largeur des ailes vestigiales (WINGL, WINGW respectivement), et longueur du tibia de la deuxième et de la troisième paire de pattes (TIBMID et TIBHIND respectivement). La différence de fonctionnalité entre des ailes atrophiées et des pattes ne souffrait d'aucune ambiguïté.

Des niveaux d'aymétrie fluctuante différents pour un caractère sur quatre

Difference in fluctuating asymmetry levels for one trait out of four

Les analyses montrent qu'un seul caractère (WINGW) sur les quatre présente des variations significatives des niveaux d'asymétrie fluctuante entre les populations ($F_{26,620}$=1,53, p=0,04). Une ANOVA avec comparaison *a priori* (*planned comparisons ANOVA*) a par conséquent été réalisée pour ce seul caractère. Les quatre contrastes considérés sont résumés dans le tableau IV. Seul le deuxième contraste n'est pas apparu significatif.

Tableau IV : Contrastes utilisés pour l'ANOVA avec comparaisons *a priori* (AF= asymétrie fluctuante)

Contrastes	Groupes comparés	Eléments testés
Contraste 1		
Bonnetianus + Solieri-INW *versus* Clairi + Solieri-C	Groupes dérivés des deux entités parentales ancestrales *versus* Hybrides évènement d'hybridation ancien	Impact de l'évènement d'hybridation ancien sur l'AF des hybrides
Contraste 2		Différences d'AF entre groupes dérivés des entités parentales de l'évènement d'hybridation ancien
Bonnetianus *versus* Solieri-INW	Parent 1 évènement d'hybridation ancien *versus* Parent 2 évènement d'hybridation ancien	
Contraste 3		
Bonnetianus + Solieri-C *versus* Curtii	Parents évènement hybridation récent *versus* Hybrides évènement d'hybridation récent	Impact de l'évènement d'hybridation récent sur l'AF des hybrides
Contraste 4		Différences d'AF entre groupes parentaux impliqués dans l'évènement d'hybridation récent
Bonnetianus versus Solieri-C	Parent 1 évènement d'hybridation récent *versus* Parent 2 évènement d'hybridation récent	

Les résultats majeurs de cette étude peuvent être résumés comme suit:

- même si un seul caractère sur les quatre étudiés présente des variations de niveau d'asymétrie fluctuante significatives, les résultats significatifs pour ce caractère indiquent tous que les hybrides possèdent des niveaux d'instabilité de développement supérieurs à ceux des parents. L'effet lié à la rupture de coadaptation génomique semble par conséquent supérieur à celui de l'hétérozygotie.

- dans le cas de l'évènement d'hybridation le plus ancien ce résultat est plus surprenant. Il a été en effet proposé que dans les situations où les zones d'hybridations étaient suffisamment anciennes on pouvait s'attendre à une "re-évolution" de la coadaptation génomique dans les populations hybrides et donc à une absence de différences de niveau d'asymétrie fluctuante avec les entités parentales (Graham & Felley 1985; Graham 1992) . Ce n'est visiblement pas le cas ici même si il est difficile de dater précisément l'évènement d'hybridation

- comme attendu les caractères vestigiaux présentent des niveaux d'instabilité de développement bien supérieurs à ceux des caractères fonctionnels. Les niveaux d'asymétrie fluctuante des caractères alaires sont au minimum 3 fois supérieurs à ceux des pattes (figure 6).

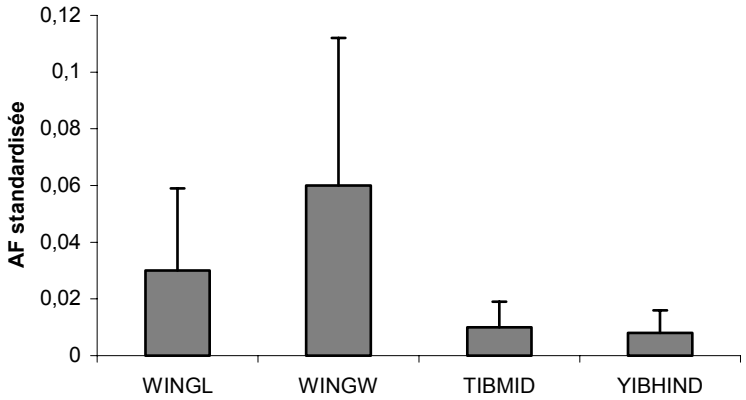


Figure 6 : Niveaux d'asymétrie fluctuante (niveaux standardisés par la taille du caractère ± écart type) pour les quatre caractères étudiés

Conformément aux prédictions les niveaux d'asymétrie des caractères vestigiaux sont supérieurs à ceux des caractères fonctionnels

As predicted asymmetry levels of vestigial traits are higher than those of functionnal traits

Ces résultats sont intéressants à deux titres au moins. Pour ce qui concerne la dynamique de l'hybridation de *C. solieri* il s'agit ici d'une confirmation de la définition des groupes effectuée à l'issue des études de génétique et de morphométrie (cf chapitre précédent) : les niveaux d'instabilité de développement sont homogènes au sein des groupes définis et les différences entre groupes sont cohérentes par rapport au scénario phylogéographique proposé (les groupes présentant les niveaux d'asymétrie fluctuante les plus élevés sont, conformément à certaines prédictions, les groupes hybrides). Pour ce qui concerne l'étude de la stabilité de développement, les différences de niveau d'instabilité de développement entre ailes et pattes confirment tout l'intérêt que peut avoir l'examen des caractères vestigiaux. Notre étude est à notre connaissance seulement la seconde ayant porté sur l'analyse comparée des niveaux d'asymétrie entre caractères fonctionnels et non fonctionnels (l'autre étude étant celle de Crespi & Vanderkist (1997) sur les Thrips *Oncothrips tepperi*). Dans les deux cas les caractères vestigiaux ont très clairement révélé des niveaux d'instabilité de développement bien supérieurs à ceux des caractères fonctionnels. Ces résultats fourniraient une illustration de la diminution de la pression de sélection s'exerçant sur les caractères vestigiaux permettant une baisse des contraintes liées au contrôle de leur développement. Dans le contexte de l'hybridation cette propriété est particulièrement intéressante puisque la diminution des pressions de sélection de ces caractères particuliers a pour conséquence une plus grande accumulation de variation liée aux mutations et à la dérive génétique (Fong *et al.* 1995). Ainsi, les systèmes de gènes codant pour ces caractères et/ou contrôlant leur développement devrait diverger plus rapidement en allopatrie et fournir de meilleurs marqueur de divergence et de dysgénèse hybride (Garnier *et al.* 2006). Il pourraient en effet être particulièrement adaptés dans le cas de faible divergence ou d'évènement d'hybridation plus ancien.

PARTIE 3

Eléments pour l'étude des mécanismes de contrôle de la stabilité de développement

Des bases mécanistiques qui restent à établir

Mechanistic bases which remain to be established

Les débats autour de la généralisation des différentes hypothèses liées à la signification et à l'utilisation de l'asymétrie fluctuante sont nombreux et vigoureux. Comme précisé plus haut les raisons des controverses sont nombreuses (cf introduction de la partie 2) mais il est évident qu'un élément favorisant est lié au fait que les bases mécanistiques de l'homéostase de développement demeurent encore très peu connues (Klingenberg 2003b; West-Eberhard 2003). L'image associée à ce contrôle qui est certainement la plus connue est celle de la métaphore du paysage épigénétique proposée par Waddington (1940 *in* Polak 2003, figure 7). Beaucoup de définitions et de concepts actuels ont incontestablement une filiation directe avec les idées développées dans le cadre de ce modèle.

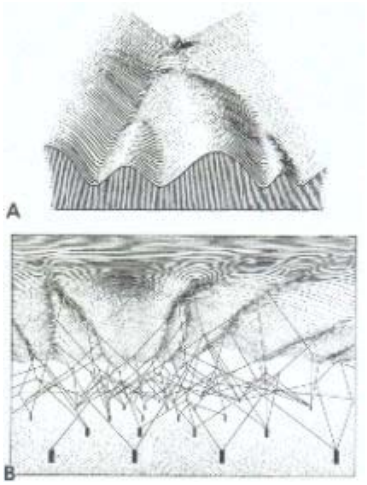


Figure 7 : Le paysage épigénétique est représenté par un paysage composé de vallées et de collines (dessin supérieur A) et dont la topographie est façonnée par l'action des gènes (dessin inférieur B). La profondeur des vallées représente les propriétés de canalisation et une balle roulant au fond d'une de ces vallées symbolise une trajectoire développementale menant à un phénotype prédéterminé. La balle résistera d'autant mieux aux pertubations (=restera dans la vallée malgré des écarts de trajectoire) que la vallée est profonde (et donc en termes développementaux, que le développement est canalisé). (D'après Waddington 1940)

Une grande confusion règne cependant dans la littérature de biologie évolutive car les mêmes termes (et celui de canalisation ne fait pas exception) sont parfois employés pour décrire à la fois des patrons et des processus ou ne font pas de distinction entre sources de variation (génétiques ou environnementales) ou entre leur nature (distinction entre conditions spécifiques ou gamme de conditions). Il semble donc nécessaire, avant toute considération sur les mécanismes de contrôle de la variation phénotypique et l'utilisation de termes très spécifiques, de les définir clairement. C'est l'objet de l'encadré 1.

Encadré 1: Des définitions et des concepts

(essentiellement d'après les synthèses de Palmer (1994) et Nijhout & Davidowitz ((2003)

Phénotype cible (ou *phénotype prédéterminé*) : phénotype qui serait spécifié par une composition génétique et des conditions environnementales données **en l'absence** complète de variation de ces facteurs et de "bruit de fond" développemental de quelque nature que ce soit.

Homéostase : terme général qui décrit les propriétés permettant à un organisme de s'ajuster à des conditions variables. La grande confusion qui règne autour de ce terme est en partie liée au fait qu'il n'est généralement pas précisé si la variation phénotypique est mesurée *au sein* d'un environnement particulier ou *entre* différents environnements (il faut entendre ici par "environnement", conditions génétiques et/ou environnementales). Des termes spécifiques décrivent ces deux situations: *homéorhésis* dans le premier cas et *canalisation* dans le second.

Homéorhésis (= *homéostase de développement*) : terme décrivant le **développement stabilisé** le long d'une trajectoire développementale aboutissant à un phénotype cible, **au sein d'un environnement donné**. Il s'agit donc ici plus de la description d'un patron que d'un processus.

Stabilité de développement : résultat des processus qui **résistent aux perturbations** affectant les trajectoires développementales (ou qui les tamponnent), au sein d'un environnement donné. La stabilité de développement serait la fonction responsable de l'*homéorhèsis*.

Instabilité de développement (= *"bruit de fond" développemental*) : résultat des processus qui **perturbent** le développement le long d'une trajectoire développementale, au sein d'un environnement donné. L'instabilité de développement fait donc référence à un ensemble d'évènements **indépendants**, **aléatoires** qui peuvent perturber la trajectoire normale du développement et mener à des variations stochastiques dans la croissance. Instabilité de développement et stabilité de développement influencent toutes les deux les niveaux d'asymétrie fluctuante mais c'est, dans l'état actuel des choses, l'instabilité de développement qui est mesuré par l'asymétrie fluctuante.

Asymétrie fluctuante : quantité, mesurée à l'échelle populationnelle et qui correspond aux déviations aléatoires entre côtés droit et gauche d'un caractère bilatéral normalement symétrique. Elle constitue la mesure presque systématiquement utilisée pour quantifier l'*instabilité de développement* car elle permet une appréciation simple de la déviation entre *phénotype cible* et phénotype réalisé. L'hypothèse sous jacente étant que les deux côtés d'un caractère homologue sont codés par les mêmes gènes et se sont développés dans le même environnement, le *phénotype cible* correspond donc au phénotype parfaitement symétrique.

Canalisation : processus par lequel une structure ou un organisme se développe "en direction" d'un *phénotype cible* **sous différentes conditions** génétiques et environnementales. Dans ce contexte il est nécessaire de distinguer entre *canalisation génétique* et *canalisation environnementale*.

Canalisation génétique : processus qui réduit la sensibilité d'une structure ou d'un organisme aux **variations alléliques** (recombinaisons, mutations, épistasie). Cette réduction est liée à la modification de l'amplitude des effets alléliques (sous l'hypothèse que le nombre et la nature des locus impliqués demeure constant).

Canalisation environnementale : processus qui réduit la sensibilité d'une structure ou d'un organisme aux **variations environnementales** (température, nutriments…). La *canalisation environnementale*, à la différence de la *stabilité de développement*, se produit sous un ensemble de conditions environnementales différentes.

Plasticité phénotypique : terme général utilisé pour décrire la variation phénotypique produite par **un génotype** en réponse aux variations environnementales. En terme de phénotype *plasticité phénotypique* et *canalisation environnementale* décrivent les opposés d'un même phénomène même si il est impossible de dire si les processus impliqués sont identiques ou différents.

Norme de réaction : type de plasticité phénotypique utilisé pour décrire l'ensemble des phénotypes produits par un génotype en réponse à différentes conditions environnementales.

1- Variation phénotypique et instabilité de développement

Les sources de variation phénotypique

Pour résumer, on distingue deux sortes de variation phénotypique (figure 8). La première correspond à la variation systématique du phénotype cible en réponse aux variations des conditions génétiques (="sensibilité" aux variations alléliques) et environnementales (=norme de réaction). La deuxième correspond à la variation autour du phénotype cible et représente l'incapacité de l'organisme à réaliser parfaitement ce phénotype. Ainsi, l'évolution de la variation phénotypique peut avoir deux origines différentes: la modification de la sensibilité du phénotype cible aux conditions environnementales ou génétiques (la courbe des phénotypes cibles s'aplatit sous l'effet de la canalisation environnementale ou génétique ; figure 8A) ou la réduction de la dispersion des points autour de la courbe des phénotypes cibles (=réduction des effets des perturbations du développement = meilleure stabilité de développement ; partie gauche figure 8A et mécanismes détaillés en figure 8B).

Les causes de l'instabilité de développement

Trois causes possibles à l'instabilité de développement

Three possible causes for the instability of development

Nijhout & Davidowitz (2003) recensent trois causes distinctes pouvant être responsables de la dispersion des points autour du phénotype cible ou, autrement dit, de l'instabilité de développement. La première est directement liée à notre méconnaissance de la totalité des variables susceptibles d'avoir un effet sur le phénotype. L'absence de prise en considération de l'effet de certains facteurs lors de l'estimation du phénotype cible entraînerait donc une définition approximative de ce dernier et donc nécessairement une interprétation erronée d'une partie des déviations entre phénotype cible et phénotype effectivement réalisé. La deuxième serait spécifiquement liée aux différences microenvironnementales auxquelles peuvent être soumis les organismes au cours de leur développement, et pouvant être à l'origine de variations (par définition non aléatoires) entre côtés droits et gauche. Cette source de variation aura ici encore pour effet d'augmenter artificiellement les niveaux d'asymétrie fluctuante. Enfin, la troisième correspond aux résultats de l'effet des perturbations stochastiques intervenant durant le développement du phénotype. Elle est considérée comme la cause principale d'instabilité de développement et d'asymétrie fluctuante. C'est précisément cette part de l'instabilité de développement que les chercheurs tentent généralement de quantifier. Les questions reliées aux origines de ces variations stochastiques restent cependant largement irrésolues. En général des variations dans les gradients morphogénétiques (qui seraient à l'origine de différences d'interprétation du signal par les cellules cibles) ou dans les processus de régulation de l'expression génique (par exemple des petites variations stochastiques, sans effet à de fortes concentrations, pourraient entraîner un comportement plus chaotique des mécanismes d'expression génique) sont avancés comme des processus probables (Palmer, 1994, Nijhout & Davidowitz, 2003, Klingenberg, 2003b). La pertinence de ces facteurs comme causes de l'instabilité de développement et les possibles mécanismes de son contrôle tout au long du développement ont été testé par le biais de différents modèles (certains s'appuyant sur des données empiriques). L'objet n'est pas ici de présenter l'ensemble de ces modèles, pour

des synthèses récentes le lecteur pourra par exemple se reporter aux articles de Klingenberg (2003b), de Kellner & Alford (2003) ou de Graham et al. (2003).

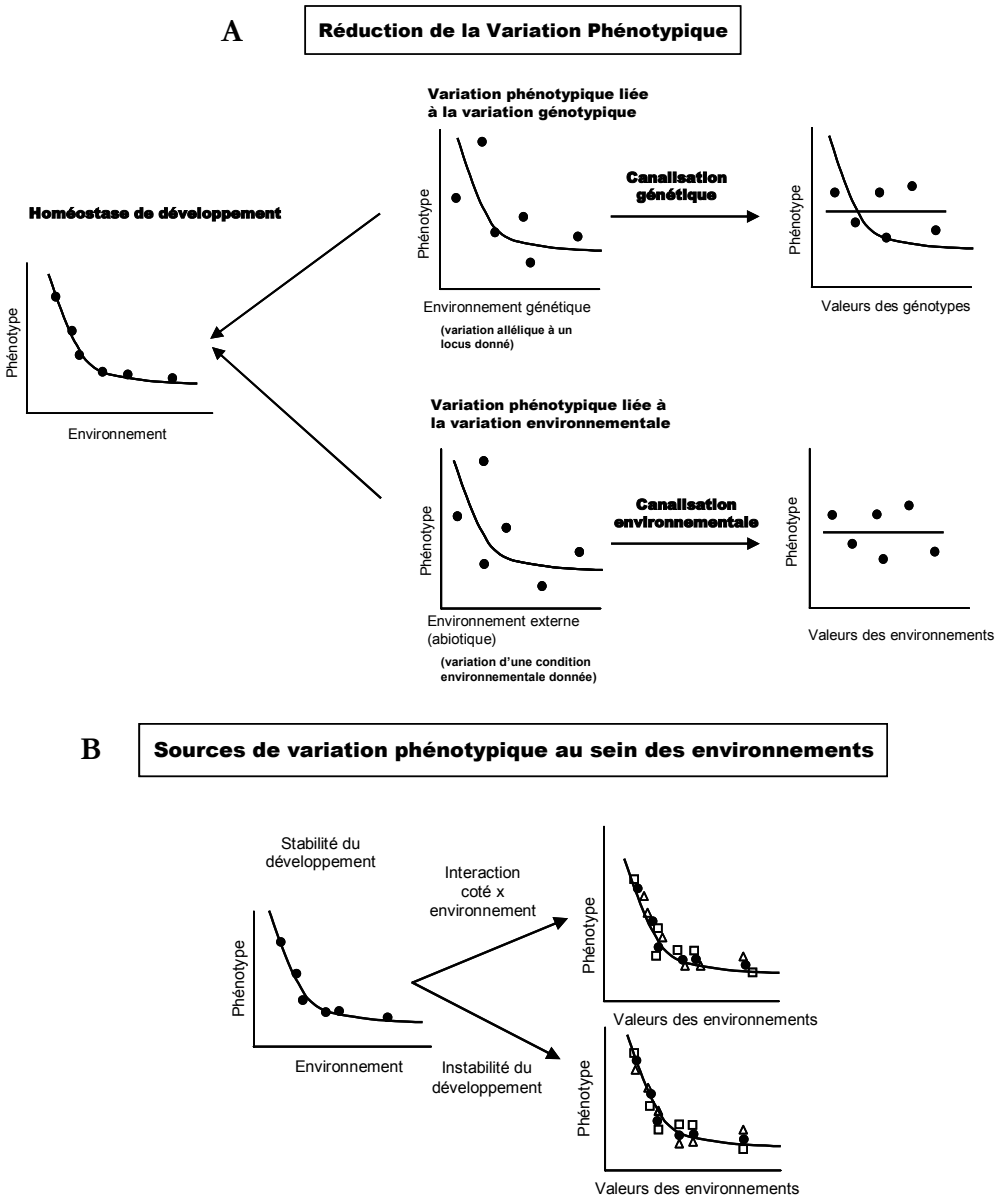


Figure 8 : Sources de variation phénotypique et mécanismes de réduction en réponse aux variations des conditions génétiques et environnementales. La ligne pleine représente le phénotype cible et les points la variation individuelle autour de ce phénotype. La canalisation génétique et environnementale réduisent la variation du phénotype cible *entre* les différents environnements, variation non affectée par la réduction de la variation autour du phénotype cible (homéostase du développement) qui intervient *au sein* d'un environnement donné. Les sources de variation *au sein* d'un environnement donné sont schématisées sur la figure B : les carrés représentent les phénotypes pour les côtés gauches et les triangles les phénotypes des côtés droits (un seul individu est représenté par type d'environnement). Dans une situation de stabilité de développement (partie gauche) la variation autour du phénotype cible est faible. Sous une situation d'instabilité de développement (partie droite en haut) les deux côtés de l'organisme, soumis au même environnement, produisent différents phénotypes. Des conditions micro-environnementales (partie droite en bas) peuvent également être responsables de variation phénotypique entre les deux côtés (voir texte). D'après Nijhout & Davidowitz (2003), modifié.

Qu'il s'agisse de l'étude de l'impact de stress environnementaux (travaux sur les oursins notamment), de sélection directionnelle ou de mutation (travaux sur les drosophiles) nous verrons que les travaux de recherche présentés ci-après trouvent tous une place dans les questionnements centrés sur l'origine et le contrôle de la variation phénotypique et notamment de l'instabilité de développement.

Le rôle des stress environnementaux

Des stress environnementaux pour "maximiser" les niveaux d'instabilité de développement

Environmental stresses to increase the levels of developmental instability

La sensibilité des organismes aux conditions environnementales constituait pour nous une propriété essentielle dans la mesure où toute étude orientée sur les mécanismes de contrôle de la stabilité de développement nécessite des situations contrastées responsables de la plus grande variation possible dans les niveaux d'asymétrie fluctuante. Ainsi en considérant des individus issus de milieux stressants (par exemple pollués comme dans le cas des oursins, cf ci-dessous) et d'autres issus de milieux *a priori* dépourvus d'agents stressant, l'on se place dans les conditions d'étude recherchées. Les travaux résumés ci-dessous se basent tous sur la comparaison d'individus ou de populations présentant des niveaux d'instabilité de développement contrastés dans l'objectif ultime, à l'exception de celui réalisé sur les gammares[5], d'étudier différents facteurs liés au contrôle de la stabilité de développement.

2- Un exemple de relation asymétrie fluctuante-stress environnemental : le parasitisme chez *Gammarus pulex*

Résultats publiés sous la référence:
Alibert, P., L. Bollache, D. Corberant, V. Guesdon & F. Cézilly (2002). Parasitic infection and developmental stability: fluctuating asymmetry in *Gammarus pulex* infected with two acanthocephalan species. *Journal of Parasitology* 88(1): 47-54. *

Une étude de l'impact de parasites sur leurs hôtes intermédiaires.

A study of the impact of parasites on their intermediate hosts.

Notre travail s'intégrait dans l'un des axes majeurs de l'équipe d'écologie évolutive, axe qui avait pour objectif général d'étudier la variation dans le degré d'homogamie pour la taille en fonction de variables externes (qualité de l'eau, température, teneur en calcium) et de variables internes (qualité des individus) chez le crustacés amphipode *Gammarus pulex* (travaux notamment de L. Bollache et F. Cézilly). Une partie de ce programme se concentrait sur l'impact de la charge parasitaire sur la qualité générale des individus. Ma contribution était de réaliser une approche comparative des niveaux d'asymétrie fluctuante des individus en fonction de leur niveau d'infestation par deux acanthocéphales parasites, *Pomphorhynchus laevis* et *Polymorphus minutus*. Dans un travail de synthèse Møller (Møller 1996) a mis en avant que la prévalence tout comme l'intensité des infections parasitaires étaient, dans la plupart des cas, associées à des diminutions significatives des niveaux d'instabilité de développement (Hoffmann *et al.* 1998; Quek *et al.* 1999). Cependant ces études concernent un nombre limité de taxons de parasites et la stabilité de développement avait été estimée uniquement sur des hôtes

[5] Ce travail avait plus pour objectif de répondre à une question précise sur l'impact potentiel du parasitisme que celui de l'étude des mécanismes plus fins de contrôle de la stabilité de développement.

définitifs. L'originalité de notre travail était (1) que la relation entre asymétrie-parasitisme été étudiée simultanément sur deux espèces de parasites proches mais soumises à des contraintes écologiques différentes et (2) pour la première fois cette relation été étudiée sur des hôtes intermédiaires et non des hôtes définitifs (ces derniers étant les oiseaux pour *P. minutus* et les poissons pour *P. laevis*). Nous savions par ailleurs que l'impact de ces parasites sur les gammares était réel puisqu'il a notamment été montré une baisse de la fertilité chez les gammares infestés par ces deux espèces de parasites (Ward 1986; Poulton & Thompson 1987). De plus, les deux parasites entraînent des modifications comportementales (géotactisme par *P. minutus*, phototactisme par *P. laevis*) chez les gammares qui ont pour conséquence d'augmenter leurs chances d'être prédaté par les hôtes définitifs des parasites (Cézilly *et al.* 2000).

Différents paramètres ont été pris en considération dans notre étude: présence ou absence de parasite, nature du parasite (*P. laevis* ou *P. minutus*), charge parasitaire, présence simultanée ou non des deux espèces de parasites. Les niveaux d'asymétrie fluctuante ont été estimés à partir de six caractères morphologiques bilatéraux (sur les antennes et sur les pattes)

Les gammares parasités plus asymétriques que les gammares sains

The infected gammarids more asymmetrical than uninfected gammarids

Nous avons trouvé une association positive entre les niveaux d'asymétrie fluctuante et les niveaux d'infection parasitaire, les gammares parasités étant plus asymétriques que les gammares sains (Alibert *et al.* 2002). Cette association concerne un index combinant deux caractères (les deux caractères méristiques) sur les six étudiés. Aucun effet de la charge parasitaire n'a été trouvé mais la présence simultanée des deux espèces de parasites semble associée avec des niveaux d'asymétrie fluctuante plus élevés des hôtes (même si cette tendance n'est pas statistiquement significative). Enfin, les mâles apparaissent, à niveau d'infection parasitaire égal, plus asymétriques que les femelles. Une première conclusion de cette étude est que les parasites considérés pourraient avoir un effet direct sur l'instabilité de développement de leur hôte. Le fait que les gammares présentant simultanément les deux espèces de parasites puissent présenter des niveaux d'asymétrie fluctuante supérieurs fournirait un argument dans ce sens. Une plus grande gamme d'individus parasités par un nombre de parasites différents ou, mieux encore, des infestations expérimentales seraient à ce stade nécessaires pour confirmer cette affirmation. Un deuxième enseignement que l'on peut tirer de ces résultats est que l'histoire sélective des caractères morphologiques pourrait expliquer les différences de réponse en terme d'asymétrie fluctuante que nous avons observées entre les différents caractères étudiés. Trois caractères sur les quatre qui n'ont donné aucun résultat significatif sont des caractères concernant les péréopodes et sont donc impliqués dans la locomotion. Il est par conséquent possible qu'ils soient soumis à de plus fortes pressions de sélection stabilisatrice que les deux caractères méristiques (nombre de soies internes sur le basipodite du péréiopode 5 et nombre de segments du flagellum de la seconde paire d'antennes) qui ont livré des résultats significatifs. Ce type de résultat rejoindrait en tous points ceux obtenus chez le carabe *C. solieri* lors de la comparaison des niveaux d'asymétrie fluctuante de caractères fonctionnels et vestigiaux.

Une relation avec la fonctionnalité des caractères?

A relationship with the functionnality of traits?

3- Asymétrie fluctuante chez les oursins : un autre éclairage sur l'instabilité de développement

Un cas de symétrie bilatérale : Pollution et instabilité de développement chez *Echinocardium flavescens* dans la baie d'Oslo

Résultats publiés sous la référence:
Saucède, T., P. Alibert, B. Laurin & B. David (2006) Environmental and ontogenetic constraints on developmental stability in the spatangoid sea urchin *Echinocardium* (Echinoidea). *Biological Journal of the Linnean Society* 88: 165-177. *

Les spatangues, oursins irréguliers fouisseurs, sont des organismes détritivores endobenthiques particulièrement sensibles aux variations d'environnement et en particulier à la nature du sédiment : granulométrie, qualité de la matière organique, degré de pollution... (voir Saucède *et al.* 2006*). En considérant des populations issues de milieux présentant des conditions environnementales contrastées, nous nous placions dans des situations potentiellement responsables de niveaux d'instabilité de développement variables. Comme précisé plus haut cette situation est nécessaire à l'étude de facteurs impliqués dans les mécanismes de contrôle de la stabilité de développement.

Une analyse de l'asymétrie fluctuante de forme

A study of shape fluctuating asymmetry

Un premier travail a été initié dans le cadre du DEA de Thomas Saucède (1997-1998) et finalisé récemment (Saucède *et al.* 2006). L'objectif général était double: (1) appliquer pour la première fois les méthodes de morphométrie géométrique pour l'analyse de l'asymétrie fluctuante chez les oursins et (2) voir si les variations de niveau d'asymétrie fluctuante pouvaient être reliées au processus de croissance des oursins, et plus précisément voir si il existait un changement graduel des niveaux d'asymétrie le long de la zone ambulacraire.

Deux populations d'*Echinocardium flavescens* ont été récoltées sur deux sites des côtes scandinaves de Norvège, caractérisés par des environnements contrastés. Le premier site (Bodø), situé près d'une petite ville au nord du cercle polaire arctique, constituait l'échantillon témoin car potentiellement très peu soumis à de quelconques pressions anthropiques. Le deuxième site, (Drøbak) est situé dans le fjord d'Oslo et correspond à l'échantillon issu d'un milieu potentiellement stressant en raison des activités humaines importantes régnant tout autour du fjord (et en particulier à Oslo). Différents descripteurs morphologiques ont été considérés: surface des plaques, distances entre points de repères, taille et forme des deux ambulacres postérieurs (ambulacres I et V, cf figure 1 dans Saucède *et al.* 2006). Conformément aux attentes, c'est la population de Bodø qui présente les niveaux les plus faibles d'asymétrie fluctuante pour les caractères de taille (surface de plaque, distances et tailles centroïdes). Cependant, et de façon assez surprenante, cette même population apparaît plus asymétrique que celle de Drøbak si l'on s'intéresse à l'asymétrie fluctuante de forme. Deux hypothèses ont été émises pour expliquer ce résultat: (1) il n'existe pas de corrélations entre asymétrie de taille et de forme quel que soit le mécanisme expliquant la plus grande asymétrie de forme dans le milieu le moins pollué (mécanisme qu'il resterait à expliquer) ou (2) l'asymétrie de forme n'est, chez cette espèce au moins, pas dépendante des conditions environnementales mais pourrait par exemple résulter de différences de conditions génétiques des individus issus des populations

Des résultats contrastés entre asmétrie de taille et asymétrie de forme

Contrasted results between size asymmetry and shape asymmetry

comparées. Cependant tout cela reste très hypothétique et nous ne disposons à ce stade d'aucun argument permettant d'appuyer l'une ou l'autre de ces hypothèses.

Une étude comparée des variations inter-inviduelles et des niveaux d'asymétrie a également été entreprise. Une question récurrente est en effet celle de l'existence ou non d'une corrélation entre variation inter-individuelle et asymétrie fluctuante, la première approche permettant de quantifier la canalisation, la seconde de quantifier la stabilité de développement (Klingenberg & McIntyre 1998; Debat & David 2001). Cette question est importante car une corrélation significative entre asymétrie fluctuante et variabilité inter-individuelle pourrait signifier que des processus identiques participent à l'expression de ces deux composantes de la variation phénotypique (Clarke 1998b; Klingenberg 2003a). Les résultats des études qui se sont penchées sur cette question sont parfois contradictoires, des corrélations positives ont été trouvées chez des insectes (Klingenberg & McIntyre 1998; Klingenberg *et al.* 2001) et chez la souris domestique (Leamy 1993) mais une absence de corrélation a également été reportée chez la souris domestique (Debat *et al.* 2000).

Dans notre étude, variation inter-individuelle et asymétrie fluctuante ont été estimées au sein de chaque échantillons ainsi qu'entre échantillons (voir encadré 2 pour un exposé du principe de l'approche). Pour les deux types d'analyses les corrélations sont apparues significatives. Ce résultat pourrait fournir un exemple supplémentaire venant supporter l'hypothèse de mécanismes développementaux partagés entre stabilité de développement et canalisation. Cependant corrélation n'est pas causalité et un patron commun de variabilité peut également être lié à des causes extrinsèques. Ici en effet les variations intra-individuelles (instabilité de développement) tout comme les variations inter-individuelles (canalisation) sont orientées selon l'axe de croissance des structures étudiées (les ambulacres postérieurs) et leur intensité semble dépendre de la vitesse de croissance des plaques. Ces deux éléments pourraient ainsi attester de contraintes architecturales exprimées au cours du développement de l'oursin et qui seraient responsables des corrélations relevées.

Encadré 2: Principe de la quantification de l'asymétrie fluctuante et de la variabilité inter-individuelle de forme

Pour la forme, l'asymétrie fluctuante comme la variabilité inter-individuelle ont été estimées, pour chaque échantillon, à partir des ANOVA (individu × côté) réalisées sur chacun des 24 résidus (correspondant aux coordonnées x, y, des 12 points de repère) issus de l'analyse Procrustes généralisée. Les valeurs des sommes des carrés, sommées et ajustées, des sources de variation "individus" et "interaction individu × coté" correspondent, respectivement, aux indices de variabilité inter-individuelle et d'asymétrie fluctuante. Les valeurs de corrélation entre les deux sont obtenues après obtention des matrices de variance-covariance issues d'une MANOVA à 2 facteurs (individu × côté) réalisée sur les 24 résidus. Des tests de corrélation de matrice sont réalisés, entre les différentes sources de variation, au sein et entre les échantillons et leurs valeurs sont testées par l'intermédiaire de tests de permutations (adaptés à ce type de données puisque conservant associées les paires de coordonnées x, y,). Cette méthode générale d'analyse a été mise au point par Klingenberg & McIntyre (1998).

Un cas de symétrie pentaradiée : Pollution et instabilité de développement chez *Paracentrotus lividus* et *Arbacia lixula* dans la baie de Marseille

Une étude de l'asymétrie pentaradiée

A study of pentaradial asymmetry

A l'instar du travail précédent il s'agissait de comparer des niveaux d'aymétrie fluctuante entre populations d'oursins issues de milieux contrastés en termes de pollution. Ce travail, réalisé dans le cadre du travail de recherche de M2 de Yoland Savriama (2004-2005), a porté sur deux espèces d'oursins réguliers. Les objectifs étaient ici (1) d'établir une nouvelle méthode permettant d'estimer les niveaux d'asymétrie fluctuante chez des organismes présentant une symétrie pentaradiée, et (2) de déterminer si les niveaux d'asymétrie fluctuante de *Paracentrotus lividus* et *Arbacia lixula* augmentaient sous l'effet de la pollution chimique et organique. Pour chaque espèce quatre populations (N=50 dans la majorité des cas) ont été considérées et différents caractères morphologiques ont été mesurés en fonction des espèces (tableau V).

Tableau V: Caractères morphologiques mesurés pour chacune des deux espèces

	P. lividus	*A. lixula*	Toutes les populations	Deux populations seulement
Longueur des ambulacres	×	×	×	
Nombre de tubercules par ambulacre	×		×	
Nombre de doublets de pores par ambulacre		×	×	
Hauteur de la lanterne d'Aristote	×			×
Largeur de la lanterne d'Aristote	×			×

L'analyse statistique des données a été réalisée à partir de la méthode proposée par Van Dongen *et al.* (1999) basée sur un modèle mixte de régression utilisant le maximum de vraisemblance restrictif (REML) comme paramètre d'estimation. Sans rentrer dans les détails, car ce n'est pas le propos ici, retenons simplement que ce modèle permet de tester la signification de l'asymétrie fluctuante par rapport à l'erreur de mesure mais aussi de tester au sein du même modèle l'hétérogénéité de l'erreur de mesure, de tester et de corriger par rapport à la présence d'asymétrie directionnelle, d'antisymétrie et/ou de dépendance par rapport à la taille du caractère. Il était également possible d'étendre ce modèle à l'étude de symétrie d'ordre n (cette extension a été réalisée par Leif Stige sous R).

Une asymétrie directionnelle généralisée et des différences de niveau d'asymétrie fluctuante pas toujours conformes aux prédictions

A generalised directional asymmetry and differences of fluctuating asymmetry levels not always conform to predictions

La présence d'asymétrie directionnelle a été détectée pour les deux espèces et tous les caractères étudiés. Ce résultat est conforme avec ce que nous avions trouvé chez l'oursin irrégulier *Abatus cordatus* (cf paragraphe suivant). Lorsque les échantillons issus de milieux présumés pollués (présomptions basées sur des indices de contamination du sédiment en différents points de la baie de Marseille, voir Perez *et al.* (2005)) sont comparés à ceux de milieux présumés sains seul le caractère "doublet de pores" (chez *A. lixula*) présente des niveaux d'aymétrie fluctuante significativement hétérogènes (supérieur dans la zone polluée). En revanche, si les sites sont regroupés, non pas par niveau de pollution supposé, mais par secteur géographique de provenance, les échantillons issus de l'Est de la baie de Marseille présentent des niveaux d'asymétrie fluctuante plus élevés pour trois des quatre comparaisons effectuées. Le secteur Est correspond au secteur de relarguage des égouts de Marseille et il est donc possible que les oursins étudiés soient sensibles à des paramètres autres que ceux ayant servi

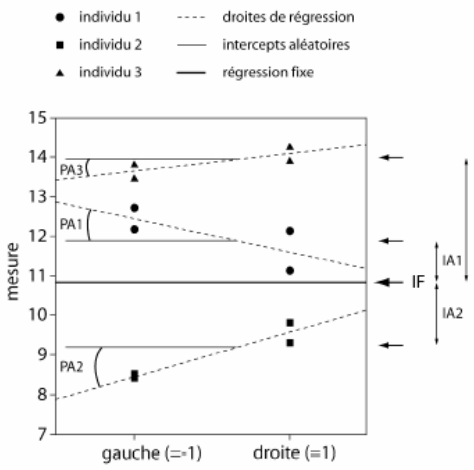
pour définir les zones comme polluées ou non polluées. D'un point de vue méthodologique ce travail a permis une avancée intéressante, celle de la mise au point d'un méthode d'analyse de symétrie d'ordre n.

Encadré 3: Principe du modèle mixte de régression utilisant le maximum de vraisemblance restrictif comme paramètre d'estimation (REML) et extension à des symétries d'ordre n (d'après Van Dogen et al. (1999) et Savriama (2005))

Modèle pour symétrie bilatérale:

Modèle complet : $Y_{ijk} = \mu + \beta + bl_i + b2_{ij} + E_{ijk}$
Modèle réduit : $Y_{ijk} = \mu + \beta + bl_i + E_{ijk}$

(les lettres grecques indiquent les effets fixes, les lettres latines les effets aléatoires)
Avec Y_{ijk} = observation d'un individu *i* pour le côté *j* (*i* =1,… ,n ; *j* = -1, 1), répétition *k* (*k* =1,… ,r), μ = intercept fixe, β = effet côté fixe (asymétrie directionnelle), bl_i = intercept aléatoire indépendant et normalement distribué ~ (ind) N (0, σ1), $b2_{ij}$ = effet côté aléatoire (AF) ~ (ind) N (0, σ 2), E_{ijk} = erreur de mesure aléatoire ~ (ind) N (0, σ 3). Dans ce modèle, les auteurs attribuent les valeurs de – 1 et 1 respectivement aux côtés gauche et droit. La significativité de la FA est testée *via* le ratio entre les REML des deux modèles.



Représentation graphique du modèle mixte proposé pour modéliser l'asymétrie fluctuante pour un jeu de données arbitraire de trois individus et de deux répétitions de mesures pour chacun des deux côtés. La ligne épaisse représente la régression fixe. Les initiales IF indiquent l'intercept fixe. La pente égale à zéro indique l'absence d'asymétrie directionnelle (AD). Les lignes de régression en pointillés représentent les effets aléatoires que sont les intercepts et les pentes, respectivement indiqués par les IA (Intercept Aléatoire) et les PA (Pentes Aléatoires). Les PA expriment le niveau d'asymétrie fluctuante individuel. Ici l'individu 2 est celui qui est le plus asymétrique (d'après Van Dongen *et al.* 1999 modifié).

Modèle pour symétrie d'ordre n:

Modèle complet : $Y_{ijk} = \mu + \beta_j + bl_i + b2_{ij} + E_{ijk}$
Modèle réduit : $Y_{ijk} = \mu + \beta_j + bl_i + E_{ijk}$

(les lettres grecques indiquent les effets fixes, les lettres latines les effets aléatoires)
Avec Y_{ijk} = observation d'un individu *i* pour le côté *j* (*i* =1,… ,n ; *j* = -1, 1), répétition *k* (*k* =1,… ,r), μ = intercept fixe (moyenne générale), β_j = effet module fixe, bl_i = intercept aléatoire (effet individu) indépendant et normalement distribué ~ (ind) N (0, σ1), $b2_{ij}$ = effet module aléatoire (AF) ~ (ind) N (0, σ 2), E_{ijk} = erreur de mesure aléatoire ~ (ind) N (0, σ 3).
L'effet module fixe représente la différence entre chaque module d'un individu (moyenne des répétitions pour un module) et la moyenne générale. Ce paramètre modélise donc l'asymétrie directionnelle pour un module.
L'effet module aléatoire est calculé à partir de la différence entre chaque module d'un individu (moyenne des répétitions pour un module) et la moyenne de l'individu (moyenne des répétitions pour tous les modules). Ce paramètre modélise l'asymétrie fluctuante pour un module.

4- De l'asymétrie directionnelle... fluctuante

Résultats publiés sous la référence:
Stige, L. C., David, B. & P. Alibert (2006) On hidden heterogeneity in directional asymmetry - can systematic bias be avoided? *Journal of Evolutionary Biology* 19: 492-499 *

L'asymétrie directionnelle peut varier entre individus

Directional asymmetry can vary among individuals

L'asymétrie fluctuante n'est pas la seule forme d'asymétrie présente chez les organismes. L'asymétrie directionnelle et l'antisymétrie, correspondent toutes les deux à des déviations systématiques entre les côtés gauche et droit, et sont considérées comme adaptatives (Palmer & Strobeck 1986). Parce que dans ces cas l'asymétrie devient la norme, les différences droite-gauche deviennent des estimateurs biaisés de l'instabilité de développement. Différents types de corrections pour l'asymétrie directionnelle ont alors été proposés, comme par exemple soustraire aux valeurs d'asymétrie individuelle la valeur de la moyenne des valeurs droite – gauche sur l'échantillon (*asymétrie directionnelle moyenne*) ou corriger *via* les ANOVA ou les régressions par le biais du facteur fixe "côté" (Graham et al. 1998, Van Dongen et al., 1999, Palmer & Strobeck 2003). De fait ces corrections qui consistent à mesurer l'asymétrie fluctuante autour de la valeur d'asymétrie directionnelle plutôt qu'autour de la valeur 0, fournissent des valeurs d'instabilité de développement non biaisées seulement dans le cas où l'asymétrie directionnelle exprimée par chaque individu (asymétrie directionnelle individuelle ou *underlying DA*[6] dans Stige *et al.* 2006) est la même pour tous les spécimens. Parce que ces variations individuelles viennent se confondre avec les variations liées à l'instabilité de développement il est généralement conseillé d'exclure les caractères présentant une asymétrie directionnelle significative.

Quand la variation est prédictible...

When variation is predictible...

L'originalité de notre travail a été de montrer qu'une partie de la variation d'asymétrie directionnelle individuelle qui peut exister entre les spécimens pouvait être détectée et éliminée. Ces variations peuvent en effet être décomposées en une partie prédictible et une partie aléatoire. La partie prédictible correspond à l'association entre les asymétries droite-gauche observées et un facteur externe donné (sexe, taille, échantillon...). Il s'agit donc de la part de variation systématique de l'asymétrie directionnelle. La partie aléatoire est la part de variation des niveaux d'asymétrie directionnelle individuelle qui ne peut être associée à aucun facteur et qui, comme l'erreur de mesure, peut affecter la qualité des estimateur d'asymétrie fluctuante et donc la puissance des analyses mais qui n'a pas de raison de conduire à des résultats faux.

L'analyse a porté sur plus de 400 spécimens de l'oursin irrégulier *Abatus cordatus* échantillonné dans quatre localités autour des îles Kerguelen. Une étude conjointe de la taille (taille centroïde) et de la forme a été réalisée. L'analyse statistique des valeurs de taille a été effectuée par la méthode REML (cf. paragraphe précédent) et celle des valeurs de forme par la méthode Procrustes adaptée aux analyses d'asymétrie (Klingenberg & McIntyre, 1998 ; voir plus haut l'étude sur *Echinocardium flavescens*). Dans les deux cas les

[6] Cela peut être définit comme l'asymétrie droite-gauche pré-déterminée (ou "cible") pour un génotype donné, dans un environnement donné. Nous utilisons ici le terme d'*asymétrie directionnelle individuelle* par opposition à celui d'*asymétrie directionnelle moyenne* qui se définit à l'échelle populationnelle.

variations systématiques d'asymétrie directionnelle individuelle ont été détectées en rajoutant aux modèles des effets d'interaction fixes entre le facteur "côté" et différents autres facteurs d'intérêt. Par exemple l'interaction entre "côté" et "population" représente les variations populationnelles d'asymétrie directionnelle ou celle entre "côté" et "taille" les changements allométriques d'asymétrie directionnelle (voir tableau I dans Stige et al. 2006* pour un détail des effets testés pour l'analyse de la forme). Les modèles permettent en outre de corriger les effets de ces facteurs sur l'asymétrie directionnelle si ils sont significatifs.

Les résultats indiquent que les niveaux d'asymétrie directionnelle dépendent de la taille des individus et de l'origine géographique (facteur population), pour la taille comme pour la forme. La figure 9 illustre les niveaux d'aymétrie (taille centroïde) pour les quatre populations ainsi que les variations systématiques d'asymétrie directionnelle individuelle.

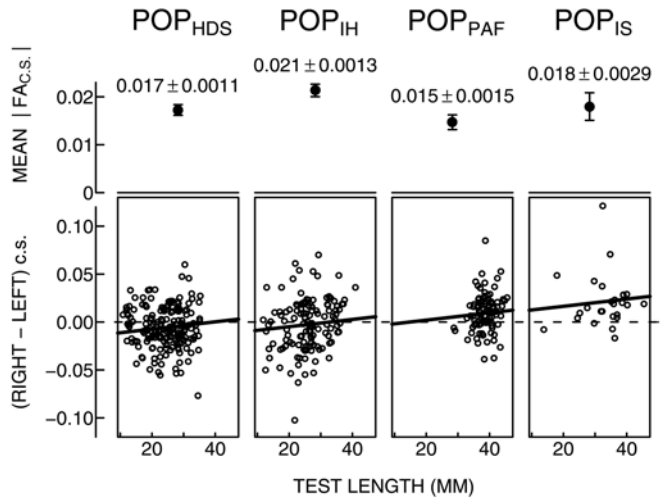


Figure 9 : Asymétrie absolue (mean |FAc.s.|) et relative [(right - left)c.s.] de la taille centroïde (c.s.). La partie inférieure de la figure montre les valeurs individuelles d'asymétrie relative (droite – gauche) en fonction de la longueur du test (test length) pour quatre populations (POP). Les droites représentent les prédictions obtenues par un modèle de régression mixte REML et correspondent aux variations systématiques d'asymétrie directionnelle (changements allométriques d'asymétrie directionnelle). La partie supérieure de la figure indique les valeurs moyennes ± écart type des valeurs d'asymétrie individuelle absolue. Ces dernières correspondent aux valeurs obtenues pour l'effet aléatoire individuel "côté" estimé à partir d'un modèle général de régression. Elles représentent l'asymétrie fluctuante corrigée pour l'asymétrie directionnelle et les erreurs de mesure (d'après Stige *et al.*, 2006).

Parallèlement, pour quantifier l'impact de la part aléatoire de variation d'asymétrie directionnelle individuelle sur la baisse de puissance de l'analyse, un modèle a été construit en estimant la corrélation entre l'asymétrie fluctuante et l'instabilité de développement en réponse à l'hétérogénéité de l'asymétrie directionnelle d'une part et de l'instabilité de développement d'autre part (voir Stige *et al.* 2006 pour une présentation plus détaillée du modèle). Ce modèle montre que la perte de puissance est probablement faible dans la plupart des cas.

5- Relation asymétrie fluctuante-valeur sélective chez la drosophile

Résultats regroupés dans le manuscrit:

Stige , L.C., P.-E. Bourgeon,, F.-X. Flotterer & P. Alibert. The effects of stress conditions on the detection of the relationship between developmental instability and fitness (in prep).

Une relation très difficile à tester

A relationship very difficult to appraise

Une des raisons de l'engouement des chercheurs pour les approches utilisant les propriétés de stabilité de développement des organismes est incontestablement liée à l'utilisation de l'asymétrie fluctuante comme marqueur de leur valeur sélective. Puisque le postulat de départ est que la symétrie parfaite (de caractères normalement symétriques) correspond au phénotype idéal, alors toute asymétrie fluctuante devrait traduire un développement non optimal et pourrait potentiellement être contre sélectionnée. La controverse autour de cette relation asymétrie fluctuante–valeur sélective existe depuis plus d'une dizaine d'année et les études présentant des résultats contradictoires sont nombreuses (Leung & Forbes 1997; Møller 1997; Clarke 1998a; Møller 1999; Simmons *et al.* 1999; Lens *et al.* 2002; Clarke 2003; Tracy *et al.* 2003). Cette hétérogénéité dans les résultats témoigne au moins d'une chose: si toutefois elle existe, la relation entre ces deux paramètres est difficile à tester. Plusieurs raisons à cela peuvent être avancées: (1) il est difficile d'estimer de façon précise les différentes composantes de la valeur sélective (donc d'un point de vue pratique les composantes de l'aptitude phénotypique) ainsi que leurs variations entre individus, (2) les niveaux d'asymétrie fluctuante sont généralement faibles (et d'autant plus si ils sont soumis à la sélection naturelle), (3) les niveaux d'asymétrie fluctuante dépendent de l'histoire évolutive des caractères (cf discussions plus haut) et (4) la relation asymétrie fluctuante -fitness n'a de sens dans le cadre du modèle des bons gènes et de la sélection sexuelle, que si l'asymétrie fluctuante est héritable, et si tel est le cas, la valeur de l'héritabilité sera très faible en raison de l'association avec la valeur sélective.

Accroitre les différences d'asymétrie et de valeur sélective

Increasing the differences in asymmetry and fitness

Dans le cas d'une association significative entre asymétrie fluctuante et valeur sélective on peut faire la prédiction que la plupart des populations naturelles doivent être « à l'équilibre », c'est-à-dire présenter des niveaux d'asymétrie fluctuante faibles car contre-sélectionnés (on peut donc comprendre qu'il soit difficile de mettre en évidence cette relation en raison de ces faibles valeurs et de la faible variabilité inter-individuelle de celles-ci). Une solution possible pour étudier cette relation est de se trouver dans une situation où les variations de niveau d'asymétrie fluctuante et de valeur sélective entre individus sont plus importantes car il devient, statistiquement, plus facile de la mettre en évidence. Dans ce cadre, une approche possible est la réalisation d'une expérience de sélection directionnelle intense sur un caractère déterminé car il a été démontré que ce type de sélection pouvait avoir pour effet d'augmenter l'instabilité de développement (voir Parsons, (Parsons 1990) pour une présentation d'expériences réalisées chez la Drosophile). En partant de ce constat, nous avons, dans le cadre du travail de recherche de M2 de Paul-Eric Bourgeon, testé la relation asymétrie fluctuante/fitness avant et après un tel évènement de sélection. Nous faisions l'hypothèse que si la relation entre les deux facteurs existait, elle devait être d'autant plus détectable que la sélection était intense. Cette étude (inscrite dans un projet plus large proposé par Leif Stige) a été réalisée chez *Drosophila melanogaster* en considérant l'évolution des niveaux d'asymétrie de caractères

morphologiques bilatéraux et leur corrélation avec certaines composantes de l'aptitude phénotypique. Les questions que nous nous posions étaient les suivantes :

- les niveaux d'asymétrie fluctuante augmentent-ils significativement au cours des générations de sélection ? ou autrement dit un évènement de sélection directionnelle entraîne-t-il un stress sur les systèmes de gènes contrôlant la stabilité de développement ?
- si oui, existe-t-il des différences suivant les lignées de sélection ?
- existe-t-il une relation entre les niveaux d'asymétrie fluctuante et les composantes de la valeur sélective étudiées ?
- si cette relation existe, est-elle plus facilement détectable en condition de stress ? ou autrement dit, évolue-t-elle au cours des générations de sélection ?

Deux lignées sélectionnées et une lignée contrôle

Two selected and one control lines

Les lignées de drosophiles ont toutes été constituées à partir d'un stock d'individus sauvages prélevés à Marsannay la Côte en septembre 2004. Le caractère ayant fait l'objet d'une sélection directionnelle est le nombre de soies sternopleurales + transverses (que nous appellerons par la suite soies sternopleurales par commodité). Ce caractère a été choisi car plusieurs travaux avaient déjà montré des niveaux d'asymétrie fluctuante significatifs sur ce caractère (Polak 1997; Indrasamy *et al.* 2000) ainsi qu'une réponse à la sélection directionnelle sur un grand nombre de générations (Barker & Cummins 1969; MacGrath *et al.* 1984; Mackay 1995). A partir du stock sauvage trois lignées ont été constituées: une lignée sélectionnée pour une diminution du nombre de soies (lignée L), une lignée sélectionnée pour une augmentation du nombre de soies (lignée H) et une lignée contrôle (lignée C). A chaque génération trois répliquats par lignée étaient réalisés et au sein de ces répliquats une partie des individus était utilisée pour des tests d'estimation d'aptitude phénotypique (succès d'appariement pour les males, fécondité et taux de survie larvaire pour les femelles) et une autre partie pour constituer les générations suivantes. La sélection a été appliquée sur cinq générations

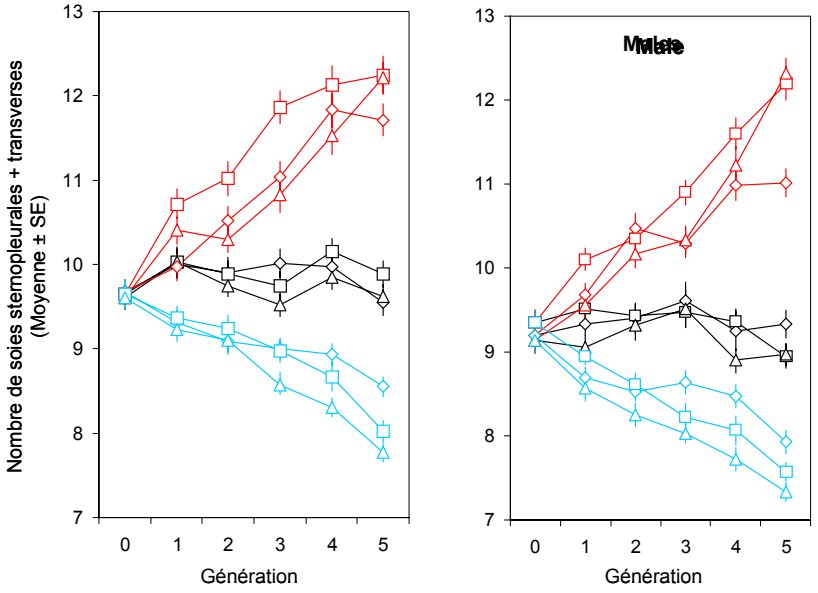


Figure 10 : Evolution du nombre moyen de soies sternopleurales au cours des générations de sélection directionnelle. Pour les deux graphiques, les trois courbes supérieures correspondent aux trois répliquats de la lignée H, les trois courbes inférieures à ceux de la lignée L et les trois centrales à ceux de la lignée C.

successives et trois caractères morphologiques (méristiques) bilatéraux ont été considérés: les soies sternopleurales, les soies frontales et les soies fronto-orbitales. Au total 3468 femelles et 3748 mâles ont été mesurés. La figure 10 montre clairement les effets forts, et conformes aux attentes, de la sélection directionnelle opérée sur les soies sternopleurales.

Une augmentation des niveaux d'asymétrie dans le lignée H

An increase of fluctuating asymmetry levels in the line H

Des tests de Levène sur les valeurs absolues des asymétries révèlent que seuls les niveaux d'asymétrie des soies sternopleurales de la lignée H présentent une différence significative entre les générations ($F_{5,562} = 4,20$; $p = 0,00093$). La figure 11 illustre ces tendances (les niveaux d'asymétrie ne différant pas selon le sexe et n'étant pas corrélés à la taille du caractère, les sexes ne sont pas distingués ici). Une tendance existe dans la lignée L pour ce même caractère mais elle n'est pas statistiquement significative. En revanche les niveaux d'asymétrie des deux autres types de soies ne semblent pas varier au cours des générations.

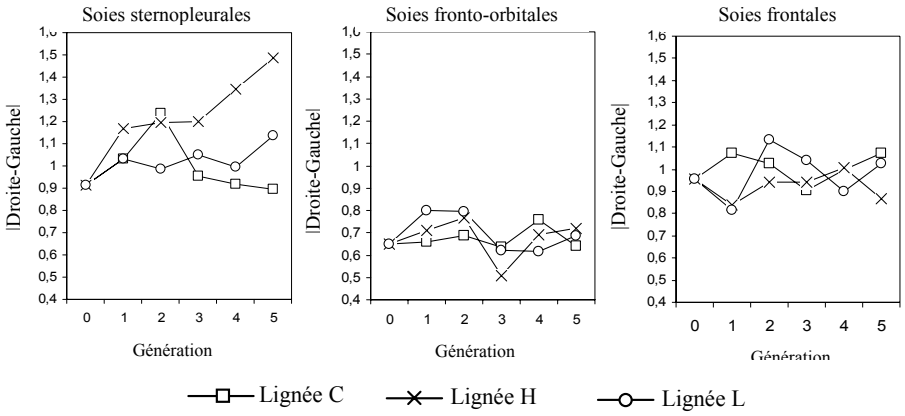


Figure 11 : Evolution des niveaux d'asymétrie |Droite–Gauche| au cours des générations de sélection pour les trois caractères morphologiques.

Seules les soies qui font l'objet d'une modification de leur niveau d'asymétrie fluctuante au cours des générations de sélection sont celles qui ont fait l'objet d'une intense sélection directionnelle. La sélection directionnelle, en provoquant une rupture de coadaptation génomique (cf partie 2), agirait comme un stress affectant la stabilité de développement (Parsons 1990; Markow 1995). L'impact de la sélection est, dans le cadre de notre expérience, limité à la stabilité de développement du seul caractère sélectionné. Cela pourrait indiquer que les gènes impliqués dans la stabilité de développement présentent une certaine indépendance avec ceux soumis à la sélection *via* les soies sternopleurales. Ainsi, cinq générations de sélection ne semblent pas suffisantes pour affecter l'homéostase de développement des individus dans sa globalité.

Les males appariés plus symétriques que les males non appariés

Paired males more symmetrical than unpaired males

Concernant la relation asymétrie fluctuante – aptitude phénotypique, nous avons trouvé une relation significative (toutes générations confondues) pour deux caractères (les soies fronto-orbitales et soies frontales) sur les trois étudiés mais pour une seule lignée (la lignée H) : les males appariés présentant des niveaux d'asymétrie fluctuante plus faibles que les males non appariés

(fronto-orbitales: $F_{119,119} = 1,47$; $p = 0,008$, frontales: $F_{119,119} = 1,60$; $p = 0,017$). Ce résultat est conforme à ceux de plusieurs études menées sur d'autres diptères (Allen & Simmons 1996; Blanckenhorn *et al.* 1998; Norry *et al.* 1998) mais il apparaît d'autant plus intéressant que contrairement à ces études il a été obtenu sur des caractères qui ne sont pas connus pour être directement impliqués dans la capacité des mâles à s'accoupler. Cet élément fournit par conséquent un éclairage supplémentaire dans le cadre du débat sur l'utilisation de l'asymétrie fluctuante comme marqueur de l'état général des mâles, notamment dans le contexte de la sélection sexuelle.

Une relation qui devient détectable

A relationship which become detectable

Si l'on s'intéresse aux différences de niveaux d'asymétrie entre les générations il apparaît que pour les deux lignées ayant subi la sélection directionnelle (lignée H et lignée L) les niveaux d'instabilité de développement des mâles appariés de la cinquième génération sont significativement supérieurs à ceux de la population sauvage de départ pour les soies frontales (aucune différence n'apparaissant significative pour les deux autres caractères). Ces résultats sont conformes aux prédictions puisque cela signifie que les niveaux d'asymétrie fluctuante ont, pour le caractère considéré, globalement augmenté mais aussi que la relation asymétrie fluctuante-aptitude phénotypique qui n'était pas détectable dans la population sauvage (ni même sur l'ensemble des générations mélangées) l'est devenue dans l'échantillon présentant les niveaux d'asymétrie les plus élevés (génération 5). En revanche aucune relation, quels que soient les échantillons considérés, n'a été trouvée entre asymétrie fluctuante et aptitude phénotypique (taux de survie larvaire ou fertilité) chez les femelles.

PARTIE 4

Perspectives de recherches
Contrôle de la variation phénotypique: apports de la morphométrie

Contexte scientifique et objectifs

Du génotype au phénotype : la voie de l'étude des mécanismes de contrôle de la variation phénotypique

From genotype to phenotype: studying the mechanisms of control of phenotypic variation

La variation est une condition *sine qua non* de l'évolution. Les mécanismes responsables de la variation génétique nécessaire à l'évolution des caractères (mutations, recombinaisons ou polyploïdie par exemple) ont été, et demeurent, très largement étudiés. Mais la sélection naturelle s'exerce sur les phénotypes et il est par conséquent crucial d'être capable de faire le lien entre l'architecture génétique sous-jacente à un phénotype et l'expression de la variabilité de ce même phénotype. Ainsi, les questions centrées sur comment les phénotypes sont générés, contrôlés et transformés sont incontournables en biologie évolutive. Ces questions illustrent le recentrage (ou tout au moins l'élargissement) récent de la biologie évolutive du développement (*evolutionary developmental biology*) ou évo-dévo vers des questions plus centrées sur les processus microévolutifs (Corley 2002).

Les relations entre variation génétique et variation phénotypique peuvent être très complexes, notamment parce que la production de variation phénotypique non délétère est contrainte à de multiples niveaux. Le défi est de déterminer comment les processus développementaux structurent la transformation de la variation génétique et des effets environnementaux en variation phénotypique (Hallgrímsson *et al.* 2005). De fait, les études des processus limitant et structurant la variation phénotypique peuvent s'intéresser à deux types de mécanismes évolutifs (Sholtis & Weiss 2005): (1) ceux responsables de la production de phénotypes prédéterminés en dépit de la variation génétique ou environnementale (par exemple la stabilité de développement, la canalisation, la variation cryptique) et (2) ceux entraînant la production de phénotypes différents à partir du même génotype dans des environnements différents (par exemple plasticité phénotypique, norme de réaction, polyphénisme). Mon programme de recherche est axé sur le premier point et s'articule autour des deux objectifs généraux suivants :

- comprendre comment la variation phénotypique est modulée par la stabilité de développement
- explorer les concepts de modularité et de contraintes morphologiques par l'étude des patrons de covariation morphologique chez différents taxons et à différentes échelles de temps et d'espace.

Ce programme, tout en assurant une continuité avec les recherches que j'ai pu développer jusqu'à présent, prend le parti de privilégier les questions liées aux mécanismes de développement notamment par le biais de l'étude conjointe des patrons de contrôle de la stabilité de développement et de covariation morphologique. Il comprend plusieurs projets complémentaires qui sont conduits sur différents modèles biologiques en fonction de la question abordée.

Méthodes

Une des raisons de l'intérêt croissant pour les approches connectant évolution à l'échelle microévolutive et développement est liée aux avancées conceptuelles et méthodologiques récentes dans les domaines de la biologie et de la génétique du développement, des techniques bioinformatiques et de la morphométrie (Hallgrímsson *et al.* 2005). Dans un article de synthèse récent Breuker et al. (Breuker *et al.* in press) montrent clairement comment ces avancées peuvent permettre de porter un regard nouveau sur des concepts et des problématiques relativement anciens. La morphométrie, outil majeur de caractérisation des phénotypes, fournit une très bonne illustration de ce phénomène. Comme nous l'avons indiqué déjà plusieurs fois dans ce mémoire, le développement des méthodes de morphométrie géométrique permet de quantifier les variations morphologiques sous tous leur aspects et d'utiliser cette information comme n'importe quelle donnée quantitative (Rohlf & Marcus 1993). Le potentiel de ces méthodes pour les études de stabilité de développement (Klingenberg & McIntyre 1998; Auffray *et al.* 1999; Debat *et al.* 2000; Klingenberg *et al.* 2002; Saucède *et al.* 2006), d'ontogénie (Zelditch *et al.* 2006), de modularité et d'intégration (Klingenberg *et al.* 2001; Klingenberg 2004, 2005; Richtsmeier *et al.* 2005) ou de développement en général qui commence tout juste à être exploré s'avère très prometteur.

Variation phénotypique et stabilité de développement

Aucun processus de développement n'est parfait et, en conséquence, la variation phénotypique ne s'exprime pas qu'entre les individus (comme la résultante de leur différences de génotype et/ou d'histoire évolutive) mais également au sein des individus (Willmore & Hallgrímsson 2005). Cette variation intra-individuelle peut être indirectement appréciée par la quantification des variations entre structures homologues répétées. Nous avons vu plus haut que chez les êtres vivants les structures homologues répétées les plus fréquentes étaient les structures bilatérales et que l'étude de l'asymétrie fluctuante constituait l'approche la plus communément utilisée. Malgré les débats évoqués plus haut (ou plutôt grâce à eux) l'intérêt du marqueur morphologique que constitue l'asymétrie fluctuante reste entier (Polak 2003). Une des raisons du succès persistant de l'asymétrie fluctuante est qu'elle peut être utilisée à la fois en tant que patron (par exemple lors de l'étude de son association avec des stress génétiques ou environnementaux) mais aussi en tant que processus (par exemple lors de l'étude des relations

entre stabilité de développement et contrôle des voies développementales). C'est dans cette deuxième perspective que s'ancrent mes recherches à venir.

- Ontogénie de l'asymétrie fluctuante chez l'oursin
[collaborateurs: B. David (Dijon,) L.C. Stige (Oslo)]

Les oursins permettent une approche originale de l'ontogénie de la stabilité de développement

Sea urchins allow an original approach of the ontogeny of developmental stability

Ce projet vise à mieux comprendre les mécanismes responsables de la stabilité de développement et en particulier de voir si un individu est capable au cours de son ontogénie de modifier et/ou d'ajuster les niveaux de cette fonction. Les enjeux sont importants puisque la façon dont la stabilité de développement varie durant le développement des organismes peut être utilisée pour déterminer les contributions relatives des facteurs primordiaux intrinsèques (contraintes, conditions génétiques) et extrinsèques (environnement). Le modèle biologique retenu - les oursins – est, comme indiqué dans la première partie de ce mémoire, remarquablement adapté à l'étude de l'ontogénie de la stabilité de développement car les plaques constituant leur test calcaire témoignent, sur l'individu adulte, des différentes étapes de son développement. La deuxième particularité de l'étude proposée réside dans la façon d'appréhender la stabilité de développement. Nous avons vu dans la partie bilan de ce mémoire que l'étude de l'instabilité de développement pouvait se faire, selon l'espèce considérée, de façon classique en estimant la variation entre structures bilatérales ou, de façon plus originale, entre les cinq parties homologues. Ce dernier type d'approche est particulièrement intéressant car il permet d'estimer une variabilité entre structures homologues, non pas à partir de deux valeurs (droite et gauche) comme c'est classiquement le cas, mais à partir de cinq. Le gain de puissance dans la détection d'un signal traduisant de l'instabilité de développement est donc non négligeable. Les travaux réalisés dans le cadre du travail de recherche de Y. Savriama (cf partie 3, étude des relations entre pollution et instabilité de développement chez deux espèces d'oursins réguliers dans la baie de Marseille) ont permis la mise au point de l'approche statistique.

Notre projet portera sur l'étude d'une espèce à symétrie bilatérale (*Abatus cordatus*), ainsi que sur les deux espèces à symétrie pentaradiaire (*Paracentrotus lividus* et *Arbacia lixula*) précédemment étudiées. Il s'agira de comprendre comment les niveaux d'asymétrie évoluent au cours de l'ontogénie d'un individu. Pour cela, et selon le matériel disponible, jusqu'à trois démarches parallèles pourront être entreprises : (1) un suivi intra-individuel de l'asymétrie des plaques le long des ambulacres, (les plaques différent en terme d'age, celles proches du péristome (région de la bouche) étant les plus vieilles), (2) un suivi intra-individuel de l'asymétrie des stries de croissance de plaques spécifiques et (3) une comparaison inter-individuelle de plaques homologues, provenant d'individus de différentes classes d'âge. Des trajectoires ontogénétiques d'asymétrie seront ainsi déterminées et pourront être confrontées aux prédictions que l'on peut établir *a priori* en fonction des mécanismes susceptibles d'être impliqués. Kellner & Alford (2003) ont récemment fait la synthèse des mécanismes proposés dans la littérature et ont établi pour chacun d'entre eux les trajectoires ontogénétiques attendues (figure 12).

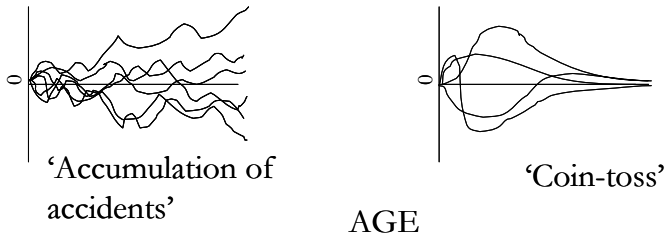


Figure 12 : Deux exemples de trajectoires ontogénétiques d'asymétrie (R=valeur du côté droit, L=valeur du côté gauche). L'axe des ordonnées indique l'asymétrie pondérée par la taille du caractère et l'axe des abcisses indique l'age. Le graphique de gauche illustre l'hypothèse où les accidents développementaux s'accumulent au cours de la vie de l'individu. Sous cette hypothèse on prédit donc une corrélation positive entre age et asymétrie fluctuante. Le graphique de droite illustre l'hypothèse considérant que l'accroissement en taille des structures consiste en l'accumulation de sous unités. Alors que la taille et la variance de la structure augmentent en proportion du nombre n de sous-unités, le coefficient de variation (CV) s'accroit lui en proportion de la racine carré de n. Comme l'asymétrie est la différence entre deux structures composites, quand le CV des deux côtés décroit, la différence relative entre eux décroit également. D'après Kellner & Alford (2003).

Une étude préliminaire a déjà été réalisée sur quatre populations antarctiques d'oursins irrégulier *Abatus cordatus* dans le cadre du stage postdoctoral de Leif C. Stige. A l'échelle du test, il apparaît notamment que les niveaux d'asymétrie fluctuante sont plus élevés pour les plaques les plus anciennement formées (celles situées près du péristome) et que ces niveaux d'aymétrie sont positivement corrélés entre plaques voisines et négativement entre plaques éloignées. A l'échelle des plaques, les niveaux d'asymétrie ne semblent pas décroître au cours de l'ontogénie et apparaissent corrélés entre stries d'accroissement. Ainsi, ces premiers résultats indiqueraient une absence de correction de l'asymétrie, à une échelle locale tout au moins, et suggèrent par ailleurs un rôle non négligeable des contraintes qui pourraient être liées à la structure du test (structure close). Ces résultats encourageants méritent maintenant d'être développés et élargis à d'autres espèces.

- Relation entre niveau de divergence génétique et instabilité de développement d'hybrides entre entités différenciées
[collaborateurs S. Garnier, (Dijon), J.-Y. Rasplus, (Montpellier)]

Quels niveaux d'instabilité de développement pour quels niveaux de différenciation?

Which levels of developmental instability for which levels of differentiation?

Nous avons vu précédemment qu'il était proposé que chez des hybrides entre entités plus ou moins différenciées, la stabilité de développement soit le résultat de deux facteurs agissant de façon antagoniste: l'accroissement des niveaux d'hétérozygotie qui tendrait à augmenter la stabilité de développement et la rupture de la coadaptation génomique qui tendrait à la diminuer. Quand le niveau de divergence entre les entités parentales est faible ou modéré on s'attend théoriquement à ce que le premier facteur soit dominant, et à l'inverse quand le taux de divergence est plus élevé l'effet de la rupture de coadaptation génomique devrait l'emporter (Graham, 1992). Nous avons vu également que dans les faits cette règle était difficile à

établir car les études comparées ne concernaient pas les mêmes groupes taxonomiques ou encore les mêmes caractères morphologiques (Alibert & Auffray, 2003). Le présent projet se propose d'étudier la relation entre niveau de divergence et niveau d'instabilité de développement des hybrides entre couples d'espèces appartenant au même groupe taxonomique, celui des carabes forestiers. Nous disposons de plusieurs espèces (celles déjà étudiées appartenant au complexe *Chrysocarabus solieri* mais également *C. splendens* et de *C. ponctuatorauratus*) et de leurs hybrides (naturels ou obtenus par croisements en laboratoire). Une étude comparée des niveaux d'asymétrie fluctuante des hybrides pourra donc être engagée parallèlement à l'échelle intra- et inter-spécifique. Les comparaisons divergence génétique-instabilité de développement des hybrides auront ainsi beaucoup plus de sens. Si il existe une corrélation positive et puisque nous savons déjà que les hybrides entre les différentes entités de *C. solieri* présentent des niveaux d'asymétrie fluctuante plus élevés que les entités parentales (Garnier *et al.* 2006), nous pouvons dès lors prédire des niveaux d'instabilité de développement encore supérieurs pour les hybrides entre *C. splendens* et *C. ponctuatoauratus*. Ce travail nous fournira une meilleure compréhension du rôle des conditions génétiques supposé agir sur la stabilité de développement., mais également un éclairage supplémentaire sur la nature de la coadaptation génomique et sur son rôle dans le processus de spéciation. Ici également nous réaliserons une étude conjointe de caractères fonctionnels et vestigiaux.

Modularité et contraintes morphologiques

La modularité, un pont entre les approches fonctionnelles et l'évo-dévo.

Modularity as a gap between functional approaches and evo-devo.

Un aspect supplémentaire de l'étude des processus limitant et structurant la variation phénotypique concerne le degré de dépendance (ou d'indépendance) des différentes parties d'une structure morphologique complexe. La morphologie d'un organisme est le résultat de systèmes de développement qui permettent à la fois une grande flexibilité (le phénotype doit être capable de répondre aux conditions environnementales) mais également une grande constance (le phénotype "final" doit être intégré et fonctionnel) (Klingenberg 2004). Un argument fréquemment avancé pour expliquer cette double propriété des organismes est celui de leur architecture modulaire (Raff 1996; Wagner & Altenberg 1996; von Dassow & Munro 1999; Bolker 2000; Schlosser 2002; Klingenberg 2004; Eble 2005). Brièvement, la morphologie des organismes (taille et forme) est le fruit du développement coordonné de ses différentes parties ou modules. Un module peut être défini comme une unité, plus ou moins individualisée, issue des interactions fortes coordonnant le développement de ses différents composants, et peut constituer une unité d'évolution relativement indépendante (Schlosser 2002; Klingenberg 2004). Cependant, même si les interactions entre modules sont par définition moins nombreuses et/ou plus faibles qu'au sein même des modules, c'est la coordination entre ces derniers qui assure un développement intégré et fonctionnel des organismes (figure 13). C'est cette double échelle d'intégration qui permettrait aux phénotypes des organismes d'être à la fois flexibles et constants.

L'identification et l'étude des modules morphologiques peut s'avérer extrêmement informative en Evolution, à la fois dans le cadre des approches

fonctionnelles traditionnellement menées en biologie évolutive (par exemple la recherche des facteurs externes qui façonnent les organismes par le biais de la sélection naturelle) et les approches d'évo-dévo dites structurelles (celles qui cherchent à comprendre comment les organismes sont construits). L'étude de la modularité est présentée comme un pont possible entre ces approches fonctionnelles et celles d'évo-dévo qui ont jusqu'à présent été considérées indépendamment (Breuker *et al.* in press).

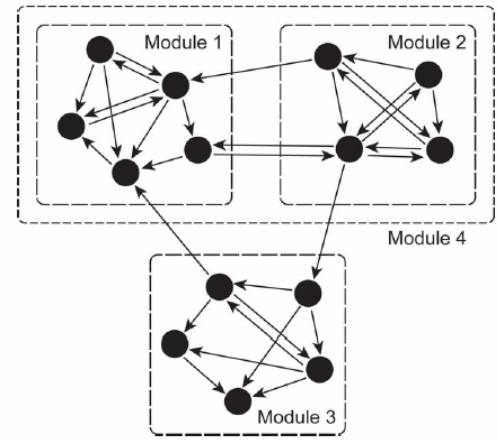


Figure 13 : Schéma théorique de quatre modules. les caractères morphologiques sont figurés par des ronds noirs et les flèches représentent les interactions entre les caractères. Un module (cas du module 4) peut être constitué de plusieurs modules. (d'après Klingenberg, 2005).

- Evolution de la modularité dans des lignées actuelles et fossiles de campagnol
[collaborateurs: S. Montuire, (Dijon), N. Navarro, (Manchester, UK)]

Des covariations morphologiques dans le temps et en regard des données génétiques.

Morphological covariations in time and in regard to genetical data.

L'objectif est, chez différentes espèces de campagnols (*Clethrionomys glareolus*, *Microtus (Terricola) pyrenaicus* ou *M. arvalis*) présentant des modes de croissance des dents différents (rhizodonte et hypsodonte notamment), de rechercher des patrons de covariations morphologiques entre les différentes parties de la dent qui pourraient correspondre à des modules de développement. Les méthodes d'étude seront issues des plus récentes adaptation des techniques de morphométrie géométrique proposées notamment par Klingenberg (2004, 2005). Soulignons ici que l'approche méthodologique comprend l'étude conjointe de la variabilité inter-individuelle et de l'instabilité de développement, cette dernière fournissant un moyen de distinguer entre deux types de mécanismes responsables de la covariation entre caractères : les interactions développementales directes et les variations parallèles (voir Klingenberg, 2004). Le principe de l'identification des modules est basé sur la comparaison des patrons de covariation des valeurs de variabilité inter-individuelle et des valeurs d'asymétrie fluctuante entre les modules définis *a priori*, et sur la comparaison des valeurs trouvées à celles calculées entre des partitions alternatives. Une étude préliminaire a été réalisée dans le cadre du travail de Master 2 Recherche de Rémi Laffont que j'ai co-encadré avec S. Montuire (Biogéosciences) et dont l'objectif était de voir si il existait une organisation modulaire au sein des deux premières molaires

inférieures (M_1 et M_2) du campagnol *Microtus arvalis* (sachant que la partie antérieure de la M_1 est connue pour être très variable à l'échelle spécifique et générique alors que la partie postérieure apparaît beaucoup plus fixe). Les résultats obtenus sont intéressants puisque deux modules ont pu être délimités dans la M_1, mais ils sont un peu différents de la séparation morphologique faite classiquement. De fait, la localisation de ces modules peut être mise en relation avec l'existence de deux cuspides ayant un rôle important dans le développement de cette dent. Par ailleurs, quand les deux molaires sont considérées simultanément la partition entre les deux est masquée par les faibles covariations caractérisant les deux modules de la M_1. Cela pourrait signifier que les processus responsables de l'intégration au sein de la première molaire perturbent ceux de la seconde.

Dans un deuxième temps cette étude sera étendue aux populations de campagnols fossiles. Il s'agira alors d'étudier l'évolution de la modularité dans l'espace et dans le temps. Nous tenterons notamment de voir si l'on peut relier certains épisodes de l'histoire évolutive des espèces étudiées (apparition, extinction, changement des conditions environnementales…) à une évolution des niveaux d'intégration morphologique et de modularité. Une partie de ce travail sera intégrée dans le travail de thèse d'Elodie Renvoisé (Encadrantes: S. Montuire & C. Tougard).

Des travaux récents combinant les connaissances sur les forces responsables des mouvements morphogénétiques à celles issues de la génétique du développement permettent dorénavant la formulation de modèles généraux de patrons de formation et de croissance de caractères morphologiques (Breuker *et al.* sous presse). Les premières applications de ces modèles d'étude de variation et d'innovation morphologiques ont porté précisément sur la formation des cuspides des dents de mammifères (Jernvall 2000; Salazar-Ciudad *et al.* 2003). Les résultats obtenus par R. Laffont dans le cadre de son stage de recherche fournissent les tout premiers tests empiriques de ces modèles et il est encourageant de constater que les deux approches apparaissent congruentes.

- Modularité axiale *vs.* extraxiale chez les oursins
[collaborateurs: B. David, G. Eble (Dijon)]

Une modularité à l'échelle de l'organisme entier.

A modularity at the scale of the whole organism.

Les modalités de la croissance post-larvaire chez les oursins sont dorénavant clairement cernées dans le cadre du modèle récent connu sous le nom de "Extraxial-Axial Theory" (ou EAT) (David & Mooi 1996; Mooi & David 1997). Très brièvement, le modèle EAT suggère que la paroi du corps des échinodermes est constituée de deux parties bien distinctes: l'axial et l'extraxial. Chez les oursins, la plupart du test est formé d'éléments axiaux organisés en cinq zones de croissance. Chez les oursins réguliers les cinq zones de croissance sont presque parfaitement équilibrées (c'est cet équilibre qui est à l'origine de la symétrie radiaire) alors que chez les oursins irréguliers une symétrie bilatérale qualifiée de secondaire apparaît au cours de la croissance et serait la conséquence d'un changement de cet équilibre. En utilisant les mêmes méthodes que celles utilisées dans le projet précédent nous testerons la présence de modules, et si il existent, leur correspondance avec les régions axiales et extraxiales. Ici encore les résultats seront analysés à la lumière des récentes découvertes dans le domaine de la génétique du développement qui montrent une très bonne correspondance entre les zones

de croissance identifiées par le modèle EAT chez les échinodermes et le domaine d'expression de gènes de développement tels que *distal less* (Mooi *et al.* 2005) où de gènes Hox tels que *HpHox5* et *HpHox11/13* (Morris & Byrne 2005).

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Articles utiles à la compréhension du mémoire

ARTICLE 1 :

Alibert, P., B. Moureau, J.-L. Dommergues, and B. David (2001) Differentiation at a microgeographical scale within two species of ground beetles, *Carabus auronitens* and *C. nemoralis* (Coleoptera, Carabidae): a geometrical morphometric approach. *Zoologica Scripta* **30**:299-311.

ARTICLE 2 :

Garnier, S., P. Alibert, P. Audiot, B. Prieur, and J.-Y. Rasplus (2004) Isolation by distance and sharp discontinuities in gene frequencies: implications for the phylogeography of an alpine insect species, *Carabus solieri*. *Molecular Ecology* **13**:1883-1887.

ARTICLE 3 :

Garnier, S., F. Magniez-Jannin, J.-Y. Rasplus, and P. Alibert (2005) When morphometry meets genetics: inferring the phylogeography of *Carabus solieri* using Fourier analyses of pronotum and male genitalia. *Journal of Evolutionary Biology* **18**:269-280.

ARTICLE 4 :

Bertin, A., B. David, F. Cézilly, and P. Alibert (2002) Quantification of sexual dimorphism in *Asellus aquaticus* (Crustacea: Isopoda) using outline approaches. *Biological Journal of the Linnean Society* **77**:523-534

ARTICLE 5 :

Alibert, P. and J.-C. Auffray (2003) Genomic coadaptation, outbreeding depression and developmental instability. In *Developmental instability: Causes and Consequences*, Ed M. Polak, New York: Oxford University Press, 116-134.

ARTICLE 6 :

Garnier, S., N. Gidaszewski, M. Charlot, J.-Y. Rasplus and P. Alibert (2006) Hybridization, developmental stability and functional significance of morphological traits in the carabid beetle *Chrysocarabus solieri* (Coleoptera, Carabidae). *Biological Journal of the Linnean Society* 89: 151-158

ARTICLE 7 :

Alibert, P., L. Bollache, D. Corberant, V. Guesdon & F. Cézilly (2002). Parasitic infection and developmental stability: fluctuating asymmetry in *Gammarus pulex* infected with two acanthocephalan species. *Journal of Parasitology* 88(1): 47-54.

ARTICLE 8 :

Saucède, T., P. Alibert, B. Laurin & B. David (2006) Environmental and ontogenetic constraints on developmental stability in the spatangoid sea urchin *Echinocardium* (Echinoidea). *Biological Journal of the Linnean Society* 88: 165-177

ARTICLE 9 :

Stige, L. C., David, B. & P. Alibert (2006) On hidden heterogeneity in directional asymmetry - can systematic bias be avoided? *Journal of Evolutionary Biology* 19: 492-499

ARTICLE 1 :

Alibert, P., B. Moureau, J.-L. Dommergues, and B. David (2001) Differentiation at a microgeographical scale within two species of ground beetles, *Carabus auronitens* and *C. nemoralis* (Coleoptera, Carabidae): a geometrical morphometric approach. *Zoologica Scripta* **30**:299-311.

Differentiation at a microgeographical scale within two species of ground beetle, *Carabus auronitens* and *C. nemoralis* (Coleoptera, Carabidae): a geometrical morphometric approach

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New morphometric methods, the geometrical morphometrics, offer promising perspectives to appraise morphological variation among organisms and open up, to a large extent, the field of morphometrics for the study of systematics and evolution. Until now, however, few studies have explored the potential of these methods at a microgeographical scale. In the present work, we applied them to quantify morphological (size and shape) differentiation among populations of two forest species of ground beetles: *Carabus auronitens* and *C. nemoralis*. We found a significant shape variation among sites, as well as among sexes, for both species. Additionally, for *C. auronitens*, we found significant positive correlations in both sexes between morphological (shape) and geographical distances between populations. In contrast, significant size differences were found between sexes, but not between sites. We conclude that geometrical morphometric methods provide valuable tools for the study of morphological variation among populations and therefore offer, on the whole, interesting perspectives for the study of biodiversity patterns.

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Introduction

Population differentiation is considered as an essential step in the process of speciation (Balon 1993; Margurran 1998), and its study is therefore crucial for the understanding of the processes underlying biodiversity. For several decades, geographical differentiation within species has essentially been viewed from a genetic viewpoint. This has been related to the concomitant development of efficient genetic markers as well as powerful statistical analyses of data obtained with these markers (Sunnucks 2000). All of these studies contributed to a significant increase in our knowledge of the definition of species and of their sensitivity to geographical dispersal and eventually to habitat clearance. In contrast, studies focusing on morphological differentiation within species are quite rare (but see Brown *et al.* 1992; Thomas *et al.* 1998). So far, morphological approaches have received only moderate attention, partly because of the lack of accuracy of traditional morphometric methods at the intra-specific level. However, the last few years have seen the development of new morphometric methods: the geometrical morphometrics (Bookstein 1991; Rohlf 1993a; Rohlf & Marcus 1993). These methods, which allow the study of shape in addition to the study of size, offer powerful analytical and graphical tools for the quantification and visualization of morphological variation within and among samples of organisms. The principle of these methods can be briefly summarized as follows. Raw data corresponding to two- or three-dimensional Cartesian coordinates of landmarks describe the form of the morphological structure under study. Differences among individual configurations of landmarks are captured using mathematical functions which fit the differences in positions of the landmarks. Then, the variation in shape within and among samples can be appraised using the parameters of these functions as variables in classical uni- or multivariate statistical procedures (Rohlf & Marcus 1993; but see also Bookstein 1996 for some restrictions). In addition, as these methods preserve the geometrical relationships among landmarks, they provide the opportunity to represent the contribution of each landmark to the shape changes directly in the space of the original specimen, e.g. by the mean of displacement vectors or deformation grids.

In the last 10 years, the number of studies using geometrical morphometrics has increased considerably. These methods have now been used and proved to be relevant in a large spectrum of fields of morphometrics, including systematics, phylogeny, ontogeny or the study of developmental stability (Loy *et al.* 1993; Zelditch *et al.* 1993, 1995; Fink & Zelditch 1995; Auffray *et al.* 1996; David & Laurin 1996; Naylor 1996; Klingenberg & McIntyre 1998). Surprisingly, few studies have explored the potential of geometrical morphometric methods at a microevolutionary level (Loy 1996, but see, for example, Laurin *et al.* 1994; Auffray *et al.* 1996; Baylac & Daufresne 1996; Adams & Funk 1997).

In the present study, we apply geometrical morphometrics to assess morphological differentiation among populations of carabid beetles at the microgeographical scale. Ground beetles (Coleoptera, Carabidae) belong to one of the best studied invertebrate families (see Lövei & Sunderland 1996 for a review). Species richness (more than 40 000 described species), abundance and the large distribution of ground beetles makes this group particularly prone to investigation in numerous topics of ecology and evolution, including landscape ecology and conservation research programmes. Carabids have proved their usefulness as environmental indicators (Heijerman & Turin 1994; Maelfait *et al.* 1994), and studies at the community (Klein 1989; Blake *et al.* 1994; Eyre & Luff 1994; Davies & Margules 1998) or population (Liebherr 1986; Basedow 1994; Cardenas 1994; Baumgartner *et al.* 1997) levels are numerous. In order to explore morphological differentiation among populations at a regional scale of up to 50 km, we chose two forest species of ground beetles, *Carabus* (*Chrysocarabus*) *auronitens* Fabricius, 1972 and *Carabus* (*Archicarabus*) *nemoralis* Müller, 1764, that differ slightly in their habitat requirements, *C. auronitens* being more stenotopic and strictly bound to forest, and *C. nemoralis* more eurytopic and less strongly associated with forest (Assmann *et al.* 1994; Kennedy 1994; Niehues *et al.* 1996). This, and the fact that the two species are wingless, explain their small dispersal power (Wallin & Ekbom 1988; Nève de Mévergnies & Baguette 1990). For instance, Mader (1984), after a 2-year mark–release–recapture experiment, reported that for several species of stenotopic forest carabids, but also for more eurytopic ones (including *C. nemoralis*), a 6-m-wide highway was almost never crossed during the whole period of the experiment and therefore constituted a very effective barrier to mobility. Similar results have been reported for *C. auronitens* (Nève de Mévergnies & Baguette 1990). At a microgeographical scale, connectivity, and its impact on population dynamics, is thus strongly related to the dispersal ability of individuals, i.e. their ability to cope with barriers as well as to move over long distances. This makes ground beetle populations particularly sensitive to geographical isolation. Therefore, our objectives were to use a geometrical morphometric approach to estimate the morphological (size and shape) differentiation in *C. auronitens* and *C. nemoralis*: (i) among populations in relation to geographical distance and to the presence of barriers between sites; and (ii) within populations, between sexes.

Materials and methods

Study area and sampling

The study took place in the vicinity of Dijon (Burgundy area, eastern France) where six sampling sites were selected (Fig. 1). The sites CIT-A and CIT-B were both located in the large forest of Cîteaux, the two sites being spaced 10 km apart. Two sites, BRAZ and VERN, were located in two presumed fragments of the Cîteaux forest (Brazey forest and Vernot forest) and were 5 km and 10 km from CIT-A and CIT-B, respectively. According to the local archives housed at the Prefecture of Dijon, Brazey forest and Vernot forest were isolated from the Cîteaux forest before the 17th century. Both are separated today from the Cîteaux forest by various barriers, such as railroads, roads, creeks, canal or agricultural fields (Fig. 1). The two other sites, FERT and CHAUX, are located 45 km south and east, respectively, in large forests (Ferté forest and Chaux forest). The forest and study area size are summarized in Table 1. All the selected forests are coppice dominated by common oak (*Quercus robur*), hornbeam (*Carpinus betulus*) and common beech (*Fagus sylvatica*). They are also similar in terms of soil and altitude. The surroundings are mainly agricultural land.

Animal capture took place from December 1997 to June 1998 using two different sampling methods according to the period. From December to February, as almost all beetles were sheltering for the winter (dormant period), they were collected directly after inspection of wood debris. From March to June, when animals became active, sampling was carried out with pitfall traps. Each trap was visited twice a week. Six hundred and sixty-two beetles were captured with, whenever possible, a minimum of 65 individuals for each site (Table 1). Both species, *C. auronitens* and *C. nemoralis*, were captured in all of the sampling sites, except for FERT, where only *C. nemoralis* was found, and CHAUX, where only *C. auronitens* was captured.

Data collection

Two-dimensional Cartesian coordinates of 50 landmarks were recorded on the ventral view of each specimen (Fig. 2) using an optic measuroscope (Nikon measuroscope 10×, Nippon KOGAKU K.K. model 0, 1/100 mm). All specimens were scored by one experimenter (BM) in a random order with regard to the site of origin. The location of the landmarks was chosen according to two criteria: reliability in terms of correspondence between specimens and the ability to best describe the geometry of the form under study. In

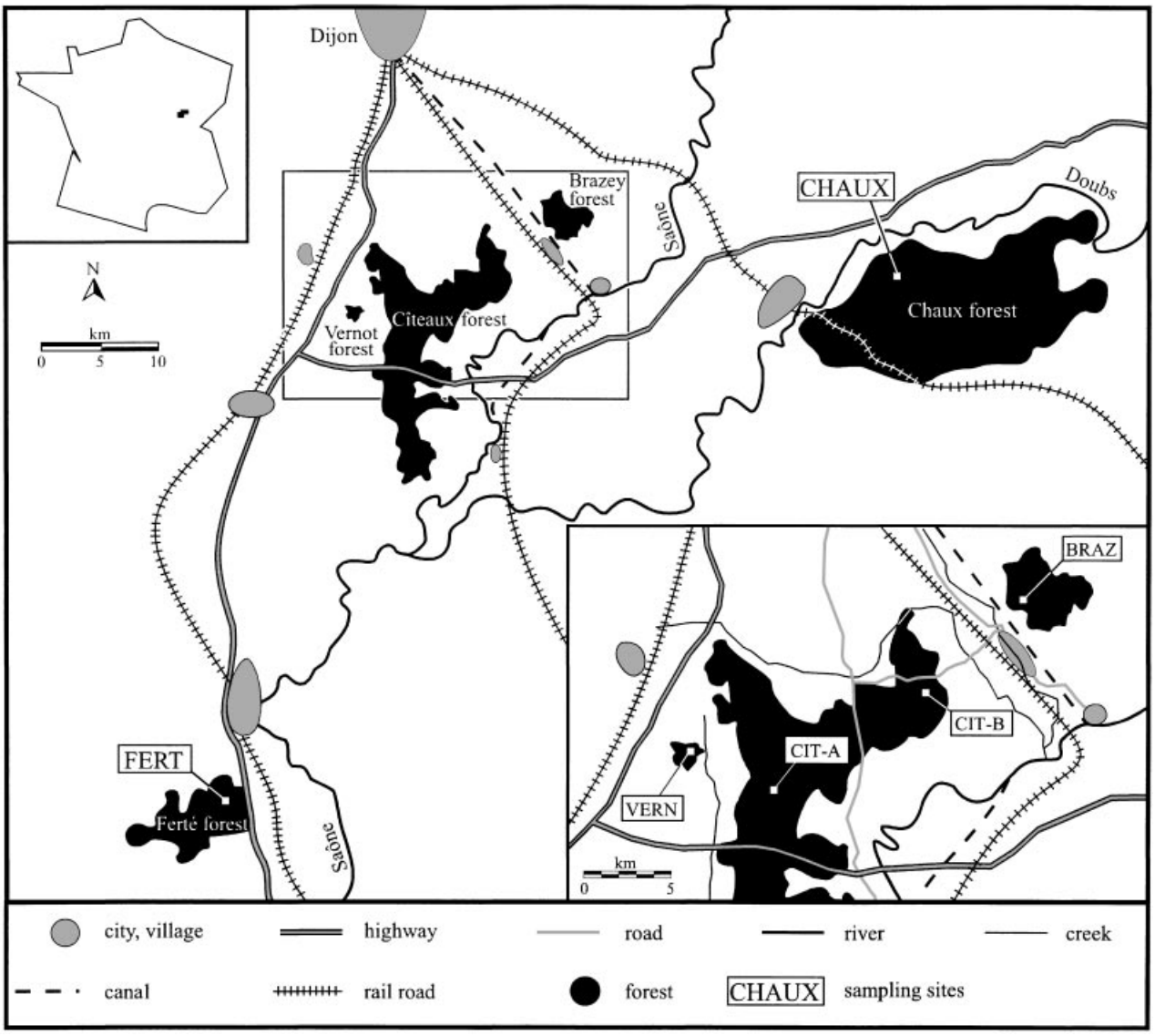

Fig. 1 Map of the study area and sampling sites.

Table 1 Location, area and size of samples.

					Sample size			
					C. auronitens		*C. nemoralis*	
Site	Forest	Locality	Total forest size (ha)	Study area size (ha)	Females	Males	Females	Males
CIT-A	Massif de Cîteaux	Argilly	10300	8	34	35	33	35
CIT-B	Massif de Cîteaux	Magny-lès-Aubigny	10300	9	36	34	31	36
BRAZ	Massif de Brazey	Brazey-en-plaine	1140	7	35	35	33	36
VERN	Bois de Vernot	Argilly	97	2.3	35	35	33	32
CHAUX	Forêt de Chaux	Bretenière	13000	5	33	10	—	—
FERT	Forêt de la Ferté	Saint-Ambreuil	7750	5	—	—	35	36
Totals					173	149	165	175
					322		340	

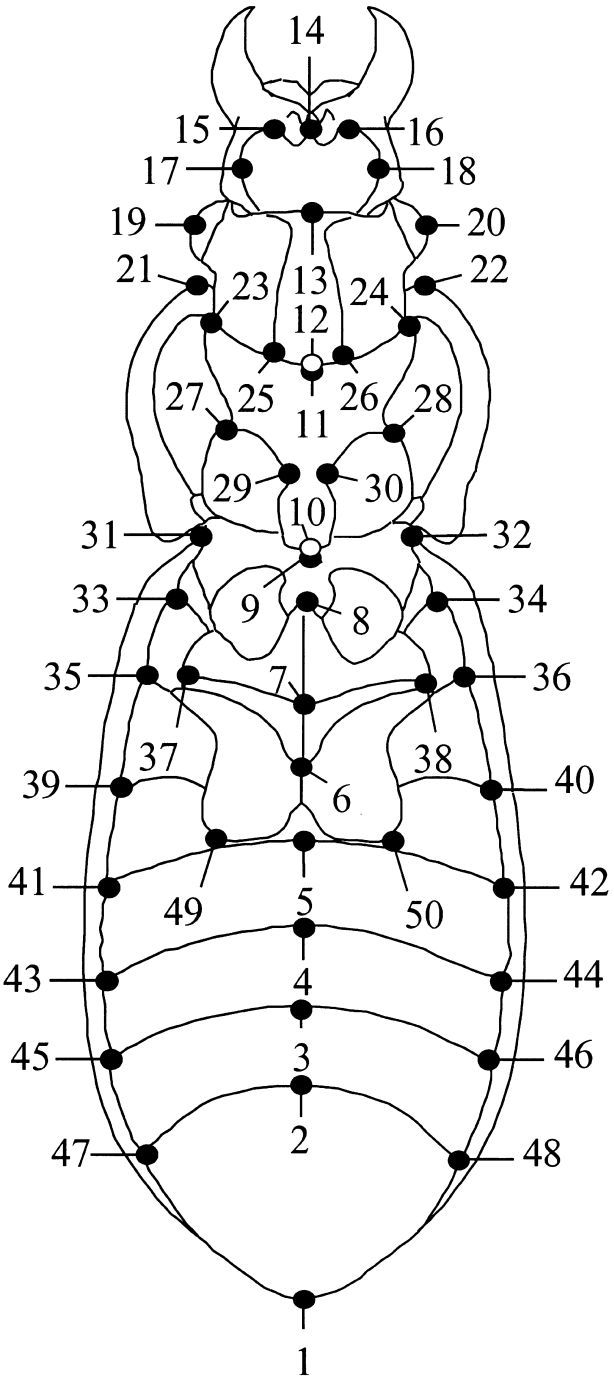

Fig. 2 Location of the 50 landmarks on the ventral view of the ground beetle. Legs, antennae and maxillae and labial palps were removed (modified from David *et al.* 1996).

order to make the location of landmarks easier, legs, antennae and maxillae and labial palps were removed. The raw data set therefore corresponded to 662 configurations of 50 (x,y) coordinates. There is no consensus among morphometricians about the interest of considering landmarks on one or both sides in bilaterally symmetrical organisms (for a discussion, see morphmet forum at http://life.bio.sunysb.edu/morph/). It is argued that the study of symmetrical landmark configurations increases the degrees of freedom without adding much new information, and leads to high correlation values in the covariance matrices. However, because in our case the whole organism (and not only part of it) was studied, we considered that it was important to take into account the whole shape variation, including that related to differences between sides. In addition, from a biological point of view, the consideration of the full body allowed a more realistic visualization of the shape variations in the space of the original specimen when using the deformation grids.

Prior to morphometric analysis, a geometrical adjustment was performed on the configurations of all the specimens in order to correct for the possible imperfection in the alignment of the three articulated parts of the body, i.e. head, prothorax and abdomen, which could have occurred during the preservation of animals. This correction consisted in aligning the segments defined by the landmarks 12 and 14 (located on the head) and 10 and 11 (located on the prothorax) to the symmetry axis defined by the abdominal landmarks 6 and 8 (David *et al.* 1996).

Shape analysis

Shape differences among individuals and populations were investigated using generalized least-square (GLS) Procrustes superimposition methods (Rohlf & Slice 1990; Bookstein 1991; Rohlf & Marcus 1993). These methods allow the description and quantification of the differences between two or more specimens after their landmark configurations have been aligned according to a procedure which ensures the best overall fit. This is performed in several steps (Rohlf & Slice 1990). First, all the configurations of landmarks are scaled by standardizing the size to a unit centroid size, the centroid size corresponding to the square root of the sum of the squared distances between the centroid (i.e. centre of gravity of the landmarks) and each of the 50 landmarks of the configurations (Slice *et al.* 1996). Then, the centroids of all the landmark configurations are superimposed and translated to the origin. Finally, the landmark configurations are rotated against a reference configuration so that the sum of the squares of the residual distances between corresponding landmarks is at a minimum. The reference configuration corresponds to a computed configuration (called consensus configuration hereafter) expressing the shape that has the least summed squared Procrustes distances to all the configurations of the studied sample (Slice *et al.* 1996).

Within both species, shape differences between each of the aligned configurations (322 and 340 for *C. auronitens* and *C. nemoralis*, respectively) and the consensus configurations

were recorded in terms of residuals (so-called Procrustes residuals) at each of the 50 landmarks. To explore the shape variation among individuals and samples, it is possible to perform a principal component analysis (PCA) on the Procrustes residuals. If variations are considered in full shape space (i.e. both uniform and nonuniform transformations, *sensu* Slice *et al*. 1996), such an analysis corresponds to a version of a more complex geometrical morphometric method, the relative warps analysis (Bookstein 1991; Rohlf 1993a). More precisely, a PCA on Procrustes residuals is technically identical to version 1 of the relative warps analysis (Bookstein 1996). The relative warps correspond to the principal components, and define a shape space in which individuals are replaced. Here, we performed a relative warps analysis on both species using the software TpsRelw v. 1.18 (Rohlf 1993b). This approach allows the expression of shape variations along the relative warps in terms of the transformation of deformation grids.

For both species, shape differences between sexes and sampling sites were tested by a two-way multivariate analysis of variance (MANOVA) on the scores obtained for all of the individuals on the relative warps representing 95% of the total variance. We retained only 95% of the expressed variance in order to reduce the number of axes involved in the computation. To test whether morphological divergence among populations was related to geographical distances, matrices of pairwise shape differences (i.e. noneuclidian distances between populations in the shape space) were compared with the corresponding matrices of pairwise geographical distances between sampling sites using Mantel tests. These tests were conducted for both sexes and both species.

Size analysis

We investigated size differences among individuals and populations using two kinds of size estimators: the centroid size and the euclidian distance between the landmarks 1 and 14. Whereas the former constitutes the size parameter almost systematically used in geometrical morphometrics, the latter provides a widespread estimator of size in traditional morphometric studies on beetles. Both centroid size and distance between landmarks 1 and 14 were calculated and extracted prior to GLS superimposition. Sexual dimorphism and variation in size among sites were tested using ANOVA. When ANOVAs were significant, pairwise comparisons among means were performed using the T′-method of Spjøtvoll and Stoline (Sokal & Rohlf 1995) for nearly equal sample size. As such an unplanned multiple comparison test is conservative, the occurrence of type I error is limited.

Measurement error

Because of the large amount of work involved in taking duplicate measurements on the 662 individuals under study, errors due to measurement were estimated from a subsample of 40 individuals of *C. auronitens* (20 males and 20 females) randomly chosen across all samples. Each of the 40 specimens was measured twice. As positioning has been shown to be an important source of error (Arnqvist & Mårtensson 1998), the second session of measurement was conducted after the specimens had been removed and replaced under the measuroscope in order to take the positioning error into account. The 80 landmark configurations obtained were then scaled, translated and rotated against the consensus configuration (computed as described above) by a GLS Procrustes superimposition method. Then, as for the study of the shape variation among individuals, a relative warps analysis was conducted. The variability in the position of the 80 configurations in the shape space was assessed using the scores obtained by each individual on the first three relative warps. For each of these axes, the variability in the scores was partitioned into 'within-individuals' (i.e. measurement error) and 'among-individuals' components using model II one-way ANOVAs, with individuals as the categorical factor (Bailey & Byrnes 1990; Arnqvist & Mårtensson 1998).

The percentage measurement error, estimated as the proportion of the total variance attributable to within-individuals variation, was found to be 0.4%, 36.5% and 4.2% for the first three relative warps, accounting for 26.3%, 12.1% and 10.7% of the total explained variation, respectively. If the proportions of measurement error for the first axis, and to a lesser extent for the third axis, are very low, the measurement error for the second axis remains fairly high, in comparison with other axes and also with other studies (Bailey & Byrnes 1990). However, it should be kept in mind that the imprecision of the measurements is expressed relative to the inter-individual variation, i.e. when variation among individuals is low, the within-individual variation (measurement error) becomes important. Thus, in studies conducted at the intraspecific level, as is the present study, higher values of measurement errors are expected. Overall, and owing to the low values of measurement error on the first and the third axes, we assumed that the variability due to the imprecision of measurements was not a source of bias in our study. Moreover, visual inspection of plots of the Procrustes residuals after GLS superimposition of the specimens measured twice revealed that the total variability was homogeneously distributed among the 50 landmarks (not shown here). Thus, none of the landmarks appeared to be associated with a greater measurement error.

Results

Figure 3 shows the result of the GLS superimpositions for the 322 specimens of *C. auronitens* (the result for *C. nemoralis*, which is quite similar, is not shown here). The relative

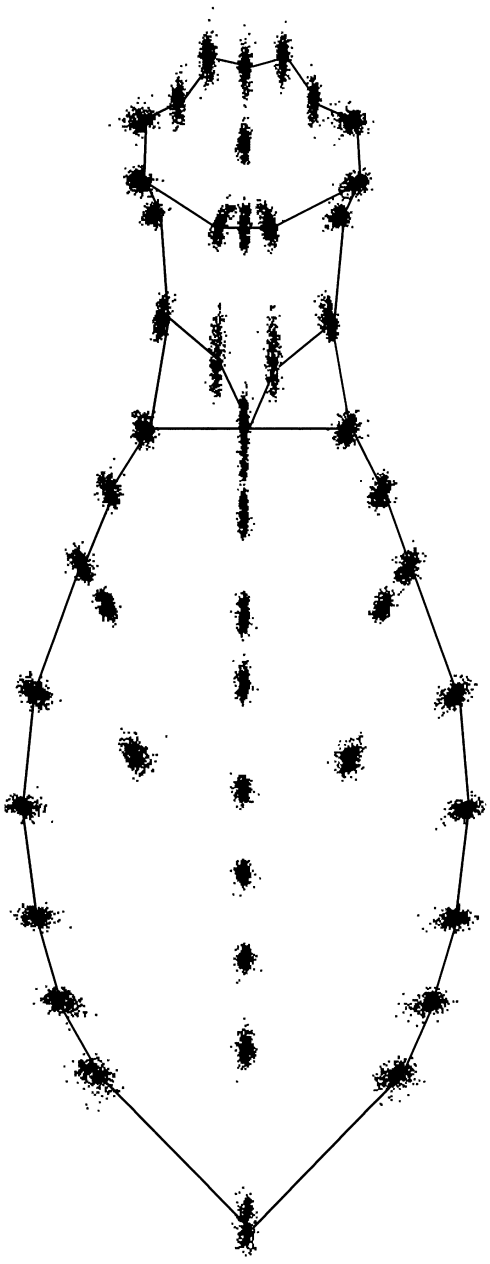

Fig. 3 Result of the generalized least-square superimposition for the 322 landmark configurations of *C. auronitens*. Points express the overall shape variation in landmark location around the consensus configuration.

warps analysis showed that 95% of the total variability was explained by the first 26 axes for *C. auronitens* and by the first 28 axes for *C. nemoralis*. For both species, much of the shape variation was captured by the first three relative warps, with 71.53% and 65.15% of the shape variation among specimens explained for *C. auronitens* and *C. nemoralis*, respectively.

Sexual dimorphism in shape

Two-way MANOVAs, performed on the scores obtained by the individuals in the relative warps analysis, revealed, for both species, a significant shape variation among sexes (Table 2). To better visualize the shape variation associated with sexual dimorphism, we only considered the consensus configuration for each sex in each sampling locality. Thus, the 10 consensus configurations per species were subjected to relative warps analysis. For both species, sexes are clearly separated in the shape space as shown by the MANOVA (Fig. 4). Interestingly, deformation grids expressing the range of shape variation along the axes indicate that sexual dimorphism concerns the same part of the body for both species. In particular, the heads of males are more elongated than those of females. In Fig. 4, grid a2 illustrates this elongation for males of *C. auronitens* and grid b2 shows the relative head contraction for the female of *C. nemoralis*. In addition, in both species, the posterior abdominal segments of females appear to be more enlarged than those of males. This trend is also visible for males in grid a2 and for females in grid b2 (Fig. 4).

Shape differentiation among populations

MANOVAs revealed, for both species, a significant shape variation among sites (Table 2). Figure 5 shows that, when the relative warps analysis is limited to the consensus configuration of each sampling locality, the individuals from the CHAUX site exhibit a clear shape differentiation. Deformation grids indicate a similar pattern of deformation for both sexes. In females, grid a1 displays strongly curved vertical lines at the level of the prothorax, which indicates a forward movement of the related landmarks and therefore the elongation of the prothorax for the specimens from the CHAUX site (Fig. 5). Correspondingly, a slight compression of the head occurred. The same morphological trends also pertained to males, as illustrated by grid b1, which expresses opposite deformations to those of specimens from the CHAUX site.

On the whole, Mantel tests revealed, for both sexes, a significant positive correlation between morphological and geographical distances between populations when considering the overall shape space (males: Mantel *t*-test: 1.798, $r = 0.84$, $P = 0.0361$; females: Mantel *t*-test: 2.091, $r = 0.95$, $P = 0.0183$). In other words, this means that geographically more distant specimens are more differentiated in terms of shape.

For *C. nemoralis*, the geographically most distant site (FERT) was not, morphologically, the most differentiated. Instead, the smaller site (VERN), and to a lesser extent the CIT-A site, appeared more distant in the shape space of the first three relative warps (Fig. 6). Deformation grids indicated a similar pattern of differentiation for both sexes, although weaker for males. As expected, Mantel tests did not reveal any significant correlation between morphological and geographical

Table 2 Results, for both species, of the two-way MANOVAs performed on the individual scores obtained on relative warps representing 95% of the total variation. Degrees of freedom of numerator and denominator are denoted 'Num. d.f.' and 'Den. d.f.', respectively.

	C. auronitens					*C. nemoralis*				
Source of variation	Wilks Λ	*F*	Num. d.f.	Den. d.f.	*P**	Wilks Λ	*F*	Num. d.f.	Den. d.f.	*P**
Sex	0.112	38.45	26	126	< 0.001	0.108	39.03	28	133	< 0.001
Site	0.004	15.0	104	502.41	< 0.001	0.008	11.10	112	530.81	< 0.001
Site × sex	0.118	3.44	104	502.41	< 0.001	0.476	0.97	112	530.81	ns

*Significance levels: ns, not significant.

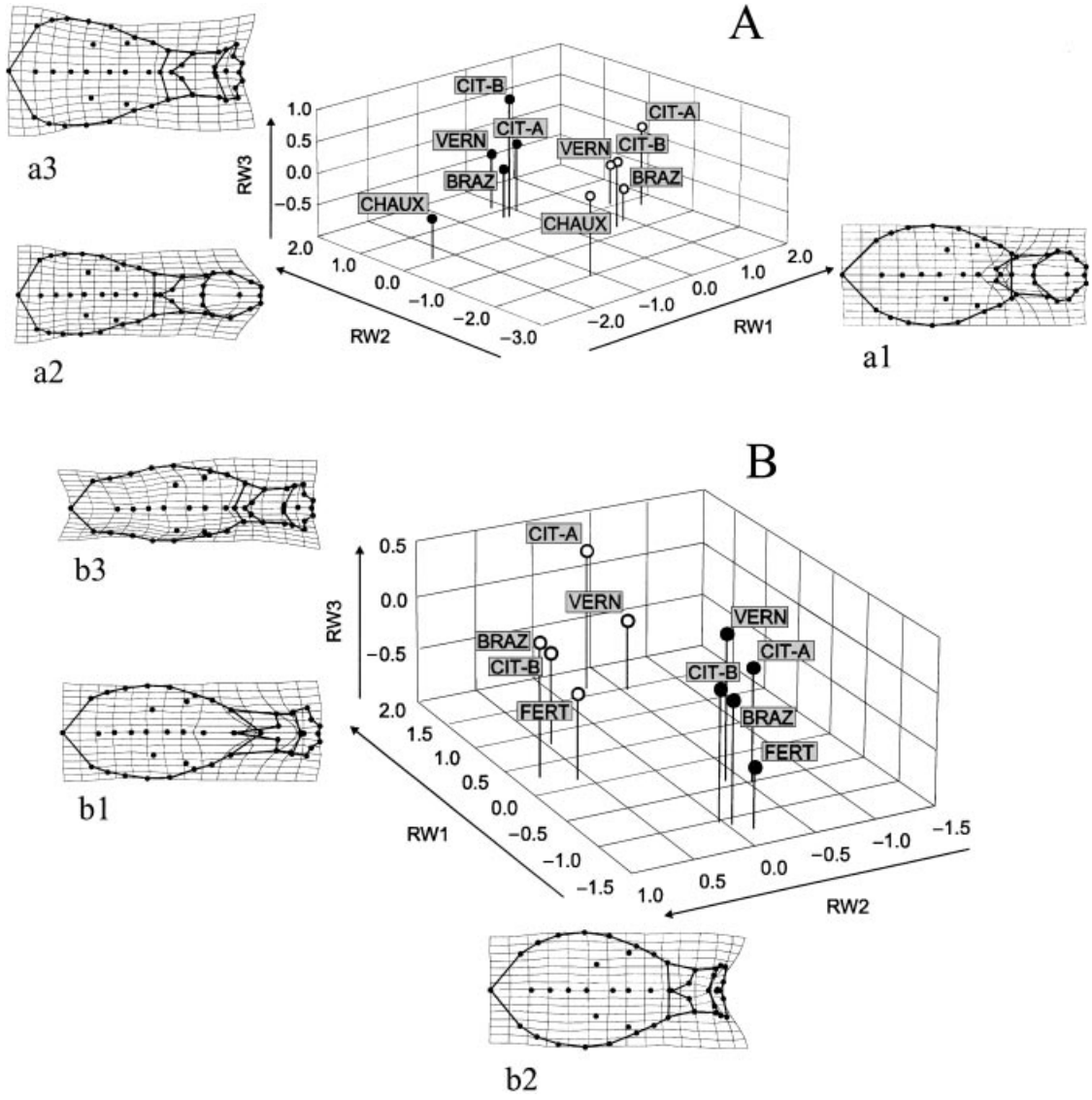

Fig. 4 Relative positions of the consensus configurations, for each site and sex, in the shape space defined by the first three relative warps. —A. *C. auronitens*. —B. *C. nemoralis*. Open circles correspond to the female samples and filled circles to the male samples. Deformation grids indicate which landmarks are implied for each axis definition and express the maximal shape variation along these axes in showing positive deformations. Negative deformations correspond to displacement of landmarks in the opposite direction on the grids. Scores on the relative warps are × 100 and deformation grids are magnified three times.

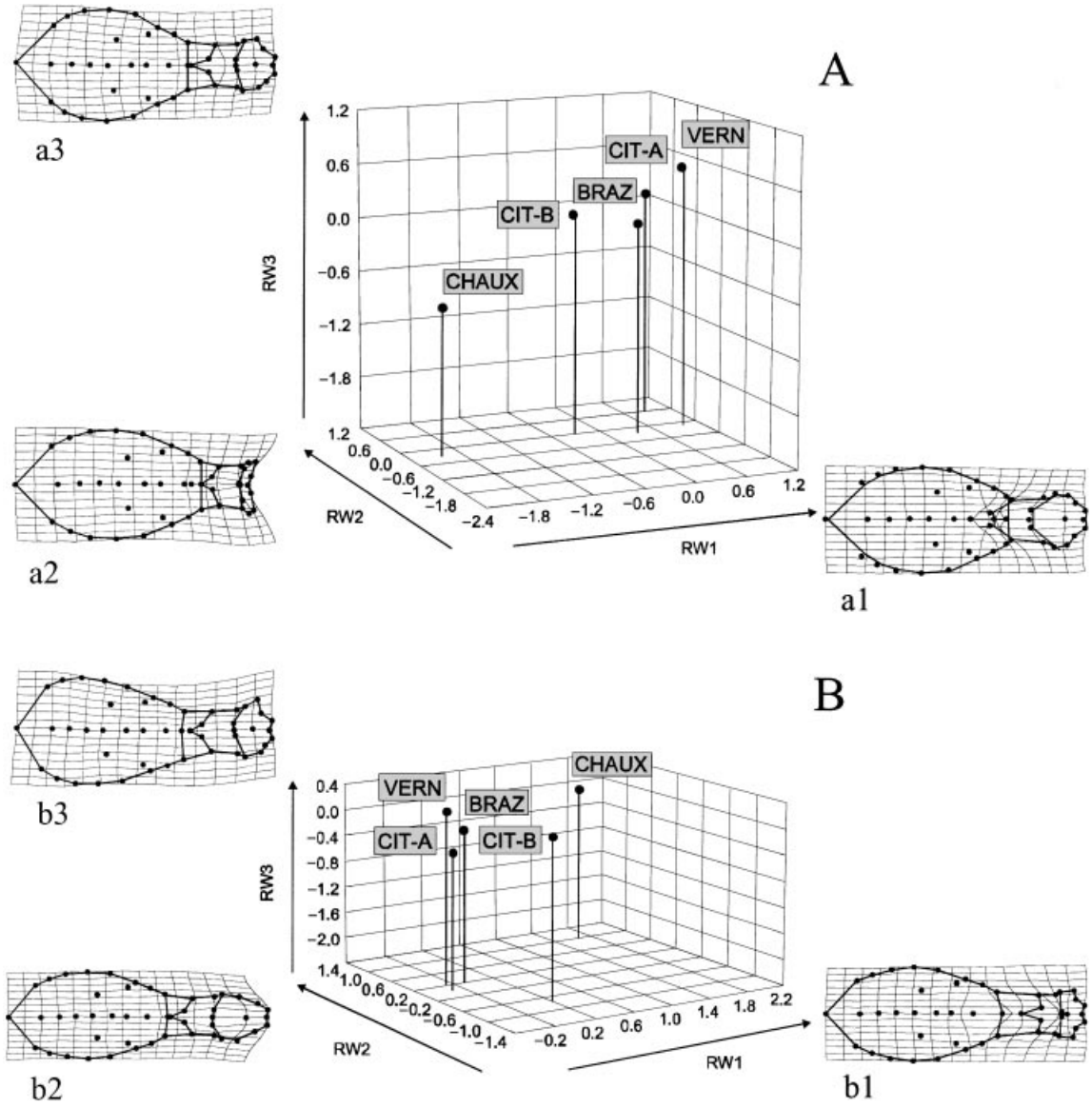

Fig. 5 Relative positions of the average configurations of the five sites for *C. auronitens* in the shape space defined by the first three relative warps. —A. Females. —B. Males. See legend of Fig. 4 for explanation of the deformation grids. Scores on the relative warps are × 100 and deformation grids are magnified three times for females and twice for males.

distances between populations (males: Mantel *t*-test: 0.334, $r = 0.12$, not significant; females: Mantel *t*-test: −0.176, $r = -0.043$, not significant).

Finally, as shown in Table 2, interaction between sex and site effects tested in the MANOVAs was not significant for *C. nemoralis*, whereas it appeared highly significant for *C. auronitens* (Wilks $\Lambda_{104,502.41} = 0.118$, $F = 3.44$, $P < 0.001$). This can be explained by a biased sex ratio for this species (see Table 1) in the most differentiated sample in terms of shape (CHAUX site).

Size variation

The two size estimators (centroid size and euclidian distance between the landmarks 1 and 14) produced rather similar results; therefore, only those concerning centroid size are presented here. Not surprisingly, a clear sexual dimorphism in size was detected. For both species, females were significantly larger than males when tested on the all-individual data set as well as within each studied site (Table 3). Figure 7 shows, for both species and both sexes, the variation in centroid size among sites. *C. auronitens* samples were homogeneous

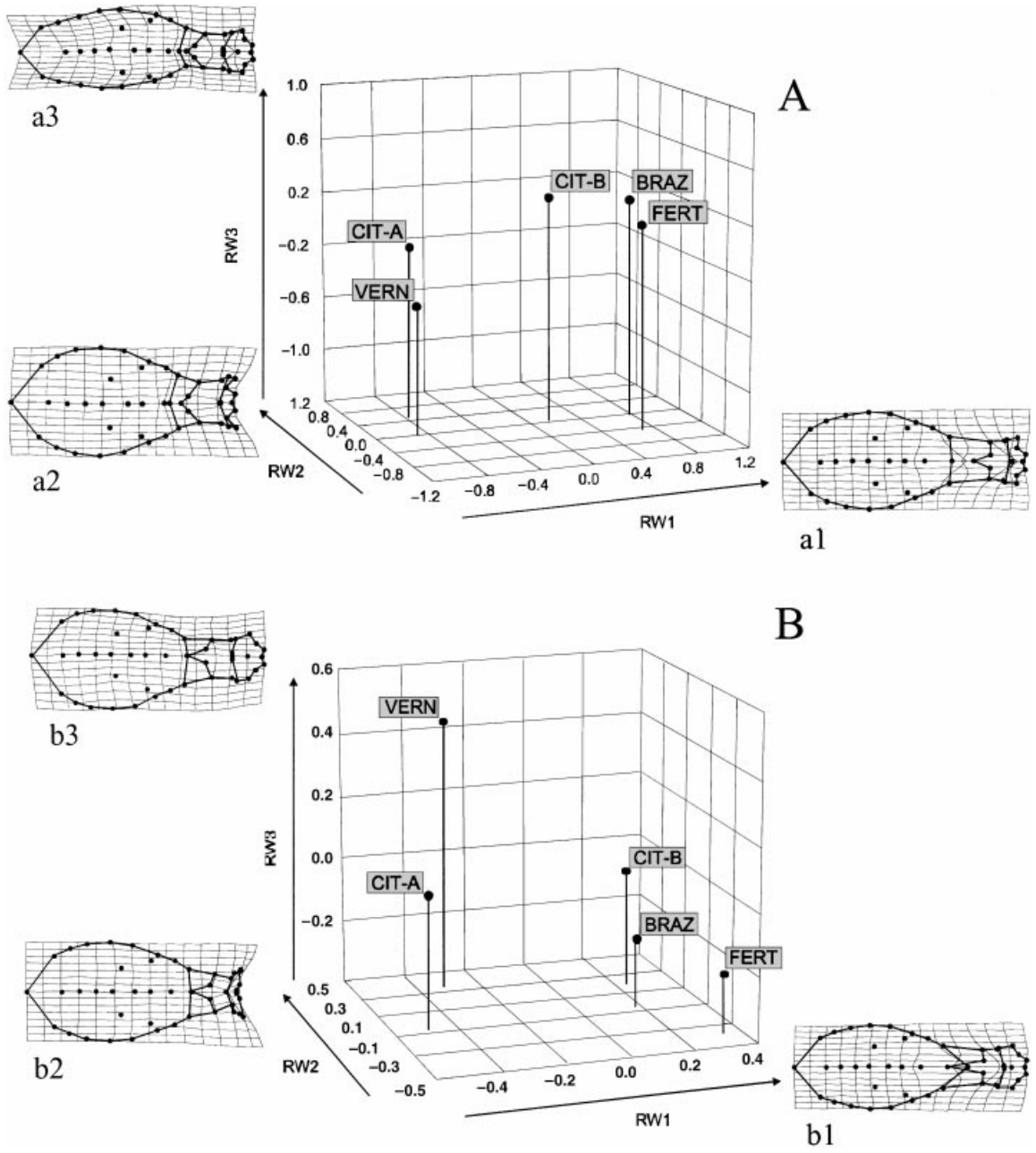

Fig. 6 Relative positions of the average configurations of the five sites for *C. nemoralis* in the shape space defined by the first three relative warps. —A. Females. —B. Males. See legend of Fig. 4 for explanation of the deformation grids. Scores on the relative warps are × 100 and deformation grids are magnified 2.5 times for females and three times for males.

with regard to size (only one comparison among sites was significant), whereas a relatively high number of comparisons (seven out of 20) were significant for *C. nemoralis*. For this species, individuals from BRAZ, and to a lesser extent those from CIT-B, were larger than the others, but differences were more pronounced for females than for males (Fig. 7).

Discussion

Morphological differentiation and geographical distance

The main objective of this study was to use a geometrical morphometric approach to detect space-related morphological changes within two species of ground beetle. We found, at least for *C. auronitens*, measurable morphological changes between populations. The body shape of specimens sampled

	C. auronitens				*C. nemoralis*			
Source of variation	*F*	Num. d.f.	Den. d.f.	*P**	*F*	Num. d.f.	Den. d.f.	*P**
All specimens	260.77	1	312	< 0.001	237.56	1	330	< 0.001
CIT-A	54.77	1	67	< 0.001	21.90	1	66	< 0.001
CIT-B	64.18	1	68	< 0.001	70.15	1	65	< 0.001
BRAZ	65.37	1	68	< 0.001	58.76	1	67	< 0.001
VERN	118.51	1	68	< 0.001	51.39	1	63	< 0.001
CHAUX	16.26	1	41	< 0.001	—	—	—	—
FERT	—	—	—	—	50.43	1	69	< 0.001

*Significance levels.

Table 3 Results, for both species, of the ANOVAs testing differences between the centroid size of males and females. Degrees of freedom of numerator and denominator are denoted 'Num. d.f.' and 'Den. d.f.', respectively.

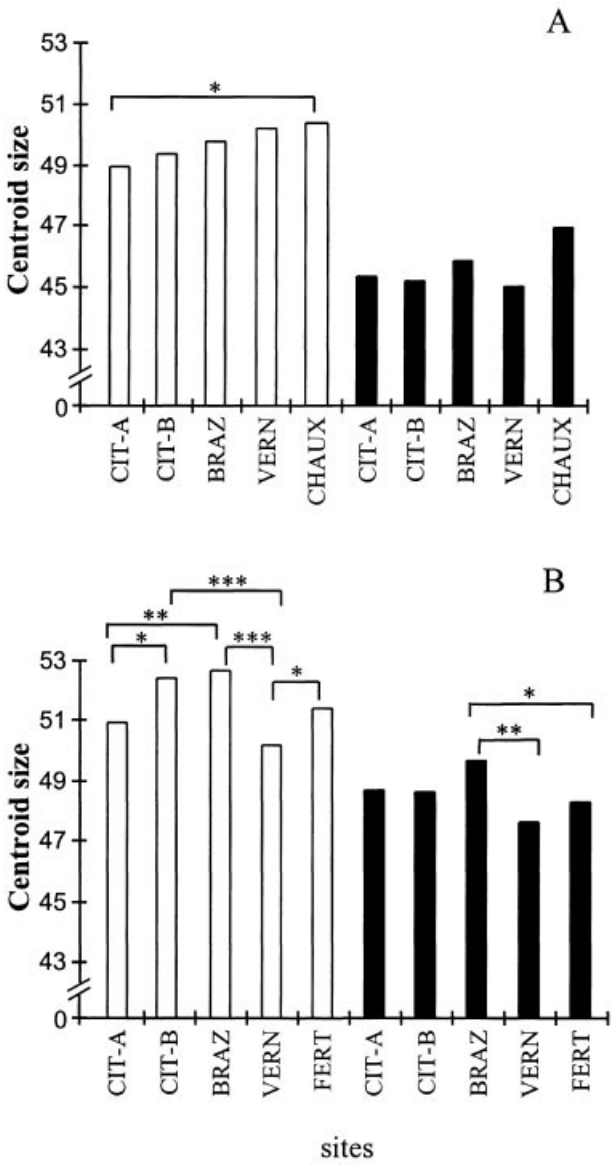

Fig. 7 Variation in centroid size among sites for both sexes. —A. *C. auronitens*. —B. *C. nemoralis*. Open bars, female samples; filled bars, male samples. Asterisks indicate when differences among sites are significant: $*P < 0.001$; $**P < 0.01$; $***P < 0.05$.

in the geographically most distant site (CHAUX site) was clearly differentiated from that of specimens originating from sites located 45 km apart; on the whole, the amount of morphological divergence between populations was significantly correlated to their geographical distance. These results were found for both sexes. We believe that such shape differences are the consequence of isolation and fragmentation, rather than simply a response to local environmental differences between sampling sites. One argument in favour of our results is that morphological differences between significantly differentiated sites concern only shape, but not size. It is generally considered that a variation in size between populations largely depends on environmental conditions, whereas a variation in shape reflects variation in the genetic constitution (see, for example, Patton & Smith 1989; Adams & Funk 1997). It is thus reasonable to consider that the shape changes reported here reflect a genetic differentiation between the Chaux forest population and those from the Cîteaux forest and its surroundings. Moreover, our results are in agreement with the available data concerning the population genetics of *C. auronitens*. On the basis of a study of variability at four electrophoretic loci, Assman *et al*. (1994) have proposed a phylogeographical scenario for the colonization of Europe after the last glaciation period. These authors postulate several refuge areas in southern France from which populations expanded their area to northern France and middle Europe. During this postglacial recolonization, the populations of *C. auronitens* would have undergone several bottlenecks, which would have been responsible for the genetic differentiation and reduced genetic variability observed in populations in central and northern France (Assman *et al*. 1994). Nowadays, the use of hypervariable genetic markers offers promising perspectives for the investigation of the genetic structure and population dynamics of beetles. In a pioneering work focusing on the population genetics of the endangered species *Carabus solieri*, Rasplus *et al*. (2000) have found, using microsatellite markers, fairly high genetic differentiation between the populations of the southern French Alps, even for those not too far apart (around 11 km apart). Fragmentation of the habitat of *C. solieri* could explain these low levels of gene flow (Rasplus *et al*. 2000). The use of such molecular markers would

allow us to test the congruence between morphological and genetic differentiation for *C. auronitens*.

At a closer scale, although we found a significant correlation between geographical and morphological distances, it is noteworthy that the various barriers separating sites do not play a major role in the differentiation of populations. Indeed, sites which are separated from the Cîteaux forest by barriers (VERN and, more conspicuously, BRAZ; Fig. 1) are not morphologically more differentiated than those located inside this continuous forest (sites CIT-A and CIT-B). Such a result may have several nonexclusive interpretations. First, differentiation among populations exists but the morphometric approach used was not powerful enough to detect it. A study of genetic markers would be helpful to test such a hypothesis. Second, the time elapsed since the sites BRAZ and VERN were isolated from the main part of Cîteaux forest has not been long enough to allow a measurable divergence between populations. As mentioned above, these sites have been isolated for at least 300 years. Most of the barriers are, of course, much more recent as the highways, railways and canal shown in Fig. 1, and the intensive exploitation of agricultural fields, all occurred during the 20th century. Third, despite the brachyptery, both species studied have a nonnegligible dispersal ability. The rare data concerning *C. nemoralis* indicate that this species does not present a strict association with forests and would be able to explore more open landscapes, such as set-aside or even arable habitats (Kennedy 1994). The fact that the geographically most distant site of our study (FERT) was not morphologically the most differentiated supports the idea that *C. nemoralis* would be able to move over relatively long distances in a heterogeneous environment. Concerning *C. auronitens*, although this species is more stenotopic and strictly bound to forest (Assman *et al.* 1994; Niehues *et al.* 1996), it could also be a better colonist than is generally believed (Niehues *et al.* 1996).

Sexual dimorphism

Another objective of the present study was to provide additional elements concerning morphological variation between sexes. As expected, we found an important female-biased sexual dimorphism in size for both *C. auronitens* and *C. nemoralis*. More interestingly, the geometrical morphometrics allowed us to show a sexual dimorphism of the shape, in particular for the posterior abdominal segments, which appeared to be more enlarged for the females of the two species. This sexual abdominal shape dimorphism, which has been reported for several groups of insects (Adams & Funk 1997), has been hypothesized to result from a positive correlation between fecundity and female abdomen size, and hence from a selection for the increase of fecundity (Wickman & Karlsson 1989; Adams & Funk 1997). This, as well as allometric trajectories, remains to be studied in the *Carabus* group.

Conclusions

Geometrical morphometrics, in identifying and quantifying biodiversity through the computation of morphospaces or disparity estimates, appears to be powerful enough to assess morphological variations at all taxonomic levels, including the intraspecific level. This latter characteristic offers a particularly interesting perspective, as intraspecific variation has until now mainly been assessed from a genetic viewpoint, and there is obviously a need to extend such an approach to phenotypic traits such as morphology. Our results demonstrate that geometrical morphometric methods provide valuable tools for the study of morphological variation among populations and thus offer promising perspectives for various problematics, such as the assessment of the impact of habitat fragmentation on species and, more generally, the study of biodiversity patterns. The present study provides additional support for the idea that geometrical morphometric methods have the potential to become one of the most powerful techniques for describing variation below the species level (Loy 1996). Moreover, in this work, we applied only one method of the several available in geometrical morphometrics. Other relative warps analyses, such as those offering the possibility to assign a given weight to large-scale or small-scale deformations on the specimen, may allow more accurate decomposition of the morphological variation in uniform and nonuniform shape transformations (Bookstein 1991; Rohlf 1993a). These techniques also allow a broader use of multivariate analyses (Bookstein 1996). Finally, it is worth reiterating that geometrical morphometrics also allows us to study conjointly numerous characters, as it proposes, in addition to the study of size, an overall assessment of the shape of organisms. The more traits taken into account, the better the estimation of morphological diversity.

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ARTICLE 2 :

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Isolation by distance and sharp discontinuities in gene frequencies: implications for the phylogeography of an alpine insect species, *Carabus solieri*

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Abstract

Analysis of genetic isolation by distance (IBD) is of prime importance for the study of processes responsible for spatial population genetic structure and is thus frequently used in case studies. However, the identification of a significant IBD pattern does not necessarily imply the absence of sharp discontinuities in gene frequencies. Therefore, identifying barriers to gene flow and/or secondary contact between differentiated entities remains a major challenge in population biology. Geographical genetic structure of 41 populations (1080 individuals) of an alpine insect species, *Carabus solieri*, was studied using 10 microsatellite loci. All populations were significantly differentiated and spatially structured according to IBD over the entire range. However, clustering analyses clearly identified three main clusters of populations, which correspond to geographical entities. Whereas IBD also occurs within each cluster, population structure was different according to which group of populations was considered. The southernmost cluster corresponds to the most fragmented part of the range. Consistently, it was characterized by relatively high levels of differentiation associated with low genetic diversity, and the slope of the regression of genetic differentiation against geographical distances was threefold those of the two other clusters. Comparisons of within-cluster and between-cluster IBD patterns revealed barriers to gene flow. A comparison of the two approaches, IBD and clustering analyses, provided us with valuable information with which to infer the phylogeography of the species, and in particular to propose postglacial colonization routes from two potential refugia located in Italy and in southeastern France. Our study highlights strongly the possible confounding contribution of barriers to gene flow to IBD pattern and emphasizes the utility of the model-based clustering analysis to identify such barriers.

Keywords: barrier to gene flow, clustering analysis, colonization, ground beetle, isolation by distance, microsatellites

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Introduction

The evolutionary potential of a species depends mainly on population structure, which results from a balance of evolutionary forces producing either local differentiation or homogeneity (Slatkin 1987). The relative importance of each factor may change in space and time, and some events can leave imprints for a long period of time (Hewitt 2000). In such a context the distinction between historical and present processes is considered a key point in studies of population differentiation. For species with low dispersal ability, a higher genetic similarity is expected between neighbouring individuals or populations than between distant ones. This pattern of genetic structure is called isolation by distance (IBD, Wright 1943). At equilibrium, under dispersal and genetic drift, IBD pattern is revealed by a positive and significant correlation between genetic

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differentiation and geographical distances (Slatkin 1993; Rousset 1997). Identification of IBD can help to show equilibrium between migration and genetic drift (contemporary processes), or to link limited dispersal ability and genetic differentiation. It can also allow the determination of the neighbouring size, the estimation of demographic parameters or the comparison of the relative influences of gene flow and drift on population structure among different regions (Slatkin 1993; Rousset 1997; Hutchison & Templeton 1999; Rousset 2000; Pogson *et al*. 2001). Although empirical studies provide numerous examples of IBD occurrence in natural populations (Slatkin 1993; Peterson 1996; Pogson *et al*. 2001 and references therein), there is growing evidence that many species have not yet reached migration-drift equilibrium and that observed patterns of genetic differentiation reflect population history rather than current levels of gene flow (e.g. Latta & Mitton 1999; Pogson *et al*. 2001; Turgeon & Bernatchez 2001). In such nonequilibrium systems, the study of the relationship between genetic and geographical distances can still be informative revealing, for instance, recent range expansion or a quasi absence of gene flow (Slatkin 1993; Hutchison & Templeton 1999). Hence, analyses of IBD patterns are of prime importance in identifying processes responsible for spatial population genetic structure and are thus used widely in case studies.

The occurrence and pattern of IBD depends on the spatial scale considered and can also change according to region and time, due to spatial and/or temporal variation in relative influences of forces moulding population structure (Slatkin 1993; Rousset 1997; Castric & Bernatchez 2003). Moreover, the assumptions of spatial and temporal stability in some models contrast with heterogeneity in terms of demography, environmental conditions or history characterizing natural populations. As a consequence, a significant IBD pattern does not necessarily mean a spatially homogeneous gene flow. Indeed, some authors have pointed out that signficiant IBD pattern could exist despite the presence of barriers to gene flow (Bossart & Prowell 1998; Lugon-Moulin & Hausser 2002). In such a context, it is of prime importance to be able to identify heterogeneity of gene flow due to such barriers. A model-based clustering method (Pritchard *et al*. 2000) can help to approach this problem. This method was developed to infer population structure and to assign individuals to populations using multilocus genotype data. Unlike previous assignment methods, the genetic composition of source populations is unknown (populations are not defined a priori). We will show that the combined analysis of IBD patterns and clustering analysis is a powerful approach to detect sharp discontinuities in gene frequencies due to physical barriers to gene flow and/or secondary contact.

In the present study we used 10 microsatellite loci to describe the genetic population structure of *Carabus solieri* Dejean (Coleoptera, Carabidae), a ground beetle distributed in the Southern Alps of France and Italy. This species exhibits high levels of diversity and differentiation for several characters including neutral genetic markers, morphology and colour (Bonadona 1973; Darnaud *et al*. 1978; Rasplus *et al*. 2001). As with many other ground beetles species, this brachypterous insect has limited dispersal abilities and is therefore susceptible to exhibit an IBD pattern if it has reached migration-drift equilibrium. However, it is suspected that current population structure still contains historical imprints and that the pattern of population structure is due partly to historical events, i.e. hybridization after a secondary contact between two differentiated entities considered as subspecies (Rasplus *et al*. 2001). In addition, the habitat structure within its range appears rather heterogeneous (due, in particular, to the mountainous relief) and provides numerous potential barriers to gene flow. Hence, our objectives were (i) to test for migration-drift equilibrium by studying the IBD pattern, (ii) to detect and identify potential barriers to gene flow, (iii) to determine the nature of such barriers (secondary contact vs. physical barriers) and (iv) to discuss plausible phylogeographical scenarios and in particular to infer recolonization routes and the origin of the different entities recognized in *C. solieri*.

Materials and methods

Species studied and sampling scheme

C. solieri occurs in a relatively restricted area in the southern and Ligurian Alps (Fig. 1). This species is associated mainly with humid forests, either deciduous or coniferous, but the species also occurs in dry Mediterranean forest and alpine grasslands. It is a spring breeder, laying eggs in spring and summer, depending on environmental conditions. Larval development occurs in summer and tenerals emerge in late summer or autumn, and overwinter in the soil. Mating will occur during the following spring. This ground beetle is endangered mainly by habitat destruction and fragmentation, especially in highly man-modified habitats in Estérel (southern France), and to a lesser extent by exhaustive collection by entomologists in specific areas. Consequently, *C. solieri* is protected in France.

Despite its limited range, *C. solieri* exhibits important variation in colour, morphology and genes (see Rasplus *et al*. 2001). As a result, intraspecific classification of *C. solieri* is unclear. Depending on authors, three to six subspecies are recognized, but some authors describe more. Rasplus *et al*. (2001) have proposed that *C. solieri* could be subdivided into two distinctive entities considered as subspecies, which differentiated during last glaciations within two different refugia, one in the south of France and one in Italy. After postglacial recolonization, these entities

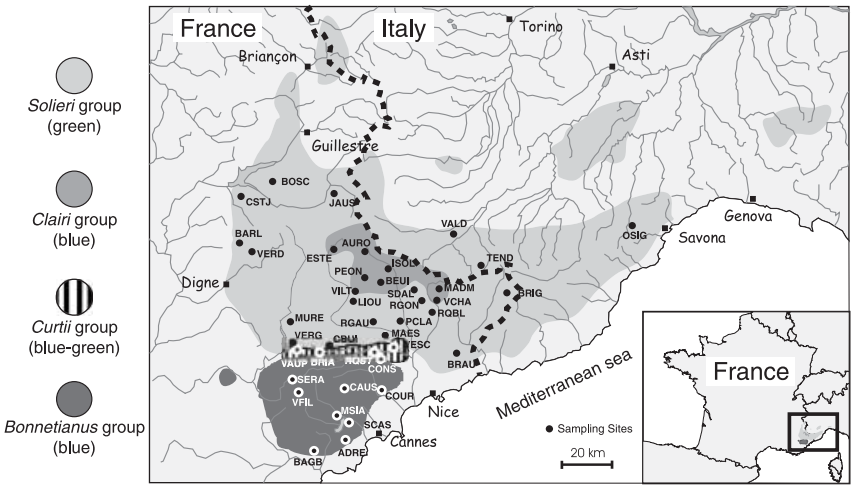

Fig. 1 Range of the different groups of *C. solieri* (see text for details) and location of sampling sites. Colour of individuals is given below the group label.

would have encountered each other, hybridized and introgressed with each other. Here, we will simply consider four groups of *C. solieri* without considering them as real taxonomic entities. Such groups are convenient because they have a geographical coherence and are consistent with the colour of individuals (Fig. 1). The *Bonnetianus* group occurs in the most southern part of the range, from the Estérel to the Montagne du Cheiron. It represents one of the two subspecies defined by Rasplus *et al*. (2001). The *Clairi* group inhabits mountain forests in the Mercantour massif. Individuals from both groups are deep metallic blue. Elsewhere a metallic green form occurs which we refer to as the *Solieri* group. Individuals with intermediate colour (blue-green) are found in the contact zone between *Bonnetianus* and *Solieri* groups. They are sometimes considered as a subspecies but are suspected to be hybrids between these two groups. We refer to them as the *Curtii* group.

Adults were collected with permission using rows of 20–60 unbaited pitfall traps during spring and summer 1997, 1998, 2000 and 2001. Pitfall traps were checked weekly or every 2 weeks all along the adult activity period (from April to August). Specimens were killed and stored in 100% ethanol at –22 °C until analysis. A total of 1080 individuals was collected from 41 localities (see details and localization of sampling sites in Table 1 and Fig. 1). We pooled samples from different years for four populations (AURO, CAUS, PEON and RQST, Table 1) because pairwise exact tests, performed with GENEPOP 3.3 (Raymond & Rousset 1995b) showed no significant genotypic differentiation between years.

Microsatellite analysis

Total DNA was extracted from muscles of a leg using standard phenol–chloroform extraction (Doyle & Doyle 1987). Individuals were genotyped at 10 microsatellite DNA loci: eight were cloned and characterized in *C. solieri*, one in *C. punctatoauratus*, and one in *C. nemoralis* (see Table 2). Primer sequences and other information are given in Garnier *et al*. (2002), except for locus Cn2B (isolated in *C. nemoralis*), whose unpublished primer sequences are ACA-GAA-CGA-CTA-AAA-TGA-ACA-C and ATA-ATC-TAC-CCA-CAA-CCG-AC (GenBank Accession no. AY464122). Genotyping was performed following two methods, depending on the locus (Table 2). In the first method, polymerase chain reaction (PCR) amplifications were carried out with radiolabelled dATP with αP^{33} (see Garnier *et al*. 2002 for details). PCR products were run on 6% denaturing polyacrylamide gels, and the fragments were visualized by autoradiography. Allele size was determined by reference to the original cloned allele. In the second method, one of the two primers was labelled at the 5′ end with one of the fluorescent dyes FAM, HEX or NED (Table 2). Loci giving sufficiently different size ranges were labelled with the same dye, in order to maximize the number of loci multiloaded. PCR amplification were performed as described in Garnier *et al*. (2002), but the reaction mix was slightly different as there was no αP^{33} and the mix contained 4 μM of the nonfluorescent primer and × μM of the fluorescent primer (× depending on the locus, Table 2). PCR products were analysed in the ABI 310 automated sequencer following the manufacturer's protocol using GENESCAN analysis 3.1 and GENOTYPER 2.5 software.

Intrapopulation genetic diversity

Intrapopulation genetic variation was estimated by the number of polymorphic loci (N_{pol}), observed (H_O) and expected (H_E) heterozygosities (unbiased estimate, Nei 1978), using GENETIX 4.04 (Belkhir *et al*. 2001). The observed number of alleles in a sample is highly dependent on sample size, consequently allelic richness was calculated using a rarefaction index, as suggested by El Mousadik & Petit (1996). Given that 2*N* genes have been sampled, the

Locality		Year of sampling	N	N_{pol}	A	H_O	H_E
Les Adrets de l'Estérel	ADRE	1997	20	7	3.0	0.43	0.41
Auron	AURO	1998, 2001	20	9	4.0	0.46	0.46
Bagnols en Forêt	BAGB	2000	24	5	1.5	0.15	0.14
Barles	BARL	2001	25	8	2.9	0.37	0.35
Beuil	BEUI	1998	27	9	4.5	0.52	0.53
Boscodon	BOSC	2001	29	8	2.6	0.28	0.30
Col de Braus	BRAU	1998	24	10	3.5	0.47	0.50
Briançonnet	BRIA	2000	30	8	2.9	0.45	0.42
La Brigue	BRIG	1998	21	10	5.3	0.60	0.67
Caussols	CAUS	1998, 2000	27	9	2.8	0.48	0.49
Col du Buis	CBUI	2000	30	8	3.3	0.43	0.45
Conségudes	CONS	2000	30	10	4.6	0.58	0.56
Courmette	COUR	1998	20	8	2.5	0.34	0.34
Col Saint-Jean	CSTJ	2001	15	8	2.8	0.30	0.31
Esteng	ESTE	1998	11	8	4.6	0.45	0.50
Isola	ISOL	1998	21	10	4.5	0.55	0.56
Jausiers	JAUS	2001	25	5	1.7	0.23	0.21
Le Liouc	LIOU	2001	41	8	3.1	0.38	0.37
La Madone de Fenestre	MADM	1998	22	10	4.2	0.50	0.51
Malaussène	MAES	2000	26	10	4.0	0.48	0.49
Montauroux	MSIA	2001	31	5	2.2	0.25	0.26
Mure	MURE	1998	18	6	3.1	0.33	0.34
Osiglia	OSIG	1997	44	10	5.2	0.58	0.64
Pont de Clans	PCLA	2000	36	8	3.4	0.42	0.44
Péone	PEON	1998, 2000	33	9	4.8	0.53	0.54
Rigaud	RGAU	1998	21	8	3.3	0.40	0.41
Rigons	RGON	2000	30	10	4.9	0.60	0.60
Roquebillière	RQBL	2000	30	9	3.9	0.54	0.57
Roquestéron	RQST	1997, 1998, 2000	26	10	3.7	0.47	0.47
Saint-Cassien	SCAS	1997	28	7	2.4	0.32	0.34
Saint-Dalmas	SDAL	2000	17	9	4.7	0.56	0.57
Séranon	SERA	2000	30	9	3.9	0.48	0.47
Tende	TEND	1998	25	10	5.1	0.58	0.63
Valdieri	VALD	1998	25	9	2.3	0.36	0.32
Vauplane	VAUP	1998	24	9	3.7	0.50	0.48
Vallon des Châtaigniers	VCHA	2000	30	10	3.8	0.43	0.45
Verdaches	VERD	2001	35	7	2.9	0.33	0.34
Vergons	VERG	1998	20	9	4.1	0.52	0.52
Vescous	VESC	2000	30	8	2.7	0.35	0.37
Vallon du Fil	VFIL	2000	30	9	2.9	0.32	0.35
Villetalle	VILT	2000	29	9	4.1	0.49	0.52

Table 1 Sampling sites and genetic polymorphism of the 41 populations studied. N, sample size; N_{pol}, number of polymorphic loci; A, allelic richness (estimated for a sample of 11 individuals); H_O, observed heterozygosity; H_E, gene diversity; A, H_O, H_E are averaged over 10 loci

expected number of alleles in a subsample of $2n$ genes ($n \leq N$) was estimated using FSTAT 2.9.3 (Goudet 1995) [n is fixed as the smallest number of individuals genotyped for a locus in a sample, i.e. $n = 11$ (ESTE sample)]. Different statistics were computed because the behaviour of these statistics can vary according to population history (Cornuet & Luikart 1996). Intrapopulation genetic diversity was assessed because it can reflect past range expansion (Le Corre & Kremer 1998; Hewitt 2000) or current habitat fragmentation.

Linkage disequilibrium between all pairs of loci and departure from Hardy–Weinberg equilibrium for each locus were tested within each population using exact tests. A global test for Hardy–Weinberg equilibrium across all loci was constructed using Fisher's method (Sokal & Rohlf 1995), providing that statistical independence of loci was established previously. Calculations were performed using GENEPOP 3.3 (Raymond & Rousset 1995b). Deviations from Hardy–Weinberg proportions were quantified by the unbiased estimator of Wright's inbreeding coefficient F_{IS} calculated according to Weir & Cockerham (1984).

Population differentiation and IBD

In order to assess the among-population variability, we first considered each sampling site as a distinct population. Differentiation for both all populations and all

Table 2 Polymorphisms at 10 microsatellite loci over 41 populations of *C. solieri*. Loci were isolated in *Carabus solieri* (Csol), in *C. nemoralis* (Cn) or in *C. punctatoauratus* (Cp). Labelling corresponds to radiolabelled dATP (= radio) or fluorescent primer [= fluo (dye group, concentration in mix reaction in μM)]. Tm is the annealing temperature in °C (TD means 'touchdown' procedure, see Garnier *et al*. 2002); range is the size in base pairs of the smallest and the largest allele; N_A is the total number of alleles found; n_A is the mean number of alleles found in one population (averaged over localities) with its standard deviation (SD); n_{min} and n_{max} are the minimum and the maximum number of alleles found in one locality, respectively

Locus	Labelling	Tm	Range	N_A	n_A (SD)	n_{min}	n_{max}
Csol 10129B	radio	TD 60	156–208	22	5.00 (2.07)	2	11
Csol 1122	fluo (FAM; 0.15)	TD 62	113–166	22	5.90 (2.60)	1	13
Csol 1259	fluo (HEX; 0.10)	TD 60	161–213	21	6.10 (2.66)	2	13
Csol 13F	fluo (NED; 0.40)	TD 60	163–171	6	1.88 (0.87)	1	5
Csol 6103	fluo (NED; 0.15)	TD 60	230–240	6	2.46 (0.92)	1	5
Csol 8155	radio	TD 55	126–192	30	5.80 (2.93)	1	14
Csol 828	fluo (FAM; 0.10)	TD 62	176–192	6	1.93 (0.96)	1	6
Csol 9170	fluo (FAM; 0.10)	54	208–250	11	2.68 (1.21)	1	5
Cn 2B	fluo (HEX; 0.15)	TD 58	272–444	62	7.93 (4.81)	1	18
Cp 1/24	radio	TD 55	133–222	30	2.95 (2.30)	1	13

population pairs was tested using a log-likelihood (G)-based exact test (Goudet *et al*. 1996). These tests were performed for each locus and then combined in a global test with Fisher's method (Manly 1985). In addition, both global and pairwise estimates of F_{ST} were computed following Weir & Cockerham (1984) to quantify levels of differentiation.

Finally, IBD over the distribution area was assessed by testing the correlation between genetic and geographical distance considering all population pairs in using the regression of $F_{ST}/(1–F_{ST})$ estimates on logarithm of distance for populations, as suggested by Rousset (1997). This model was tested using Mantel's tests. For geographical distances, we considered straight-line distances between all pairs of sampling sites. IBD was also tested in each cluster of populations identified according to the results of the clustering analysis (see Results section) with the same procedure. All these tests and calculations were performed with GENEPOP 3.3 (Raymond & Rousset 1995b).

Genetic model-based clustering

We used the model-based clustering method described by Pritchard *et al*. (2000) to infer population structure and assign individuals to populations using multilocus genotype data, as implemented in the program STRUCTURE. The model assumes K populations (or clusters; K may be unknown, as in the present case) modelled each by its own set of allele frequencies at each locus. The genetic composition of these populations and the assignation of individuals are both unknown. Assuming Hardy–Weinberg equilibrium and complete linkage equilibrium between loci within populations, population allele frequencies and assignation of individuals to populations were inferred simultaneously using a Bayesian approach.

Generally, the number of clusters (K) in the data is inferred from the posterior probability distribution $\Pr(K \mid X)$ calculated from the posterior probability of the data $\Pr(X \mid K)$ (X being the genotypes of the sampled individuals). Choosing K that maximizes the posterior probability of the data (PPD) can be difficult to apply for complex data sets including many groups (Rosenberg *et al*. 2002). In this case of highly structured data, as K is increased the most divergent groups separate into distinct clusters first, in some cases analogously to the hierarchical branching of tree diagrams (Pritchard *et al*. 2000; Rosenberg *et al*. 2002). As we should aim for the smallest value of K that captures the major structure in the data, a second way to choose K is to consider the successive increase of the PPD for increasing values of K, which can be regarded as the gain of information at each addition of a set of allele frequencies. However, it should be emphasized that the PPD is not an accurate estimate and should be regarded as a heuristic guide to which models are most consistent with the data (see Pritchard *et al*. 2000). In the simple case of individuals sampled from two differentiated locations, the more differentiated these two populations, the more important would be the increase of PPD between $K = 1$ and $K = 2$. Suppose now a set of differentiated populations exhibiting an IBD pattern. As runs are performed with increasing K, the study area would be partitioned into smaller and smaller subareas, until each sampling location, and populations would be clustered according to their geographical proximities. Differences in allelic frequencies between clusters would be less important as K is incremented. Thus, we would expect the PPD increase from $K = 1$ to K to be equal

to the number of sampling sites but to a lesser and lesser extent. In other words, the information brought by each successive additional set of allele frequencies would gradually decrease. By adopting this approach, it is then theoretically possible to identify clusters of populations separated by barriers to gene flow (or vicariant events), as strong allele frequency changes are usually evident on either side of these barriers. In such a context, the gain of PPD would be high until the value of K is equal to the number N of such clusters; then, this gain would drop for a value of $K = N + 1$ and eventually gradually decrease for greater values of K if IBD is occurring in some clusters. We then paid attention both to values of the PPD for each run and to the importance of the increase of the PPD for successive values of K.

Independent runs of the program were carried out for the total data set for values of K comprised between one and 40 (almost the number of sampling sites). As three main clusters were identified (see Results section), STRUCTURE runs were then performed within each cluster. All runs were based on 100 000 iterations after a burn-in period of 20 000 iterations. A minimum of five independent runs were conducted for each situation (number of cluster — data set combination) in order to assess the consistency of the results across runs, using admixture model and uncorrelated allele frequencies model without incorporating population information (see Pritchard *et al*. 2000 for details). We used the program DISTRUCT (Rosenberg 2002) to display individuals' membership coefficients for each cluster. Finally, we also performed an analysis of molecular variance (AMOVA, Excoffier *et al*. 1992) as implemented in ARLEQUIN 2.0 (Schneider *et al*. 2000) to quantify the different genetic variance components (among groups, among populations within groups, within populations).

Results

Intrapopulation genetic diversity

Data relative to polymorphism of each locus are presented in Table 2. Considering all the loci, a total number of 216 alleles were found. The amount of polymorphism varied greatly among loci, ranging from six to 62 alleles (Table 2). The mean number of alleles per locus found in one locality varied from 1.88 to 7.93. Some loci presented high intrapopulation polymorphisms, e.g. 18 alleles found in the locality OSIG (44 individuals) for the locus Cn 2B (Table 2).

Thirty of 41 localities had a fixed allele for at least one locus, some of which BAGB, JAUS and MSIA showed fixed alleles for five of the 10 loci (Table 1). Allelic richness averaged over loci ranged from 1.5 to 5.3 (Table 1). Gene diversity also varied among localities, ranging from 0.14 for BAGB to 0.67 for BRIG (Table 1). Both allelic richness and gene diversity exhibited the same clear spatial pattern,

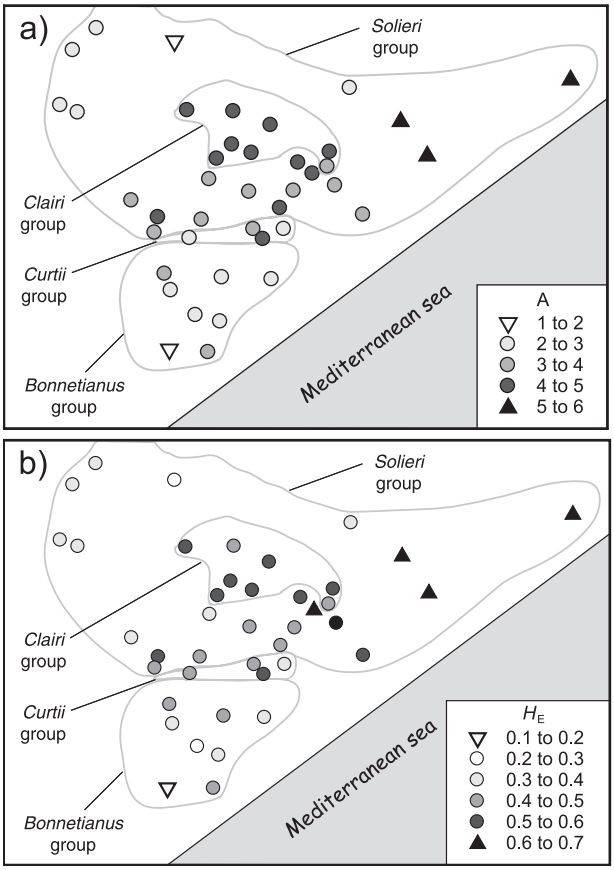

Fig. 2 Geographical distribution of (a) mean allelic richness estimated for a standardized sample size of 11 individuals [A] and (b) gene diversity [H_E]. Limits of the four groups of *C. solieri* are enclosed by grey lines.

i.e. an increase from south to northeast and from northwest to east (Fig. 2).

Out of the 1335 exact tests performed (population–loci pair combinations) for genotypic disequilibrium, only 57 (4.27%) were significant at the 0.05 level. This was less than the 5% expected to be significant by chance alone. Moreover, a single test remained significant after Bonferroni correction. There was therefore no evidence of linkage between loci, which were then considered statistically independent.

Hardy–Weinberg equilibrium was tested for each locus in all populations. A total of 335 exact tests was performed and 23 (6.9%) were significant at the 0.05 level, which is slightly over the proportion expected by chance alone. These significant tests concerned eight of the 10 loci and 16 of the 41 localities (results not shown). Four tests remained significant after Bonferroni correction: loci Csol1259 for BRIG, Csol13F and Cn2B for OSIG, and Csol13F for RQBL. Global test was significant for nine populations (two heterozygote excess and seven heterozygote deficiency) at the 0.05 level and only two remained significant (BRIG and OSIG) after Bonferroni correction. Amplification of locus

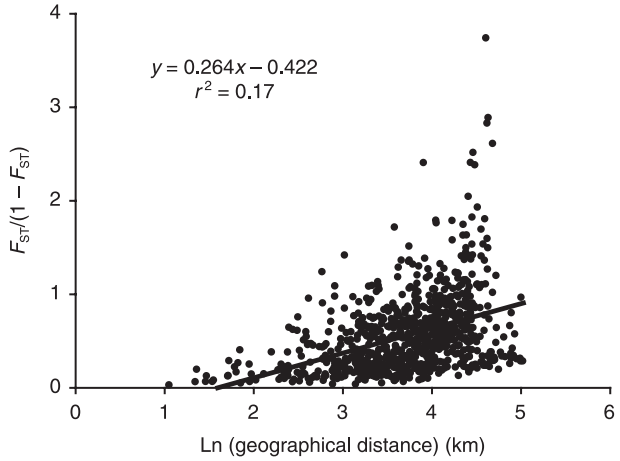

Fig. 3 Isolation by distance pattern in the distribution area. Regression of genetic differentiation [estimated by $F_{ST}/(1 - F_{ST})$] against logarithm of geographical distances (km) for all pairs of sampled populations.

Csol13F could not be obtained for five individuals from the OSIG population. Microsatellite isolation used an individual from SCAS population, one of the furthest populations from OSIG and belonging to a different subspecies (Rasplus *et al*. 2001). Therefore, we cannot exclude the presence of null alleles, e.g. due to mutation in the flanking region as a cause of the heterozygote deficiency observed. However, as there was no tendency for a particular locus to present systematically a heterozygote excess or deficiency, all loci were included in the following analyses.

Population differentiation and IBD

Genetic differentiation across all populations was highly significant for each locus and over all loci ($P < 0.0001$). Values of F_{ST} ranged from 0.256 for locus Csol1259 to 0.550 for locus Csol6103, and F_{ST} was 0.335 over all loci.

Of 8200 exact tests for single-locus genotypic differentiation between population pairs, 7090 (86.5%) were significant at the 0.05 level. The proportion of significant tests varied from 61.34% for locus Csol 13F to 98.90% for locus Csol 1122, which is much more than expected under the null hypothesis of identical genotypic distribution across populations. All pairwise tests (820 population pairs) over all loci were highly significant, even after Bonferroni correction. Multilocus F_{ST} ranged from 0.022 (SDAL vs. RGON) to 0.789 (BAGB vs. JAUS). The overall level of differentiation is high as nearly 50% of the pairwise multilocus F_{ST} were over 0.3 and 11% were greater than 0.5, whereas only 5% were lower than 0.1. As seen previously, this level of differentiation was not due to one or two particular loci. Finally, genetic differentiation between population pairs increased significantly with geographical distance ($r = 0.41$, $P < 10^{-5}$; Fig. 3).

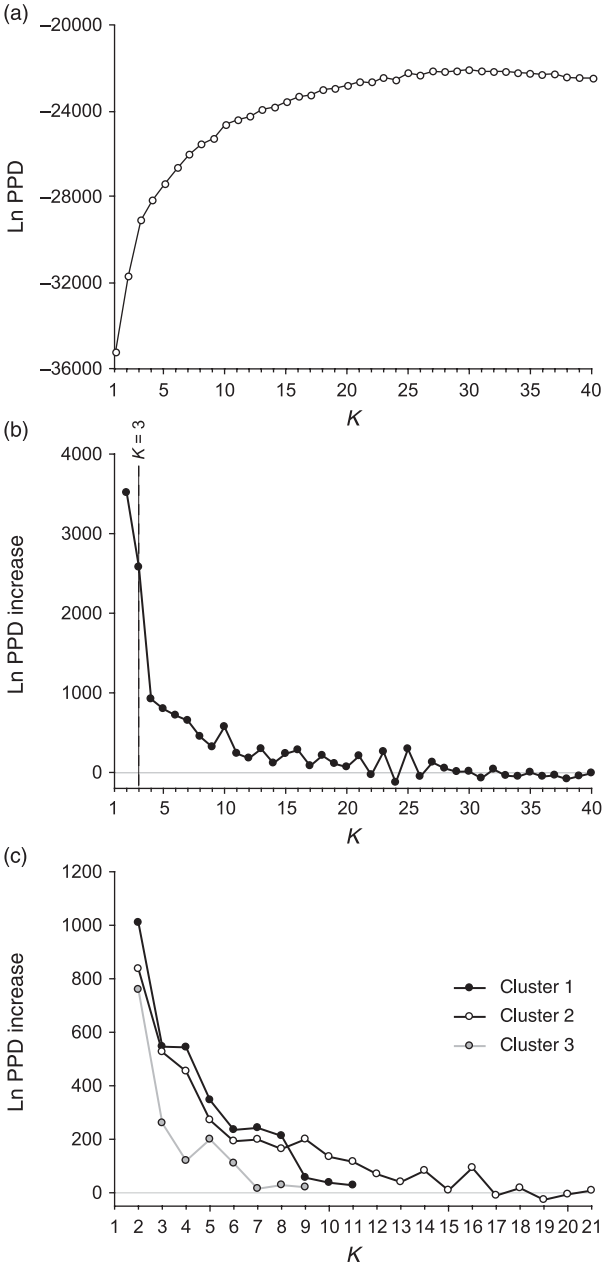

Fig. 4 Posterior probability of the data (PPD) against the maximum number of clusters (K) considered (a) and increase of PPD given K (b and c). For K clusters, this increase is calculated as (Ln PPD_K – Ln PPD_{K-1}). (a and b) Analysis for all data. (c) Analysis for the three main clusters (clusters 1, 2 and 3).

Genetic model-based clustering

Genetic structure over the whole distribution range. When considering all individuals, the PPD increased from $K = 1$ to $K = 30$, where it reached its maximum value and exhibited a plateau (Fig. 4a). This result indicates that differentiation occurs between most of the sampling sites. Concurrently, almost all individuals from the same population had

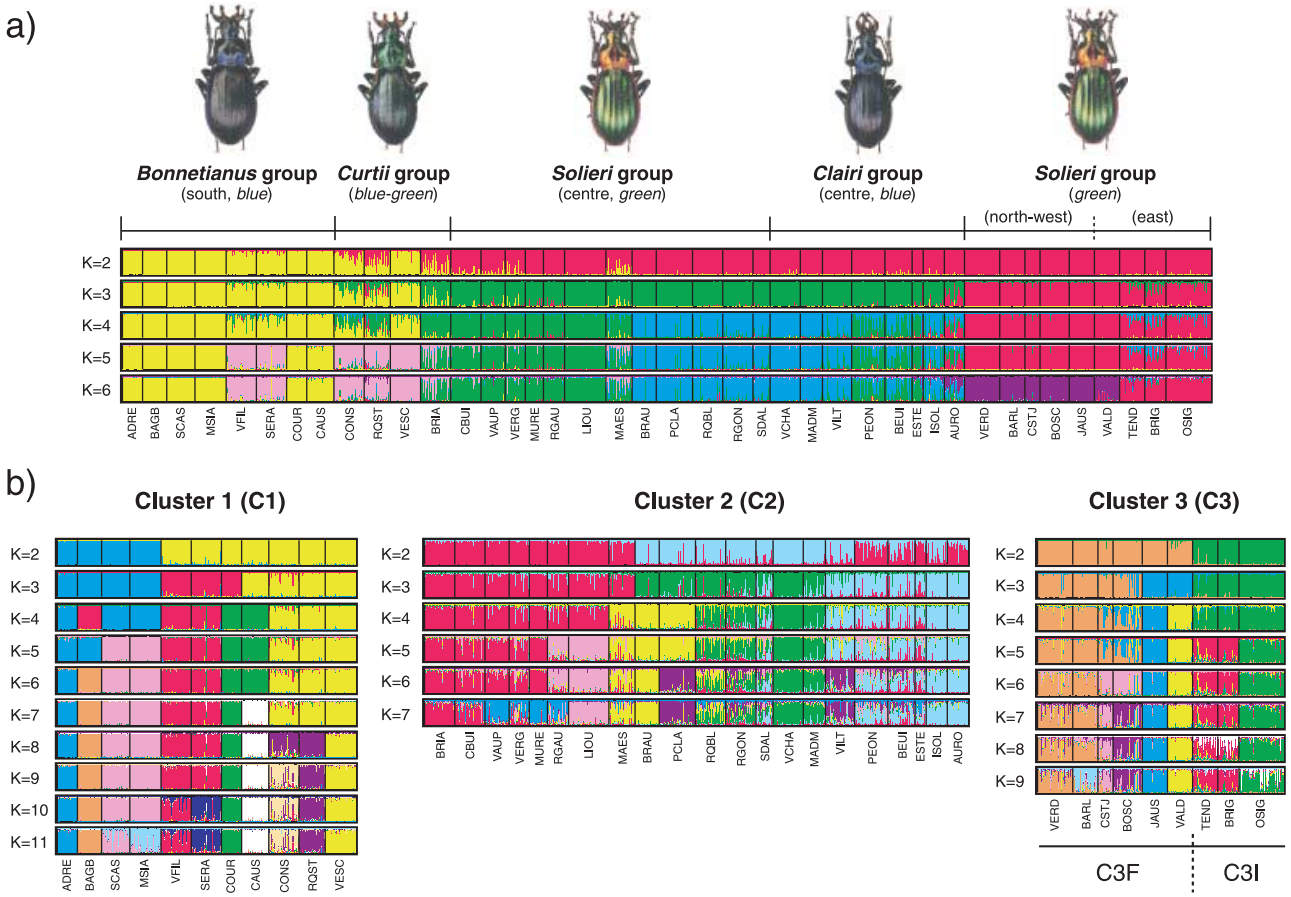

Fig. 5 Clustering results (a) for all populations sampled (for $K = 2$–6) and (b) for the three main clusters (clusters 1, 2 and 3 for $K = 2$–11, 7 and 9, respectively). Each individual is represented as a vertical line partitioned into K coloured segments, whose length is proportional to the individual's estimated membership coefficients in the K clusters. Individuals of different populations are separated by a black line. Populations are labelled below the figure and are approximately sorted from south to north (from the left to the right of the figure). Group affiliation (see text for details), localization in the distribution range and colour of individuals are given above the figure. For each data set and each K, figures are based on the run with the highest posterior probability of the data. There is no colour correspondence across figures based on different data sets.

similar membership coefficients (Fig. 5a). Generally, one of the clusters of individuals obtained for a given value of K is split into two clusters for $K + 1$. Furthermore, clusters always corresponded to geographical groups of populations in a smaller and smaller spatial scale. Theoretically, the PPD would always increase for growing values of K. However, as specified by Pritchard *et al*. (2000), the PPD is not really estimated but rather approximated in an *ad hoc* way, which explains the presence of a more or less plateau for values of K equal or superior to the actual number of populations in the sample.

Whereas these results are consistent with an IBD pattern, we could nevertheless identify three main clusters of populations. First, the increase of the PPD is high for $K = 2$ and $K = 3$, then for $K > 3$ the gain of information was definitely less and exhibited gradually decreasing values (Fig. 4b). This result means that the information brought by the fourth cluster (and the following) is much less important than the information brought by the former three. Second, once two populations have been assigned to different clusters for $K = 3$, they never belonged to the same cluster for greater values of K (partially shown in Fig. 5a). Third, whereas several clustering solutions appeared (with similar PPD) for $K = 2$, 10 runs gave exactly the same clustering solution for $K = 3$. When trying to characterize more gene pools than there are sets of allele frequencies in the model, several solutions can be found.

The three main clusters, hereafter called C1, C2 and C3, corresponded to the *Bonnetianus* and *Curtii* groups (except BRIA) for C1, the *Solieri* group from the centre of the range and the *Clairi* group and population BRIA for C2, the *Solieri* group from the northwest and the east of the range for C3 (Figs 1 and 5a). When considering the total sample, results of the AMOVA (Table 3) show that the among-cluster

Table 3 Results of the analysis of molecular variance (AMOVA). C1, C2 and C3 are the three main clusters defined according to the clustering analysis

Data set	No. of populations	Variance components		
		Among clusters	Among populations within cluster	Within populations
All populations	41	19.5	18.7	61.8
C1 + C2	32	16.5	19.9	63.6
C1 + C3	20	26.1	21.3	52.6
C2 + C3	30	19.0	15.3	65.7
C1	11		34.7	65.3
C2	21		18.0	82.0
C3	9		20.8	79.2

variance component (19.5%) is nearly equal to the among-population within-cluster component (18.7%). Thus, the within-population variance component accounted for around 60% of the genetic diversity of *C. solieri*.

Genetic structure at regional scale. The clustering analysis was conducted independently for each of the three main clusters. Individuals from RQST and CONS (*Curtii* group) had partial membership in two or three clusters (Fig. 5a). Two reasons enabled us to include these populations in C1: (i) individuals were assigned mainly to C1 (average membership coefficients to C1, C2 and C3 were 0.55, 0.26 and 0.19 for RQST and 0.68, 0.29 and 0.03 for CONS); and (ii) for $K > 4$, individuals were assigned with other populations of C1. Similarly, AURO was included in C2.

For each cluster, we found similar patterns: (i) the PPD increased with increasing value of K; (ii) similar membership coefficients were observed for individuals sampled in the same locality; and (iii) nearby populations were always clustered. However, several elements show that population structure is different according to the cluster considered. Individuals from C1 were more strongly assigned to populations than individuals from C2 and C3 clusters (Fig. 5b). Furthermore, the among-population variance component accounted for a third of the genetic diversity of C1, while it accounted for only a fifth for C2 and C3 (Table 3). Finally, the increase of PPD in C3 was important between $K = 1$ and 2, but sharply diminished from $K = 3$ (Fig. 4c). Populations assigned to different clusters for $K = 2$ never clustered together for greater values of K. As a result, C3 could be subdivided into two subgroups: C3F (for France) and C3I (for Italy) (Fig. 5b). On the other hand, such subdivision was not feasible in C1 and C2.

Within- and between-cluster IBD patterns. The relationship between genetic differentiation and geographical distance was assessed in each cluster independently, and between adjacent clusters. Within each cluster, genetic differentiation was correlated positively and significantly to geographical distance ($P = 0.035$, $P < 0.001$ and $P = 0.011$ for C1, C2 and C3, respectively). However, the slope of the regression was three times higher in C1 than in both C2 and C3 (Fig. 6). We plotted population pairs belonging to different clusters on the same figures. In the case of C1 and C2, for the same geographical distances, between-cluster differentiation (C1C2) was higher than within-C2 differentiation (C2C2) but was equivalent or slightly lower than within-C1 differentiation (C1C1, Fig. 6a). This indicates that the strong allele frequencies changes between C1 and C2 separated a homogeneous cluster (C2, among-population variance component = 18.0%, Table 3) and a heterogeneous cluster (C1, among-population variance component = 34.7%, Table 3). C1 seems more prone to genetic drift compared to gene flow than C2.

In order to assess the spatial variation of the relative influence of drift and gene flow in the contact zone between C1 and C2, we defined six 'latitudinal groups of populations' (LG, Fig. 7). We assessed trends of the regression slopes from IBD analysis within each of these groups (Fig. 6c). Slopes of the regressions were gentle for LG at the north of the *Curtii* group (LG4, LG5, LG6). The slope was steep for LG3, and even steeper when considering southern groups (LG1 and LG2), both belonging to the *Bonnetianus* group.

In the case of clusters 2 and 3, we distinguished pairs implicating populations from C3F and C3I subgroups in the intercluster population pairs because they clearly exhibit a different pattern. When considering C3I and C2, between-cluster differentiation (C2C3I) is the same as within-cluster differentiation (C2C2 and C3C3, Fig. 6b) for comparable geographical distances. Slopes of the regressions are also similar. On the other hand, when considering C3F, slopes of regressions are similar but between-cluster differentiation (C2C3F) is much higher than within-cluster differentiation (C2C2 and C3C3, Fig. 6b) for similar geographical distances, thus clearly indicating the presence of a barrier to gene flow between C2 and C3F. Neither comparison of regression slope in IBD analysis in the two subgroups C3F and

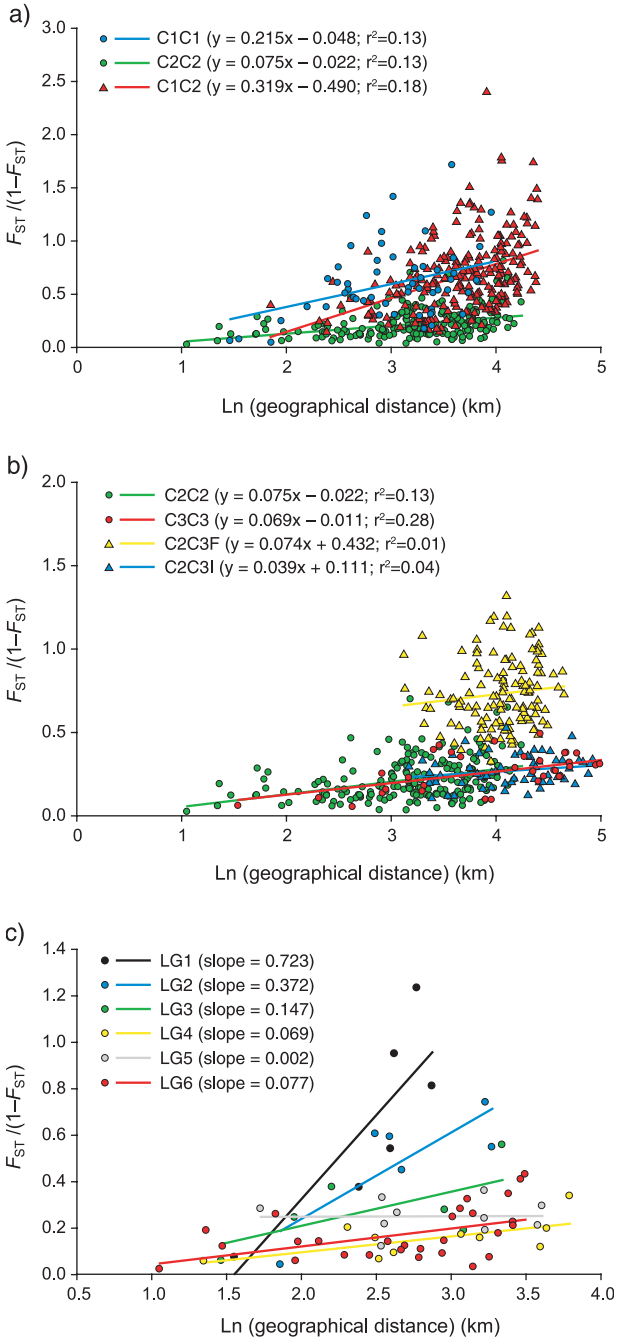

Fig. 6 Relationship between genetic differentiation [estimated by $F_{ST}/(1\text{-}F_{ST})$] and logarithms of geographical distances (km) in parts of the distribution area. Scatterplots and regression for (a) clusters 1 and 2: within-cluster 1 population pairs (G1G1), within-cluster 2 population pairs (G2G2) and between-cluster population pairs (G1G2); (b) clusters 2 and 3: within-cluster 2 population pairs (G2G2), within-cluster 3 population pairs (G3G3), and between-cluster 2 and subclusters 3F (G2G3F) and 3I (G2G3I), respectively. (c) Latitudinal groups of populations (LG): populations were pooled according to their latitude, from the south (LG1, within-LG1 population pairs) to the north (LG6, within-LG6 population pairs) (see Fig. 7 for the definition of these groups).

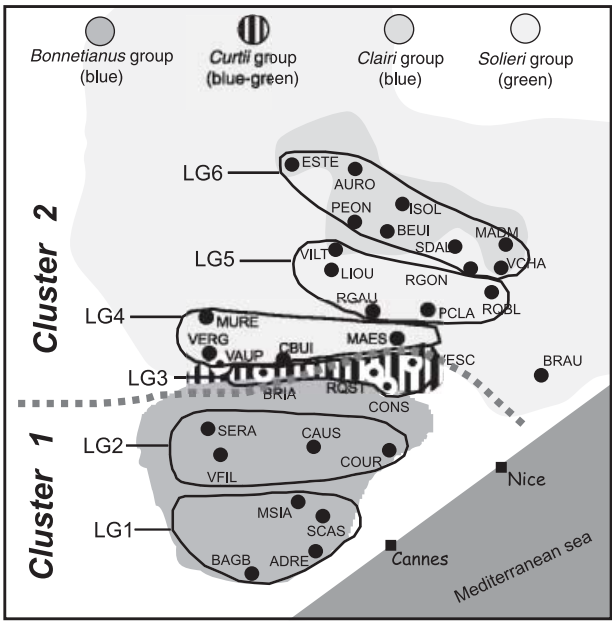

Fig. 7 Definition of six latitudinal groups of populations called LG1 to LG6 from the south to the north. BRAU population was included in any group because of its outlying position.

C3I nor comparison of intra- and intersubgroup regressions were performed because C3I subgroup is constituted of only three populations and intra- and intersubgroup spatial scales were different.

Discussion

Population differentiation and IBD

An important first result of our study is that there is significant genetic differentiation among *C. solieri* populations and that these populations are geographically structured according to an IBD pattern. All populations pairs were significantly differentiated, even at a geographical distance as small as 3 km (e.g. between RGON and SDAL). As a whole, levels of differentiation were high, as 40% of the genetic variance was due to differences among populations (Table 3), and pairwise values of F_{ST} reached a maximum value of 0.789 (between BAGB and JAUS). Previous studies on ground beetles have reported population differentiation at local scale (less than 15 km) even in relatively homogeneous areas (Assmann & Weber 1997; Brouat *et al*. 2003; Keller & Largiadèr 2003) and identified roads and nonforest areas as effective barriers to gene flow. Here, however, differentiation levels are much higher. For instance, for similar geographical distances (less than 15 km), pairwise F_{ST} range from 0.07 to 0.28 for *C. solieri*, whereas they range from 0.01 to 0.06 for *Carabus punctatoauratus*, another forest brachypterous ground beetle (Brouat *et al*.

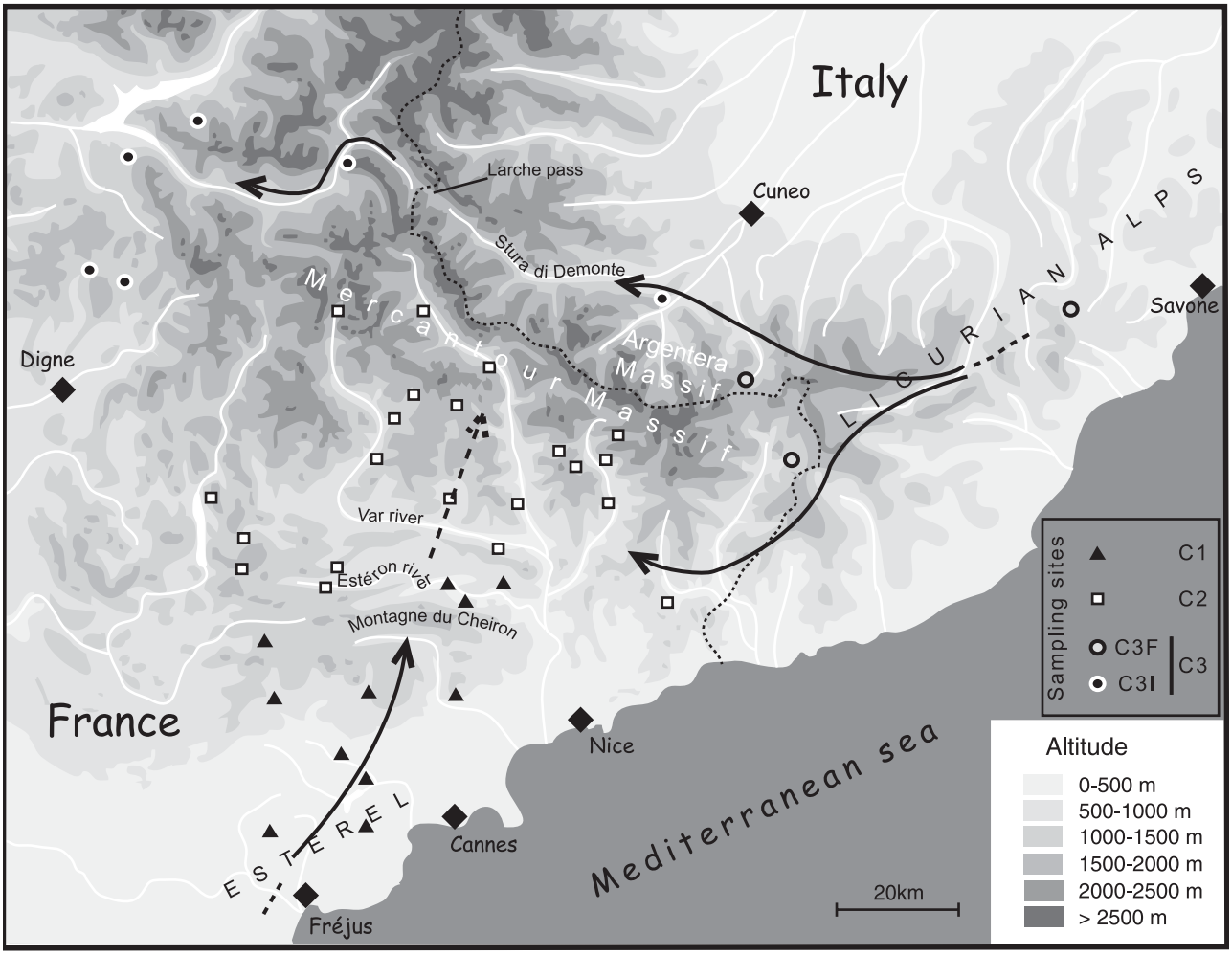

Fig. 8 Hypothetical postglacial colonization routes of *C. solieri*. Black arrows indicate the two colonization routes from the Italian refuge and the northward expansion from the French refuge (the possible colonization of the Mercantour from this latter refuge is shown by the dashed arrow). Symbols of sampling sites indicate the cluster to which they were assigned (see text for details).

2003). However, this result is not surprising because the habitat of *C. solieri* is heterogeneous and potential barriers to gene flow are numerous (e.g. relief, nonforest areas, roads, rivers).

IBD in the whole range of this beetle was revealed through a significant positive and monotonic relationship between genetic differentiation and geographical distance (Fig. 3). Similar conclusions can be drawn from the clustering analysis. A decline of gene flow among populations in relation to geographical distance has been shown for numerous insect species (Peterson 1996 and references therein), although not always at all geographical scales. For instance, Peterson (1995) reported the absence of correlation between gene flow and geographical distances in the butterfly *Euphilotes enoptes* over distances up to 30 km, and in contrast a significant IBD over a larger spatial scale (Peterson 1996). No data related to dispersal exist for *C. solieri*. However, this species is brachypterous and has clearly limited dispersal abilities, as with many other large ground beetles. Data published for other *Carabus* species range from less than 1 m to several tens of metres in 24 h and a few hundred metres in longer time-spans (den Boer 1970; Thiele 1977; Niehues *et al*. 1996). Because of this limited dispersal power and the heterogeneity of its habitat, *C. solieri* probably approaches a stepping-stone model of regional population structure.

Genetic model-based clustering and hierarchical population structure

A second important result is that although being consistent with an IBD pattern, clustering analysis clearly identified three main clusters of populations isolated by barriers to gene flow and exhibiting different patterns of differentiation.

Within-clusters population structure. A significant IBD pattern was revealed within each of the three clusters (C1, C2 and C3). However, population structure is different according to the cluster considered. First, population subdivision is stronger in C1 than in C2 and C3, as shown by a greater among-population variance component, a stronger assignment of individuals from C1 in clustering analysis and a steeper slope of the regression from the IBD analysis in C1. Moreover, intrapopulation genetic diversity is low in C1 relatively to C2 and C3 to the exception of the northwestern populations. Hedrick (1999) pointed out that levels of within-group variation could heavily influence measures of differentiation between groups for highly variable loci such as microsatellites, the maximum value of F_{ST} being at maximum less than the average within-population homozygosity. It could thus be argued that the difference of levels of differentiation observed between C1 and C2 would be due only to their difference of polymorphism (higher for C2, Table 1 and Fig. 2). However, levels of differentiation similar to those found in C2 were observed in samples originating from the northwest of the range and were associated with low levels of polymorphism (as those observed in C1). The high level of differentiation and the low genetic diversity in the southern part of the distribution of *C. solieri* are thus certainly related to the strong habitat fragmentation, which probably reduces both favourable habitat size and gene flow between habitats.

Among-cluster population structure. When considering C1 plus C2, we found between-cluster levels of differentiation higher than within C2 alone and about the same order as within C1 (Fig. 6a). Gene flow seemed to be more important in the north of the *Curtii* group range — which corresponds to the limit between C1 and C2 — and decreases strongly towards the south (Fig. 6c). This pattern could be due partly to the relief in this region. The *Curtii* group is located in the valley of the Estéron river (see Fig. 8), lined in the north by a crest, orientated east–west, and ranging from 1100 to 1550 m above sea level. This mountain range exhibits dry and open habitats unfavourable to *C. solieri* on its south slope and is also the southern limit of the Var river valley, another potential barrier to dispersal for the beetle. In the south of the Estéron valley a succession of mountain ranges, with an east–west orientation and a maximal elevation of 1777 m, probably restrict north–south gene flow. These ranges are covered by forests on their northern slopes and by dry Mediterranean vegetation on their southern slopes, with numerous cliffs. However, it remains to be determined whether the barrier between C1 and C2 is due to the present habitat structure and/or to a secondary contact between two genetically differentiated entities (see below).

When considering pairwise population differentiation between C2 and C3, results are different depending on the C3 subgroup considered. Obviously, a strong barrier to gene flow occurs between C2 and C3F but not between C2 and C3I (Fig. 6b). Even though the difference of genetic diversity between C3F and C3I (low and high, respectively) could cause a bias in the observed differentiation with C2 — the theoretical maximum value of F_{ST} being higher for C3F than for C3I (Hedrick 1999) — this is not sufficient to explain this result. If one considers two populations of C2 and C3F with similar genetic diversity level, for instance BRIA and BARL, respectively, pairwise differentiation with any population from C2 is always higher when considering BARL (pairwise F_{ST} from 0.30 to 0.46) than when considering BRIA (pairwise F_{ST} from 0.13 to 0.30) for similar geographical distances. The barrier to gene flow evidenced between C2 and C3F fit to a crest ranging from 2350 to 3300 m elevation, from the Argentera massif to the north of the Mercantour Massif (see Fig. 8). Also, the absence of barrier to gene flow between C2 and C3I is consistent with the clustering analysis. Indeed, individuals from C3I sites have partial mean memberships also in cluster C2 (probabilities of 0.07, 0.11 and 0.25 for OSIG, TEND and BRIG, respectively; $K = 3$, Fig. 5a). This result should be related to the absence of a strong barrier between the Italian part and the centre of the range of *C. solieri* and provides interesting insights to the phylogeography of the species.

Phylogeography of C. solieri

We have shown that if IBD is the basic process of population structuring in *C. solieri*, the current population structure is likely to also show historical imprints. According to morphological and molecular markers, Rasplus *et al*. (2001) recently proposed that *C. solieri* has been isolated during the last Quaternary ice age into two refugia, one probably located in Italy and the other in south of France (Estérel and/or Maures massifs). The Iberic peninsula, Italy and the Balkans are often identified as important refugia for numerous species in Europe (Taberlet *et al*. 1998; Hewitt 1999), but other places have also been proposed, in particular in the south of France (Pons 1981; Konnert & Bergmann 1995; Blondel & Aronson 1999; Vogel *et al*. 1999; Kropf *et al*. 2002). In the case of *C. solieri*, location of the refugia suited the fact that the most southern extension of the Alpine ice sheet during the last glacial period (Würm) was in the Alpes Maritimes and Ligurian region (see Kropf *et al*. 2002).

Postglacial recolonization would have then occurred northward as the blue form differentiated in the south of France and westward as the green form differentiated in Italy, probably following two ways as suggested by the present results (see Fig. 8). A first postglacial colonization route from Italy could have occurred in the north of Argentera and Mercantour massifs following the Stura di

Demonte valley, and crossed the Larche pass (1991 m elevation) to reach the present northwest part of the range of *C. solieri*. This colonization area corresponds to the range of the C3 cluster. Genetic variability estimated either by allelic richness or genetic diversity declines from east to west (Fig. 2). This is consistent with theoretical and empirical works reporting a general pattern of gradual loss of genetic diversity produced by colonization (Austerlitz *et al*. 1997; Le Corre & Kremer 1998; Hewitt 1999; but see Comps *et al*. 2001). The most frequent alleles in populations from northwest of the range (C3F) were also among the most frequent in populations from Italy (C3I), whereas most alleles with low frequencies observed in populations from C3I were absent in populations from C3F. The loss of diversity in C3F relative to C3I could be explained by the cross of the Larche pass and/or the colonization process through the narrow Stura di Demonte valley. A second postglacial colonization route from Italy probably progressed between the Mercantour massif and the Mediterranean Sea. The range from the Argentera massif to the north of the Mercantour massif (2350–3300 m high) could have prevented the mix of populations originating from the two colonization routes. This is illustrated by the barrier to gene flow identified between C3F and C2.

Hybridization and introgression between the two differentiated entities are supposed to have occurred following their secondary contact (Rasplus *et al*. 2001). The Alpine barrier is indeed one European region concentrating a high number of hybrid zones resulting from postglacial secondary contact (the so-called suture zones, Taberlet *et al*. 1998; Hewitt 2000). However, there is still uncertainty about the exact location of the first contact zone between the two original subspecies of *C. solieri*. It could correspond to the present *Curtii* group range. This would mean that the blue colour of the *Clairi* group results from an independent acquisition. An alternative hypothesis could be that the initial contact zone corresponds to the current limit between C2 and C3I. In this case the *Clairi* group would illustrate the northernmost expansion of the blue entity differentiated in south of France. Even though the present study does not show more genetic proximity between the *Clairi* and *Bonnetianus* groups than between the former and the *Solieri* group in the centre of the range, as expected under the alternative hypothesis, this does not allow us to firmly reject this second scenario. Additional studies are required.

Conclusions

In the present study, we showed empirically that a significant IBD pattern suggesting gradual and steady change in gene frequencies can nevertheless hide sharp discontinuities in gene frequencies. Indeed, we identified two major barriers to gene flow in the distribution area of *C. solieri*. If the physical nature of the first (i.e. a crest range) is evident, it remains to determine the origin of the second (secondary contact and/or habitat structure). IBD patterns are quite different when considering clusters of populations defined by such barriers independently or together. The slope of the regression of genetic differentiation against geographical distances is much higher when considering populations altogether (for instance 0.173 for C2 and C3) than when conducting independent analyses for each cluster (0.075 and 0.069 for C2 and C3, respectively). This result highlights strongly the possible confounding contribution of barriers to gene flow to IBD pattern and emphasizes the utility of the model-based clustering analysis used in this study to investigate genetic population structure more effectively, and in particular to detect barriers to gene flow or secondary contact. The three main clusters identified were characterized by different population structures, suggesting that relative influences of evolutionary forces are quite different between the corresponding regions. Here, the combined analyses of IBD patterns and clustering provided new and valuable insights to the study of *C. solieri* phylogeography, allowing in particular the identification of colonization routes from the Italian refuge. Whereas the main clusters and subgroups identified are probably related to its colonization history, the significant IBD pattern determined within each cluster suggests that the current equilibrium between migration and genetic drift is mainly responsible for the observed population structures.

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This work constitutes part of S. Garnier's PhD thesis. He studied differentiation and hybridization in *Carabus solieri* using population genetics and geometric morphometrics approaches. P. Alibert is a senior scientist working on population differentiation and speciation. J.-Y. Rasplus is a senior scientist working on systematic, evolution and conservation genetics of insects.

ARTICLE 3 :

Garnier, S., F. Magniez-Jannin, J.-Y. Rasplus, and P. Alibert (2005) When morphometry meets genetics: inferring the phylogeography of *Carabus solieri* using Fourier analyses of pronotum and male genitalia. *Journal of Evolutionary Biology* **18**:269-280.

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When morphometry meets genetics: inferring the phylogeography of *Carabus solieri* using Fourier analyses of pronotum and male genitalia

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Fourier analysis;
geometric morphometrics;
ground beetles;
phylogeography;
shape;
speciation.

Abstract

Population differentiation is a crucial step in the speciation process and is therefore a central subject in studies of microevolution. Assessing divergence and inferring its dynamics in space and time generally require a wide array of markers. Until now however, most studies of population structure are based on molecular markers and those concerning morphological traits are more scarce. In the present work, we studied morphological differentiation among populations of the ground beetle *Carabus solieri*, and tested its congruence with genetic population structure. The shape of pronotum and aedeagus was assessed using Dual Axis Fourier Shape Analysis. MANOVA on Fourier coefficients revealed highly significant morphological variation between populations and a similar geographical pattern of differentiation for both structures. On the whole, morphological and genetic patterns were also found to be congruent. Our analysis confirms the phylogeographical scenario proposing that two entities of *C. solieri* differentiated during the last glaciation events before recolonizing the actual range of the species. It also indicates a large introgression between the two differentiated entities in the centre of the range.

Introduction

Population differentiation is a crucial step in the speciation process (Rice & Hostert, 1993; Foster *et al.*, 1998; Turelli *et al.*, 2001). Recently, several authors have stressed that speciation defined in a broad sense is not restricted to the evolution of reproductive isolation (as assumed under the biological species concept) but includes the diversification of all aspects of the phenotype (see Barton, 2001). This means that it is essential to (i) study the relative influence of the evolutionary forces (e.g. gene flow, natural selection, genetic drift) that interact to produce a given pattern of differentiation and variability before complete reproductive isolation and, (ii) conduct studies with a wide array of markers.

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In such a context, studies of morphological differentiation are essential. First, most organismal taxonomy, including intra-specific variation, is based on morphological traits. Thus, one is able to appraise and interpret morphological variation at all levels of integration. Secondly, it is likely that morphological traits are, to a large extent, under polygenic control. Studying differentiation of such characters then provides a good assessment of the amount of divergence between different entities. It has even been reported that morphology could exhibit clear patterns of differentiation where molecular markers failed to detect population structure (Nice & Shapiro, 1999). Thirdly, most morphological traits are the target of selection; their study is central in the evaluation of its strength and its impact in the differentiation process. Finally, the understanding of phylogeographical history of species, or the evaluation of the action of the different evolutionary forces, all need a comparison of the patterns of geographical variation obtained from different markers, for example genetic, morphological,

physiological or behavioural markers (Long & Singh, 1995; Magniez-Jannin *et al.*, 2000; Drotz, 2003).

In this paper, we study morphological differentiation among populations of the ground beetle *Carabus (Chrysocarabus) solieri* Dejean (Coleoptera, Carabidae). This species is a suitable model to study forces driving differentiation. Despite its relatively small distribution area in the Southern Alps of France and the Ligurian Alps in Italy (Fig. 1), it exhibits important genetic and morphological variations (Bonadona, 1967; Darnaud *et al.*, 1978; Rasplus *et al.*, 2001). Numerous taxonomic entities (subspecies, races and natios) have been described on the basis of morphological variation, but the precise number varies depending on the authors (Bonadona, 1973; Darnaud *et al.*, 1978; Deuve, 1994). The high level of variation among populations observed in this species can be related to two main factors. First, brachypterous ground beetles have limited dispersal abilities, and genetic and morphological differentiation have often been reported even at a local spatial scale (Assmann & Weber, 1997; Alibert *et al.*, 2001; Rasplus *et al.*, 2001; Brouat *et al.*, 2003; Keller & Largiadèr, 2003). Secondly, in France, the genus *Carabus* was probably affected by the Pleistocene glaciations in Europe, as were other species in this area (Hewitt, 1999). Indeed, it has been proposed recently that *C. solieri* differentiated into two distinct subspecies following isolation in two refuges, one of green colour in Italy and the other of blue colour in the South of France (Rasplus *et al.*, 2001). Post-glacial recolonization then led these entities into secondary contact, where hybridization occurred. A recent study of population genetic structure of *C. solieri* with microsatellites markers allowed the identification of three main groups of populations (Garnier *et al.*, 2004). The first one occurs in the southernmost part of the distribution area, the second inhabits the north-west and the east, and the third occupies the middle part of the range. The first two could correspond to populations derived from each refuge whereas the exact origin of the third one remains uncertain, all the more so as this group contains both blue and green individuals whereas the first and second groups are represented by blue and green individuals respectively. Moreover, detection of barriers to gene flow suggested two routes of colonization from the Italian refuge. However, the exact location of the secondary contact remains to be determined, as well as the origin of populations at the centre of the range. In such a context, a morphological survey of population differentiation can be very informative because the geographical distribution of morphological differences between two hybridizing entities may reveal the position of the contact zone.

As traditional morphometrics seem to be of limited interest according to the varying number of subspecies defined by authors who have used this approach, we chose to assess the pattern of population differentiation of *C. solieri* using geometrical morphometric methods. Compared with traditional morphometry, they allow description of more complex forms by integrating the complete geometry of objects studied (Bookstein, 1991; Rohlf & Marcus, 1993; Marcus *et al.*, 1996; Lestrel, 1997). In addition, they appraise both shape and size of organisms. Geometric morphometrics methods have proved to be powerful at detecting subtle shape changes even at the intra-specific level (e.g. Baylac & Daufresne, 1996; Adams & Funk, 1997; Alibert *et al.*, 2001; Renaud & Millien, 2001; Bertin *et al.*, 2002). Finally, these

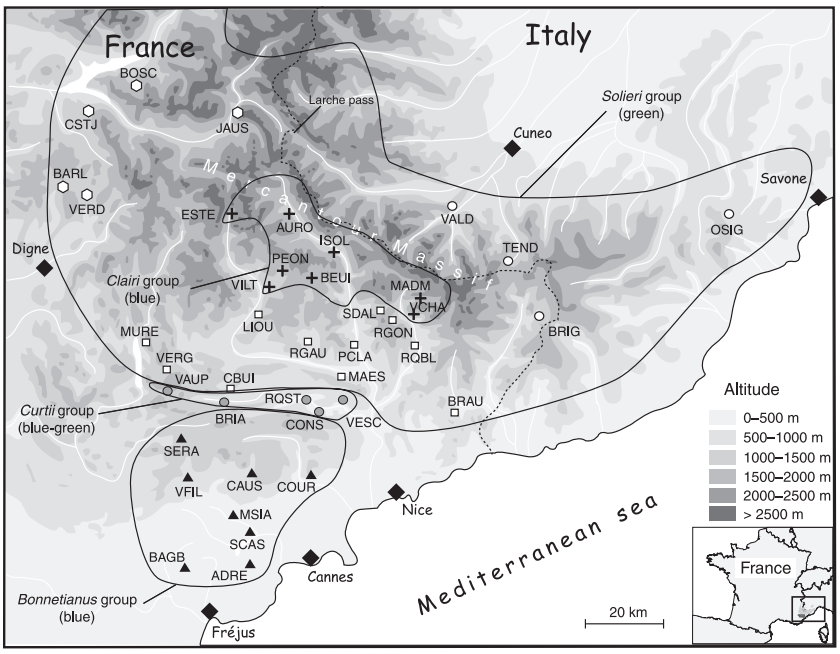

Fig. 1 Distribution area of *C. solieri*, sampling locations, and limits of the groups of populations defined according to the colour of individuals (indicated in parentheses). Sampling sites are represented by distinct symbols according to the group: Bonnetianus group (black triangles), Curtii group (grey circles), Clairi group (crosses), and Solieri group divided into Solieri-C (open squares), Solieri-NW (open hexagons) and Solieri-I (open circles).

methods also allow the direct visualization of shape difference of objects studied. Here, we considered two different morphological structures. One is the pronotum, which is a hardened plate on the dorsal side of the thorax. Leg muscles are attached to this surface. The second is the aedeagus, which is a sclerotized part of the male genitalia. The genital morphology of insects and other animals with internal fertilization has often been used as a discriminant character between closely related species, because of a rapid and divergent evolution (Eberhard, 1985). Both structures are usually considered in systematics of ground beetles and are suitable for morphological study because they are strongly sclerotized and not prone to deformation. As no landmarks are easily identifiable for either structure, their shape was studied using Fourier outline analyses. These analyses fit mathematical functions to outlines, and parameters of these functions are used for statistical appraisal of shape differences (Rohlf, 1990). Our objectives were therefore to (i) test for morphological differentiation within the range of *C. solieri*, (ii) test for congruence between morphological and genetic population structure, and (iii) investigate the implications of congruencies and/or discordances of patterns of population structure from morphological and genetic markers for the phylogeography of *C. solieri*.

Material and methods

Study area and sampling

Carabus solieri is an endangered species distributed in a relatively restricted area in the Southern Alps of France and the Ligurian Alps in Italy (Fig. 1). This ground beetle is mostly associated with coniferous or deciduous humid forests, but it can also occur in Mediterranean dry forests and alpine grasslands. Populations of *C. solieri* are threatened mainly by habitat destruction and fragmentation, particularly in the southern part of its range where habitats are highly anthropized. Moreover, entomologist's trapping can be locally sizeable. This insect is a spring breeder, laying eggs in spring and summer, depending on environmental conditions. Larval development occurs in summer and tenerals emerge in late summer or autumn, and over winter in the soil. Mating occurs during the following spring. No precise data on longevity of this species is available. However, Baumgartner *et al.* (1997) reported a lifespan of 5 years for the related species *C. auronitens*.

For convenience in the text, we considered groups of populations defined according to colour of individuals and geographical location. Note that these groups have no taxonomic value, even if they correspond more or less to subspecies described by some authors (Bonadona, 1973; Darnaud *et al.*, 1978; Deuve, 1994). Bonnetianus group occurs in the most southern part of the range (Fig. 1) and corresponds to the entity differentiated in the French refuge. Clairi group inhabits mountain forests in the Mercantour massif. Individuals are deep metallic blue in both these groups. Metallic green individuals belonging to the Solieri group occur everywhere else, and we divided these into three geographical subgroups: Solieri-I in Italy, Solieri-NW in the north-west, and Solieri-C in the centre of the distribution area (Fig. 1). Finally, individuals with intermediate colour (blue-green) occur in the contact zone between Bonnetianus and Solieri groups. They constitute the Curtii group, and, while sometimes considered to be a subspecies, members of this group are suspected to be hybrids between the two preceding groups.

Adults were collected with permission using rows of 20 to 60 pitfall traps during spring and summer 1997, 1998, 2000 and 2001. Pitfall traps were checked weekly or every 2 weeks during the adult activity period (from April to August). Pronotum shape analysis was performed for a total of 1094 individuals from 41 sampling sites (Table 1 and Fig. 1). A subsample of 24 populations was considered for studying the shape of male genitalia (310 individuals). Sex ratio was strongly biased in favour of males. However, as most of samples contained <10 females, sex was not distinguished in the analyses. The sex ratio being roughly constant across samples, it is therefore unlikely that shape sexual dimorphism, if any, would introduce a bias in the results.

Fourier analysis of outlines

Pronotum as well as aedeagus are particularly smooth and landmarks were quite rare on both structures. We were able to find only landmarks of type 2 (extrema of curvature) and type 3 (extrema of single coordinates) (*sensu* Bookstein, 1991). Because these types of landmarks are not the most accurate in term of measurement error (ME) and homology, and because they were rare we preferred to assess shape of the pronotum and the genitalia from their outline analysis. Outlines studied correspond to the two-dimensional projection of the dorsal view for the pronotum and of the left lateral view for the aedeagus (Fig. 2). A video camera coupled to a binocular stereomicroscope was used to obtain numeric pictures. Then, after manual cleaning of images, outlines were automatically extracted using an image analysis software (Optimas 6.0; Media Cybernetics, Silver Spring, MD, USA). We used Dual Axis Fourier Shape Analysis (Moellering & Rayner, 1981, 1982; Bertin *et al.*, 2002) to decompose periodic signals corresponding to outlines in a sum of trigonometric functions. A total of 128 points equally spaced on the outline were sampled for both morphological structures, and their X, Y Cartesian coordinates were considered as a complex signal, $Z_n = X_n + iY_n$ (with $n = 0$–127). The original outlines were aligned so as to have the same orientation. Starting points ($n = 0$) were defined as the maximum curvature at the right posterior lobe for the pronotum and the maximum curvature on the apex of the aedeagus (see Fig. 2). Using discrete Fourier transforms, 128 harmonics

were calculated, each one characterized by its Fourier coefficient C_k:

$$C_k = \frac{1}{128}\sum_{n=0}^{127} Z_n e^{-\frac{i2\pi kn}{128}},$$

with k the rank of the harmonic. This coefficient can be expressed by two real numbers corresponding to its real and its imaginary part ($C_k = a_k + ib_k$), which are the variables used in statistical analysis of shape (we will thereafter refer to them as real coefficients). Amplitude of harmonic corresponds to the modulus of the Fourier coefficient C_k:

$$A_k = \sqrt{a_k^2 + b_k^2}$$

and thus provides less information than the two real coefficients a and b (the difference corresponding to the phase of harmonics).

A good approximation of the outline is generally obtained with the first few harmonics (Crampton, 1995). However, when harmonics are derived from the complex signal, the conjugates of the first harmonics must also be retained for the outline approximation because of conjugate asymmetry. We thus refer to harmonic pairs consisting of harmonic k and its conjugate, harmonic $128-k$ (for $k \geq 1$). In order to obtain real coefficients independent of size, all the real coefficients were divided by the square root of the structure surface. As the zero harmonic is dependent on translations, it was excluded from the analyses. The number of harmonics to retain was determined on the one hand by assessing ME linked to each harmonic and on the other by estimating visually the quality of a series of inverse Fourier reconstructions using increasing number of harmonics, as suggested by Crampton (1995). Coordinates extraction and Fourier coefficients calculations were performed using the Matlab Toolbox CDFT 2.7 (Dommergues, 2001). Data acquisition was made by a single operator (F. M.-J.) in order to minimize ME sources.

Measurement error

The ME was assessed for three reasons. First, it allowed us to evaluate the reproducibility of our measurements. Secondly, ME can be associated to different geometric scales. Position and parallax error produce effects at a large geometric scale and then possibly affect all harmonics. We verified through preliminary experiments that this source of error was negligible in our case. Besides, as harmonics of increasing rank describe finer and finer details of the outline, ME associated with each one was expected to increase. Then, the rank of the first harmonic displaying high ME can be used to determine the maximum number of harmonic to consider in the analyses. Thirdly, phase of harmonics strongly depends on the orientation and on the starting point, whereas the amplitude does not. As the information contained in the real coefficients (a and b) is the same as in the amplitude plus the phase, a weak ME on real coefficients allows their use in statistical analyses. However, high ME on real coefficients and weak ME on amplitude reflect high ME on phase, and therefore restrict analyses on the harmonic amplitudes. In this latter case, the information is only partial.

Table 1 Sampling sites and sample size: total number of males and females for pronotum, and number of males for aedeagus.

Site	Locality	Sampling year	Sample size	
			Pronotum	Aedeagus
ADRE	Les Adrets de l'Estérel	1997, 1998	18	11
AURO	Auron	1998, 2001	20	11
BAGB	Bagnols en Forêt	2000	23	14
BARL	Barles	1997, 2001	36	14
BEUI	Beuil	1998	27	–
BOSC	Boscodon	2001	29	14
BRAU	Col de Braus	1998	24	–
BRIA	Briançonnet	2000	30	13
BRIG	La Brigue	1998	20	10
CAUS	Caussols	1998, 2000	26	12
CBUI	Col du Buis	2000	30	18
CONS	Conségudes	2000	29	–
COUR	Courmette	1998	20	–
CSTJ	Col Saint-Jean	2001	15	–
ESTE	Esteng	1998	10	–
ISOL	Isola	1998	21	8
JAUS	Jausiers	2001	25	–
LIOU	Le Liouc	2001	41	14
MADM	La Madone de Fenestre	1998, 2000	30	14
MAES	Malaussène	2000	26	–
MSIA	Montauroux	2001	31	–
MURE	Mure	1998	18	–
OSIG	Osiglia	1997	36	16
PCLA	Pont de Clans	2000	35	16
PEON	Péone	1998, 2000	33	9
RGAU	Rigaud	1998	21	14
RGON	Rigons	2000	30	–
RQBL	Roquebillière	2000	30	13
RQST	Roquestéron	1997, 1998, 2000	26	9
SCAS	Saint-Cassien	1997, 2000, 2001	36	20
SDAL	Saint-Dalmas	2000	16	–
SERA	Séranon	2000	30	–
TEND	Tende	1998	25	9
VALD	Valdieri	1998	25	15
VAUP	Vauplane	1998	24	9
VCHA	Vallon des Châtaigniers	2000	29	–
VERD	Verdaches	2001	35	14
VERG	Vergons	1998	20	–
VESC	Vescous	1997, 1998, 2000	36	13
VFIL	Vallon du Fil	2000	30	–
VILT	Villetalle	2000	28	–
Total			1094	310

Note that effective differences between pronotum and aedeagus do not correspond to female number, as several males were not exploitable for aedeagus shape analysis.

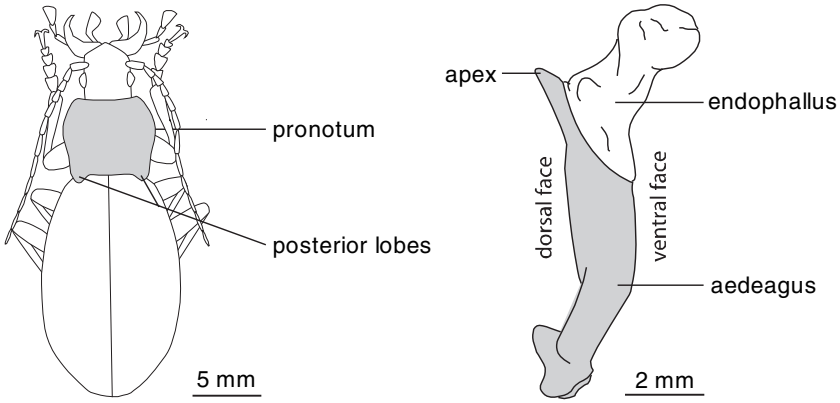

Fig. 2 Schematic representations of a ground beetle (left) and of male genitalia (right). Characters studied (pronotum and aedeagus) are shown in grey.

A subsample of 36 and 20 individuals was measured twice for the pronotum and the aedeagus respectively, in order to assess ME associated to each shape variable. Using model II one-way ANOVAS, with individuals as the categorical factor, the percentage of ME was estimated, for each shape variable separately, as the proportion of the total variance attributable to within-individual variation (Bailey & Byrnes, 1990). For the pronotum, an increase of ME for real coefficients was detected after the fifteenth harmonic pair [ME was <15% before that pair, except for the real coefficients of the first harmonic (ME = 25.2% and 25.6% for coefficients *a* and *b* respectively)]. ME remained roughly constant and <10% for amplitude of the first 16 harmonic pairs, before increasing. Note that in the ME calculations, the measure variability is partitioned into within-individual (ME) and among-individual components. When differences between individuals are moderate, ME increases. Therefore, we considered levels of ME for real coefficients of the first 15 harmonic pairs (i.e. ME < 15%) to be reasonable. As we also verified that 15 harmonic pairs allowed a good outline reconstruction, the real coefficients of these harmonics, i.e. 60 variables, were retained for statistical analyses. Concerning the aedeagus, ME exceeded 40% for at least one real coefficient of the first, the second and the fourth pairs of harmonics. However, ME was <10% for the amplitude of the first 12 harmonic pairs and then gradually increased. This difference of level of ME is due to an important ME for the phase of the harmonics, even for the first ones. Consequently, only amplitudes were considered for the study. As 12 harmonic pairs allowed a good outline reconstruction, harmonic pairs from the thirteenth onward were excluded in further analyses. Male genitalia shape was therefore described with 24 variables.

Morphological appraisal of population structure

Prior to shape analysis we extracted the square root of the surface of the structure studied as an estimator of the size of this structure. Size variation among populations (populations corresponding to the sampling sites) was tested using ANOVA.

Pronotum shape (described by the set of 60 Fourier coefficients) and aedeagus shape (described by the set of 24 amplitudes) were considered independently in the following analyses. For each structure, a multivariate analysis of variance (MANOVA) was performed on shape variables in order to test the among-population mean difference. Canonical discriminant analyses were also performed with population as the dependent variable, and mean scores of populations were plotted to illustrate the pattern of morphological differentiation in the shape space. Mahalanobis distances (D^2) were also calculated between pairs of populations.

Visualization of shape changes

A major advantage of geometric morphometry is the possibility to visualize shape variation directly on the structure studied. For instance, in the case of outline analyses, the outline can be reconstructed from any set of Fourier coefficients using the inverse Fourier transform. This can be used to describe shape variation associated with a particular direction of shape space, e.g. any multivariate factorial axis. We used multivariate regression (Krzanowski, 2000) of Fourier coefficients successively upon the two first canonical axes. Parameters of the regression were used to predict values of Fourier coefficients corresponding to theoretical individuals, here the maximum and the minimum projections on the first two canonical axes. As Fourier coefficients *per se* are very difficult to interpret (Kaesler, 1997), this approach allowed to depict outline deformation along canonical axis (Rohlf & Archie, 1984; Monti *et al.*, 2001). Average shape for some populations (which illustrates general tendencies) was also reconstructed using mean values of Fourier coefficients. These populations were chosen to represent main clusters of populations identified. The information born by these reconstructions is complementary to those described above as they summarize shape changes in the whole

shape space (and is not restricted to a particular axis). This last approach was the only one possible with the aedeagus because multivariate analyses were performed on the amplitude of the harmonics, which was insufficient for outline reconstruction through inverse Fourier transform.

Relationships between morphologic and genetic differentiation

The relationship between morphological differentiation, genetic differentiation and geographical location of populations was assessed by testing the correlation between morphological, genetic and geographical distance matrix. Morphological distances correspond to Mahalanobis distances (D^2). Genetic distances were based on allele frequency data of 10 microsatellite loci. This dataset corresponds to the one used by Garnier *et al.* (2004) to investigate the population genetic structure of *C. solieri*. We used Cavalli-Sforza & Edwards (1967) chord distance D_{CE} as the genetic distance because it has been shown to be one of the most efficient distance measures to obtain correct tree topology from allele frequency data (Takezaki & Nei, 1996). Finally, we considered geographical distances as straight-line distances between all pairs of sampling sites. Simple Mantel tests were performed to test for pairwise relationships between the three distance matrices. However, independent variables may be correlated. Thus, we used partial Mantel tests in order to assess (i) the association between morphological and genetic differentiation while taking into account the effect of geography and (ii) the association between morphological differentiation and geography while taking into account genetic differentiation. Permutation of the residuals of a null model was used because it has been shown to be applicable in most cases (Thorpe *et al.*, 1994; Legendre, 2000; but see Raufaste & Rousset, 2001; Castellano & Balletto, 2002; Rousset, 2002, for a debate). Each test was based on 10^5 permutations.

Results

Size differentiation

Size variation between populations was highly significant (ANOVA, $F_{40,1053} = 36.55$, $P < 0.0001$ and $F_{23,286} = 36.38$, $P < 0.0001$, for the pronotum and the aedeagus respectively). About half of the variation of the population mean size of the pronotum was explained by variation of altitude ($R^2 = 0.49$, $F = 36.89$, $P < 0.001$), whereas this relationship was less evident for the aedeagus ($R^2 = 0.18$, $F = 4.75$, $P = 0.04$). However, there was no clear pattern of size variation according to the six groups of populations considered. Hence, mean size for both characters appeared to be variable among populations of the same group.

Shape differentiation

Pronotum

The MANOVA on the Fourier coefficients indicated a highly significant difference between populations (Wilk's lambda $= 5.4 \times 10^{-5}$, $F = 4.80$, d.f. $= 2400$ and 33514.18, $P < 0.0001$). The first 20 canonical axes were statistically significant. However, the first two axes explained 24 and 13.6% of variance while the percentage of variance explained by the following axes was <10% and gradually decreased. Moreover, examining the projections onto canonical axes, other than the first two, revealed that they provided no major additional information. Projections of population mean scores onto the first two canonical axes (Fig. 3) showed a clear morphological differentiation between three main groups of populations: the first one corresponding to the Bonnetianus group, the second to the Solieri-NW group and the last one to all the other populations. Projections of population mean values onto the first axis were globally sorted according to latitude, from the Bonnetianus group in the south to the Solieri-I in the north-east and the Solieri-NW in the north-west of the distribution area. The Bonnetianus group appeared to be more heterogeneous than the other groups: morphological variation between its populations was as large as that between all other populations (except the Solieri-NW group), despite the Bonnetianus group occurring in a much smaller geographical area (see Fig. 1).

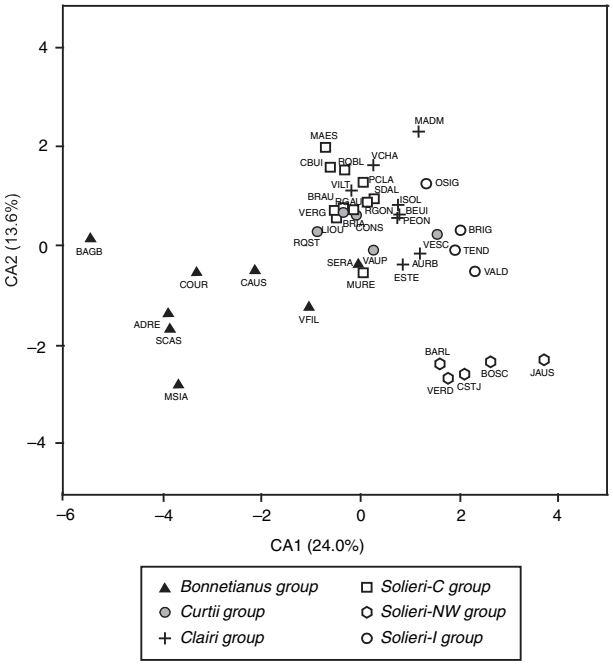

Fig. 3 Plot of the 41 population centroids onto the first two canonical axis (CA1 and CA2) for the pronotum (see text for details about groups and Table 1 for population abbreviations).

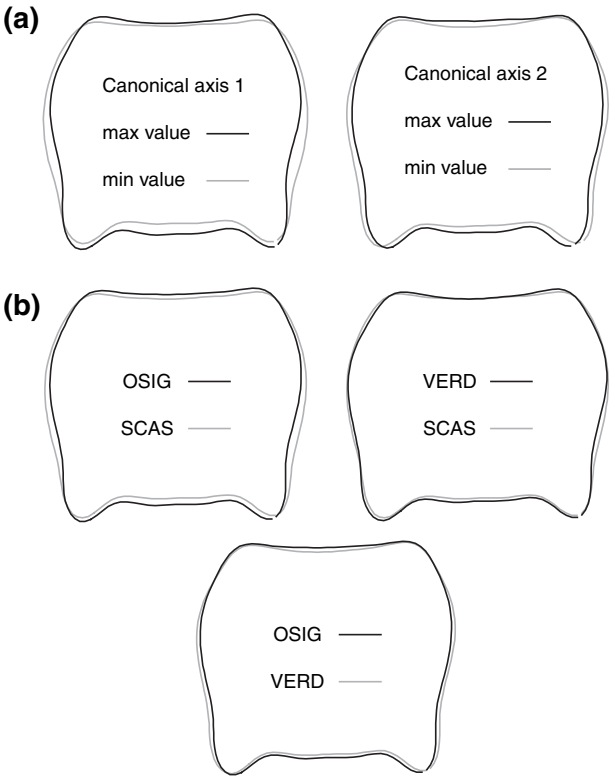

Fig. 4 Pronotum shape variation. Outlines were reconstructed either for minimum and maximum projection values on the first two canonical axes (a) or from average shape for some populations (b), and were superimposed to facilitate the visualization of shape variations.

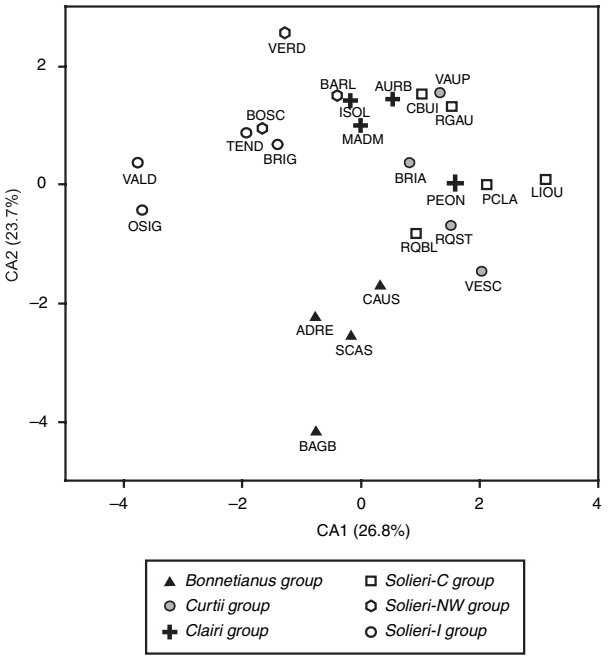

Fig. 5 Plot of the 24 population centroids onto the first two canonical axes (CA1 and CA2) for the aedeagus (see text for details about groups and Table 1 for population abbreviations).

Shape changes associated with the first axis mainly affect lateral edges of the pronotum (Fig. 4a): they appear more convex for negative projections (Bonnetianus group) and S-shaped for positive projections (Solieri-NW and Solieri-I groups). In addition, the ratio length/width seems to be higher, and the anterior edge looks more concave for positive projections. For the second canonical axis, the ratio length/width increases for positive projections but this change seems to mostly involve a decrease in the width of the posterior part of the pronotum (Fig. 4a). The anterior edge of the pronotum also looks more concave for negative projections. All these shape variations are congruent with those expressed on average shape for some populations (Fig. 4b).

The highest values of D^2 occurred between populations from the Bonnetianus group on the one hand, and populations from the Solieri-I and Solieri-NW groups, on the other. The values of D^2 are not shown here but this trend is visible on Fig. 5.

Aedeagus

Morphological differentiation between populations was highly significant, as shown by the result of the MANOVA performed on the amplitude of the first 12 harmonic pairs (Wilk's lambda $= 3.2 \times 10^{-4}$, $F = 5.03$, d.f. $= 552$ and 4468.34, $P < 0.0001$). While the first 12 canonical axes were statistically significant, only the first two were retained. Indeed, the percentages of variance explained by these axes were 26.8 and 23.7%, whereas the other axes did not exceed 11%. The scatterplot of population mean scores on the first two canonical axes showed three groups of populations that were not completely separated (Fig. 5). The first one corresponds to the Bonnetianus group, the second includes the Solieri-I and Solieri-NW groups and the last one included populations belonging to the three other groups (Curtii, Clairi and Solieri-C).

Reconstruction of average outline of some populations showed only subtle differences which were not easy to interpret (Fig. 6). The three populations compared (SCAS, OSIG and RGAU) represented a general trend for shape changes among the three groups identified from the projections onto the first canonical plan. Aedeagus apex is thicker for RGAU, especially when compared with OSIG. In the ventral face, the zone of evertion of the endophallus is more convex and the corresponding dorsal part is more concave for OSIG, than for the two other populations. Finally, the basal part of the aedeagus appears thinner for SCAS.

In the whole shape space, the highest values of D^2 corresponded to population pairs implicating either one population from the Bonnetianus group and one from

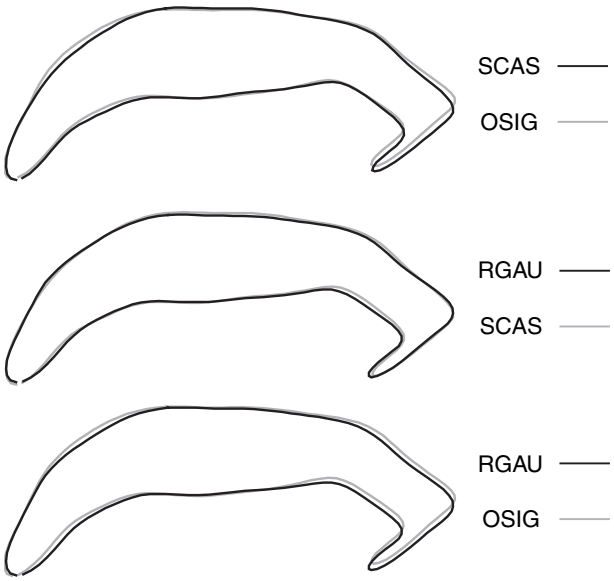

Fig. 6 Aedeagus shape variation, illustrated by superimposed outlines corresponding to average shape of three populations.

Table 2 Correspondence between morphological distances (Morpho, dependent variable), and genetic (Genet) or geographic (Geo) distances (independent variables), measured either by correlation coefficient (r) or partial correlation coefficient (r').

	Pronotum		Aedeagus	
	r	P	r	P
Simple Mantel tests				
Morpho – Geo	**0.46**	**0.0001**	**0.40**	**0.0150**
Morpho – Genet	**0.69**	**<0.0001**	**0.59**	**<0.0001**
	r'		r'	
Partial Mantel tests				
Morpho – Geo–Genet	0.09	0.1800	0.12	0.1700
Morpho – Genet–Geo	**0.58**	**<0.0001**	**0.48**	**<0.0001**

Partial Mantel tests considered for the correspondence between the first two matrices while controlling for the third. All tests were one-tailed and based on 100 000 permutations. Significant correspondences are indicated in bold. Tests were performed across 41 populations for the pronotum and 24 populations for the aedeagus.

another group, or one population from the Solieri-C group and one from the Solieri-I group (not shown).

Relationships between morphologic and genetic differentiation

Distance matrix correspondence tests provided similar results for the pronotum and for the aedeagus (Table 2). Morphological and geographical distances were positively and significantly correlated, as were morphological and genetic distances. However, partial Mantel tests showed that geographical proximity has no impact on morphological differentiation once genetic differentiation has been accounted for. Conversely, genetic differentiation carried significant additional information about morphological differentiation even when geographical proximity had been accounted for (Table 2).

Discussion

Pattern of morphological differentiation

We found highly significant morphological differentiation between populations of *C. solieri*. Overall, patterns of morphological changes assessed from the shape of the pronotum and the aedeagus are congruent, although analyses of the latter structure, performed from reduced sample sizes, should be considered more cautiously. Previous studies on morphological differentiation in this species have reported a rather different spatial pattern of variation. Bonadona (1967, 1973) studied several morphological characters based on traditional measurements on the pronotum and the abdomen and on the elytral sculptures. From his results, he defined three subspecies. The first one – *Chrysocarabus solieri bonnetianus* – corresponds to the most southern populations of the Bonnetianus group defined in the present work. The second one – *C. s. clairi* – occurs in two distinct areas, one corresponding to the north of our Bonnetianus group plus the Curtii group and the other corresponding to the Clairi group. Finally, the third subspecies – *C. s. solieri* – matches the Solieri group as defined here. Rasplus *et al.* (2001) reanalysed data from the studies of Bonadona (1967, 1973) and, not surprisingly, obtained similar results. The major discordance between our findings and previous studies concerns the Solieri-C group. Our results show that this group is not clearly differentiated from the Clairi and the Curtii groups, whereas Bonadona (1967, 1973) clustered the latter into the same subspecies (*C. s. clairi*) and considered populations of the Solieri-C group as a distinct subspecies (*C. s. solieri*). Several features in the studies of Bonadona (1967, 1973) could explain this disagreement: for example the sampling scheme which was restricted to some part of the range and the lack of estimation of ME. In addition, measures (length, width…) used by Bonadona (1967, 1973) concerned a rather limited part of the geometry of the structure studied in contrast to outlines appraisal through Fourier transforms. Finally, subspecies defined by Bonadona (1967, 1973) match the colour pattern of populations, contrary to our morphological groups. In *C. solieri* however, colour is structural and probably produced by multilayer reflectors (Neville, 1977; Parker, 2000). If there exist a strong covariation between elytral striation and cuticule microstructure (and thus colour), and if other morphological characters studied by Bonadona (1967, 1973) are weakly, if not, discriminant, then subspecies defined by Bonadona (1967, 1973) would mainly mirror variations of colour rather than variations

of individuals' shape. Unfortunately, we have no idea of the weight of each character in Bonadona's (1967, 1973) results. The study of elytral sculpture would help to test this hypothesis, even if an objective and quantified assessment of this character seems difficult.

Rapid and divergent evolution of male genitalia is one of the most widespread patterns of animal evolution (Eberhard, 1985), and three main hypotheses, i.e. the lock-and-key hypothesis, the pleiotropy hypothesis, and the sexual selection hypothesis (see Arnqvist, 1997 for a review) have been proposed to explain this pattern. In our case, it is noteworthy that the shape of male genitalia does not appear more divergent than shape of the pronotum. A first explanation could be linked to incomplete assessment of the information about the shape of aedeagus because of the technique used. For example, the three-dimensional assessment of the shape could be more relevant than the two-dimensional projection. A second hypothesis concerns the functionality of the aedeagus. Not all genital parts have the same functional importance for purpose of genital coupling and sperm transfer (e.g. Goulson, 1993), and hence are not subjected to the same evolutionary forces. In fact, aedeagus is just partly inserted into female genitalia during copulation, and other genital parts such as the copulatory piece (a chitinized apophysis on the endophallus of the male) have been reported to be functionally very important for genital coupling (Sota & Kubota, 1998). However, whereas this piece exists in some *Carabus* subgenus, it is absent in the subgenus *Chrysocarabus* and therefore in *C. solieri* (Deuve, 1994).

Morphology *vs.* genetics

On the whole, morphological and genetic patterns of differentiation were correlated, independently of the influence of geographical proximity (Table 2). A previous, related, study using a Bayesian clustering analysis of genotypes at 10 microsatellites loci, identified three main clusters of populations in the distribution area of *C. solieri*, and barriers to gene flow between them (Garnier *et al.*, 2004). The first one corresponds to the Bonnetianus group plus three populations (CONS, RQST, VESC) of the Curtii group. The second cluster is constituted by the Clairi and the Solieri-C groups plus two populations from the Curtii group (BRIA and VAUP). Finally, the third cluster can be further divided into two parts: one matching the Solieri-I group and the other the Solieri-NW group (except population VALD which clusters with the Solieri-NW group). Overall, the morphological characters studied allow the identification of the same clusters of populations, even if less differentiated. Morphological variation seems therefore to reflect the underlying population genetic structure of *C. solieri*.

A major issue of studies of differentiation and speciation is the relative importance of drift and natural or sexual selection in the evolution of reproductive isolation (Coyne, 1992). The role of natural selection in promoting speciation through ecological processes becomes more and more documented (Orr & Smith, 1998; Schluter, 2001), even if genetic drift has also been shown to be a possible important factor promoting divergence (Wlasiuk *et al.*, 2003). Interestingly, the morphological divergence between populations within the Bonnetianus group is associated with a strong genetic differentiation between populations and a weak genetic diversity within populations (Rasplus *et al.*, 2001; Garnier *et al.*, 2004). Populations of Bonnetianus groups are isolated from each other because of the fragmentation of the forest habitat, mainly because of anthropogenic activities and a high frequency of forest fires. Ground beetles have often been shown to be very sensitive to habitat fragmentation (Assmann & Weber, 1997; Keller & Largiadèr, 2003) because of their weak dispersal power. Alibert *et al.* (2001) reported a clear morphological differentiation between two forests located 45 km apart and a correlation between morphological and geographical distances in the species *C. auronitens*. Differentiation of neighbouring populations has also been revealed from molecular markers (Brouat *et al.*, 2003). In our case, the quite low level of gene flow and the local fixation of alleles observed in the Bonnetianus group area (Garnier *et al.*, 2004) suggest that drift could play a significant role in the morphological divergence between populations of the Bonnetianus group. Indeed, local environmental conditions do not appear to be very different among sites; at least they are less different than they are between sites of Solieri-C and Clairi groups which nevertheless appear less differentiated morphologically. Here for instance, the area of the sampling site of population BAGB was particularly limited, i.e. a few hundred metres of humid grove within a dry area, and not surprisingly, this population was highly distinct from the others according to both morphological characters studied and to its very low genetic diversity (Garnier *et al.*, 2004). However, we cannot exclude natural selection as a process promoting a part of the morphological variation observed. Comparison of genetic variance components (within and among populations) between neutral markers and morphological characters could help to resolve the question.

Size did not exhibit a clear pattern of differentiation between groups of populations, in contrast to shape. It is often argued that size can also significantly depend on environmental conditions (Patton & Smith, 1989; Adams & Funk, 1997; Tatsuta *et al.*, 2001). The correlation reported here between size of both morphological structures and altitude could illustrate this potential contribution of environmental factors to size. However, the relative contribution of genetic and nongenetic factors to size is clearly impossible to estimate from our data.

Implications for phylogeography of *C. solieri*

The phylogeographic scenario proposed by Rasplus *et al.* (2001) postulates that during the last glacial events,

C. solieri differentiated into two refuges, involving one blue and one green subspecies, in the south of France and in Italy respectively. The results of the study of pronotum and aedeagus shape are consistent with this hypothesis as Bonnetianus and Solieri-I (the groups supposed to derive from refuge areas) are morphologically the most differentiated. Postglacial re-colonization occurred westward for the Italian entity, probably following two routes (Garnier *et al.*, 2004). The first one was in the north of the Mercantour massif, through the Larche pass (Fig. 1). In this context, the genetic proximity of populations from Solieri-I and Solieri-NW groups (Garnier *et al.*, 2004) is in agreement with their proximity according to aedeagus shape, but contrasts with their strong differentiation according to pronotum shape. Two explanations can be proposed for this apparent discordance. First, a low level of genetic diversity in populations from the Solieri-NW group relative to those of the Solieri-I group could have resulted from successive founder effects during the expansion process (Garnier *et al.*, 2004). Indeed, both theoretical and empirical studies suggest that colonization events are often characterized by one or several founder effects resulting in a loss of genetic diversity (Le Corre & Kremer, 1998; Hewitt, 1999). Neutral or weakly selected morphological characters could have been more affected by these founder effects. As compared with aedeagus, this could be the case for the shape of the pronotum. Second and unlike the first hypothesis, the pronotum shape may be under selection. In this context, variation between Solieri-I and Solieri-NW groups could result from adaptation to different local environmental conditions acting directly or indirectly on the shape of the pronotum. However, to our knowledge, until now there is no study reporting (or suggesting) selection on this morphological structure.

The second re-colonization route from Italy occurred in the south of the Mercantour massif and led to a secondary contact with the entity differentiated in southern France, which probably expanded northward. Whereas a first hypothesis proposes that this contact could correspond to the Curtii group range, a second hypothesis postulates that the initial contact zone corresponds to the transition zone between Solieri-I group on the one hand and Solieri-C plus Clairi groups on the other (Rasplus *et al.*, 2001; Garnier *et al.*, 2004). In this second case, the Clairi group would reflect the northernmost expansion of the blue subspecies preceding the spread in the centre of the range of the entity differentiated in Italy. Our results could give arguments in favour of this second hypothesis as they show that Clairi and Solieri-C groups are relatively little differentiated and appear morphologically intermediate between Solieri-I and Bonnetianus groups. This is therefore in accordance with the idea that Clairi and Solieri-C groups could originate from hybridization and introgression between the two original subspecies. The case of *C. solieri* would then add to the few cases where introgression between differentiated entities has been reported over large geographic zones (e.g. Largiadèr *et al.*, 1994; Sota *et al.*, 2000; Turgeon & Bernatchez, 2001).

Conclusions

Outline analysis of the shape of the pronotum and of the aedeagus provided a clear pattern of morphological differentiation within the range of *C. solieri*, even at a local scale. However, this pattern is different to that found in previous studies. Outline reconstructions show that the shape changes implicated were subtle, particularly for the aedeagus. The use of powerful methods such as geometric morphometrics, but more importantly the use of different approaches and markers (morphological and molecular markers), enable us to clarify the phylogeographic history of *C. solieri*. Not only does this 'multi-marker approach' allow a better evaluation of the divergence between different entities, but it also allows the possibility of assessing evolutionary forces involved in the history and the dynamic of divergence.

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Quantification of sexual dimorphism in *Asellus aquaticus* (Crustacea: Isopoda) using outline approaches

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A marked sexual dimorphism is often observed in arthropods species in which males perform precopulatory mate guarding. It is generally thought to reflect the influence of sexual selection. Until now, sexual dimorphisms associated with mate guarding have mainly been qualitatively described. However, assessing the effects of sexual selection on sexual dimorphims requires a preliminary quantitative assessment of differences in morphology between sexes. Using Fourier analyses, we tested if morphological dimorphisms could be quantitatively assessed in the isopod *Asellus aquaticus*. In addition, we checked whether sexual dimorphism in shape was exclusively related to mate guarding through considering characters that are not, *a priori*, implicated in mating behaviour. To assess the potential role of sexual selection in shaping morphology, we then examined how dimorphic characters could influence males' pairing success. Three characters (pleotelson, paraeopods 4 and 5) differed significantly in shape between males and females. In addition, two characters (pleotelson and paraeopods 4) differed in shape between guarding males and non-guarding males, with the latter being closer in shape to females. This suggests that sexual selection may be partly responsible for the observed morphological divergence between sexes in *A. aquaticus*.

ADDITIONAL KEYWORDS: allometry – complex series – Fourier – geometric morphometrics – head – mate guarding – multivariate analyses – paraeopods – pleotelson – sex – sexual selection – shape – size.

INTRODUCTION

Sexual dimorphism is a widespread phenomenon that affects morphological, physiological and behavioural traits (Philip & Foster, 1971; Andersson, 1994; Walker & Rypstra, 2001). Many theoretical and empirical studies have focused on the adaptive significance of these sexual dimorphisms (Gould, 1974; Slatkin, 1984; Hedrick & Temeles, 1989; Katsikaros & Shine, 1997; Abraham, 1998; Green, 2000; Walker & Fell, 2001). While some secondary sexual characters are quite obvious, such as the exaggerated tail of peacocks, others are more subtle and quantitative analyses are then required to identify them and to appraise the selective forces responsible for their evolution. This is often the case when the divergences between males and females concern the shape of characters, given that such differences between sexes can be very subtle and difficult to describe. Since morphological dimorphic structures are common, attention has been paid to such traits in a wide range of organisms (David, Laurin & De Ridder, 1988; Abell *et al*., 1999; Forslund, 2000; Wellborn, 2000; Bonnet *et al*., 2001) but the magnitude of the dimorphisms has mainly been examined using conventional metrical approaches that consist of linear distances. Such traditional morphometric methods provide an accurate description of the structure of interest when it is regular (such as circles, rectangles, etc.), but do not account for the overall form (Lestrel, 1997).

When shape is more complex, which is a frequent situation in biology, some crucial information could be lost. To improve on traditional techniques, new morphometric methods have been developed over the last 20 years (Kuhl & Giardina, 1982; Lestrel, 1989;

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Bookstein, 1991; Rohlf & Marcus, 1993; Marcus & Corti, 1996; Lestrel, 1997). These methods consider the whole geometry of the studied structures and provide an accurate measure of the shape of complex morphological forms. Two main approaches are available in relation to the nature of the descriptors: landmark approaches and outline approaches. Landmarks are specific points of the biological structure that are supposed to be equivalent or homologous between each specimen. Thus, shape variation among individuals could be assessed from the differences in the geometry of landmark configurations. With outline approaches, the shape is reduced to the outline of the structure that is defined by a set of constructed points located on the boundary. Outlines are then fitted by mathematical functions and the parameters of these functions can be employed to compare individual shapes between themselves. Geometric morphometric methods have proved to be powerful tools for comparing shapes at different taxonomic levels, including the intraspecific level (e.g. Lestrel, Bodt & Swindler, 1993; Loy, 1996; Arnqvist, 1998; Crônier *et al*., 1998; Hard *et al*., 2000; Alibert *et al*., 2001; Monti, Baylac & Lalanne-Cassou, 2001), suggesting that such methods could be particularly useful for detecting and quantifying hidden morphological sexual dimorphisms.

In the present study, we investigated shape dimorphism in the freshwater crustacean *Asellus aquaticus* Linné (Isopoda). In this species, copulation is preceded by a period, called mate guarding, during which a male guards a female by carrying her until insemination becomes possible. Mate guarding is a competitive strategy used by males when a female's fertilization is time limited (Grafen & Ridley, 1983; Yamamura, 1987). Morphological dimorphisms associated with this mating behaviour are well documented in crustaceans, particularly in Gammaridae (Conlan, 1991). Such dimorphisms have also been reported in *A. aquaticus*. For example, males first pair of paraeopods, which is used to grasp their mate, bears apophyses that are absent in females. Similarly, the fourth pair of paraeopods of males, which allows them to carry females during mate guarding, is reduced and curved (Vandel, 1926; Balesdent, 1964). However, as for many other species of animals, these morphological dimorphisms have only been qualitatively described, which limits the possibility of assessing how variation in these traits influences individual fitness. The first aim of the present paper was to investigate if reported morphological dimorphisms could be quantitatively demonstrated. For that purpose, geometric methods were required since the first and fourth pairs of paraeopods are, obviously, characters for which traditional morphometric approaches would lead to an incomplete description of shapes. Since few landmarks were available in the structures of interest, the outline approach was preferred. In addition, we intended to check whether sexual dimorphism in shape was exclusively related to *A. aquaticus* mating behaviour. In this species, reported morphological dimorphisms concern exclusively characters that are used by males during the mate guarding. Therefore, we also examined other characters that are not, *a priori*, implicated in mate guarding, thus allowing comparison of shapes of these characters between guarding and non-guarding males.

MATERIAL AND METHODS

Sampling and data acquisition

Sampling was performed in the Ouche river at Echenon (Echenon: 47°19′N, 05°03′E; Burgundy, Eastern France) in April 2000. *Asellus aquaticus* were collected with hand nets using the kick sampling method (Hynes, 1954) and immediately preserved in an solution containing 70% ethanol and 5% glycerol for later measuring. Each precopula pair (i.e. a guarding male and the guarded female) was isolated in distinct tubes.

We measured the body size of all captured animals. This measure (estimated as a linear measurement from the front of the head to the tip of the pleotelson, Fig. 1) was taken by gently flattening the specimens between two microscope slides (Steel, 1961) and using an optic measuroscope (Nikon measuroscope 10×, Nippon KOGAKU K.K. MODEL 0.1/100 mm).

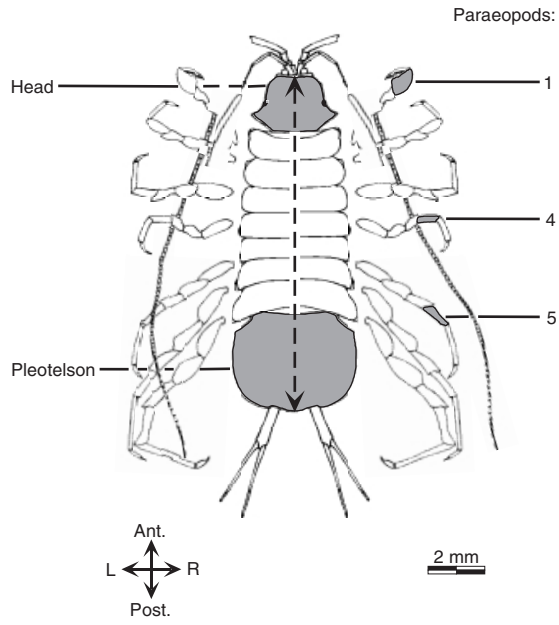

Figure 1. Dorsal view of *Asellus aquaticus* showing the studied characters (shaded areas) and the body size measurement (dotted line) (redrawn from Balesdent, 1964).

Sexual dimorphism in shape was appraised on the basis of five character measurements (head, pleotelson, propodos of the paraeopods 1, carpos of the paraeopods 4 and 5, Fig. 1). For bilateral characters (i.e. paraeopods), shape was only studied on the right appendages in order to avoid variations due to bilateral asymmetry. The 54 precopula pairs collected, and a sample of 50 males and 50 females randomly chosen from the pool of unpaired individuals of reproductive size (appraised by the body size of the smallest male and female in precopula pairs), were retained for shapes analysis. Carpos were removed from the paraeopods in order to access the whole outline of these structures. In the same way, pleotelsons were isolated from the rest of the body. For all characters, the studied outlines correspond to the two-dimensional projection of the dorsal view. They were carefully computer-drawn from numeric photographs.

Fourier analysis of outlines

Most of the methods for outline analyses consist in expressing outlines in periodic signals. Using Fourier transform, such signals are then fitted by a sum of trigonometric functions (or harmonics) that have different amplitudes and phases. Generally, periodic signals are produced by considering the x and y cartesian coordinates of N sampled points on the boundary. The complex notation ($z = x + iy$) of the coordinate pairs is advantageous because it enables the presentation of x and y Fourier transforms in a more compact and elegant form than if they are fitted separately (Moellering & Rayner, 1981). For this reason, we defined a periodic complex series z_n using the complex pairs of 256 points equally spaced on the outline:

$$z_n = x_n + iy_n \text{ with } n \text{ varying from 0 (the starting point) to 255.}$$

Since outlines were described from a finite number of their sampled points and not by a continuous function, discrete Fourier transforms (DFT) were applied to decompose the complex discrete series. Each harmonic is defined from the 256 complex pairs and Z_k, the coefficient of the k harmonic of this Fourier decomposition is:

$$Z_k = \frac{1}{256}\sum_{n=0}^{255} z_n e^{\frac{-i2\pi kn}{256}}$$

The DFT of a periodic series with 256 points is composed of 256 harmonics (k ranging from 0 to 255) and the modulus of Z_k corresponds to the amplitude of k harmonic:

$$A_k = |Z_k|.$$

DFT can be applied to complex series as well as to real series (that is when the imaginary part is fixed to 0). This method allows the description of any outline with a finite number of harmonics. Generally, a shape is adequately described by few harmonics (Crampton, 1995), and, in the case of a complex series, an accurate description is ensured by the first ones and by their conjugates where the harmonic $256 - k$ is, for $k \geq 1$, the conjugate of the harmonic k (Moellering & Rayner, 1982; Kincaid & Schneider, 1983). A reconstruction of any outline can be performed using the formula for discrete inverse Fourier transforms. For each point, a reconstruction can be obtained by summing its complex pairs estimated from all harmonics or by summing its complex pairs calculated from the few harmonics that correctly describe the outline. The complex pair of the point n, calculated from the P retained harmonics is:

$$z_n = \sum_{k=1}^{P} Z_k e^{\frac{i2\pi kn}{256}}$$

Shape variations were studied using amplitudes of the harmonics. In order to consider shape alone, all amplitudes were standardized in size by dividing them by the square root of the character area. The zero harmonics were removed from analyses since they are dependent on translations. The number of harmonics required to ensure a satisfactory fit to outlines was appraised from the Fourier power spectrum (Crampton, 1995). For all studied characters, harmonics 1–3 and their conjugates (that is harmonics 255–253) accounted together for more than 99% of the total power. Because the set of intermediate harmonics had a relatively low contribution to the amount of shape information (less than 1%), amplitudes of these harmonics (harmonics 4–252) were excluded from the statistical analyses. Coordinate extractions and calculations were performed using the software CDFT 2.7 (Dommergues, 2001) developed on MATLAB 5.2.

Measurement error

Many factors such as light or operator-error in the positioning of characters during the photograph sessions could affect the accuracy of measurements. In order to estimate the reproducibility of our measures, a second repeated measurement was performed on a random sample of 30 specimens (15 females and 15 males) for each character. Measurement error was estimated on the standardized amplitudes of the six retained harmonics as the proportion of the total variance attributable to within-individual variation (Bailey & Byrnes, 1990). Percentages of measurement error associated with the shape measures of the pleotelson and paraeopods 4 and 5 (Table 1) were acceptable for such an intraspecific study since the majority of them were less than 10%. In contrast, percentages of measurement error estimated for head and the paraeopod 1 were rather important since the variabil-

Table 1. Percentage of measurement error associated with the five studied characters. Measurement errors were calculated on standardized amplitudes of the six retained harmonics as the proportion of the total variance attributable to within-individual variation

	Harmonics					
Character	A1	A2	A3	A253	A254	A255
Head	9.37	32.17	25.11	13.21	57.98	3.28
Paraeopod 1	0.39	25.67	37.35	0.51	47.38	0.21
Paraeopod 4	3.69	4.42	14.77	7.79	3.81	0.98
Paraeopod 5	1.05	18.65	9.78	1.42	10.07	0.33
Pleotelson	1.20	1.26	6.03	8.49	3.39	2.34

ity within individuals can be higher than the variability between specimens (amplitude 254 for head, Table 1). Hence, we chose to exclude these two characters from our study.

Measurement error was also appraised on body size. To do this, all collected specimens were measured twice and the percentage of measurement error was estimated to account for less than 0.1%.

Sexual dimorphism

Differences in shape between males and females were illustrated by principal components analyses (PCA) performed on the standardized amplitudes of the six retained harmonics. Since males are larger than females in *A. aquaticus* (Steel, 1961; Balesdent, 1964), it was required to control for the possibility that sexual dimorphism in shape is simply an allometric consequence of the sexual body size dimorphism. Therefore, shape dimorphism was assessed, for each character, using multivariate analyses of covariance (MANCOVA) on standardized amplitudes (dependent variables) with sex as the main effect (i.e. males vs. females) and body size as the covariate. Because homoscedasticity of variances was not always fulfilled, we used Pillai's trace for testing significance of MANCOVAs.

Comparing paired and unpaired males

We compared body size between paired and unpaired males since differences in mean size in relation to pairing status have been reported in *A. aquaticus* (Steel, 1961; Ridley & Thompson, 1979). Variations in shape between both categories of males were investigated with the same methodology used to assess sex differences in shape.

Since apparent differences in the shape of a character between the two categories of males might be induced by correlations between traits (Lande & Arnold, 1983; Endler, 1986; Bell, 1997), we also examined the patterns of covariations between the shapes of the different studied characters for both paired and unpaired males. In order to do this, we used scores on the first principal axis (PC1) of the PCA performed for males on the retained six standardized amplitudes as shape variables. The degree of association between the shape of characters was assessed from the partial correlation of their scores on PC1 adjusted for body size.

Visualizing shape variation

An average shape (consensus) was calculated for each character and for the different groups of interest. These consensus may help to detect which parts of the studied structures differ between males and females and between paired and unpaired males, but they do not account for the overall differences demonstrated through MANCOVAs. Consensus were generated from all the reconstructions (obtained with the six retained harmonics) using the Generalized Least-Square (GLS) Procrustes superimposition method (Rohlf & Slice, 1990; Rohlf & Marcus, 1993). While this method is generally used to compare landmarks configurations, it also allows the comparison of a sequence of semi-landmarks that are evenly distributed along outlines provided that terminal landmarks of the sequence are homologous (Bookstein, 1997 in Pavlinov, 2001). Since a precise starting point had been defined for each character, this method was appropriate for our purpose.

The GLS Procrustes superimposition method involves three successive steps: translations in order to superpose centroids of all objects, scaling of the configurations centroid size to unity and rotation to minimize the overall distance between landmarks (Rohlf & Marcus, 1993). The consensus is calculated such that

its landmark configuration minimizes the summed squared of landmark distances (so called Procrustes distance) for all studied configurations (Slice *et al*., 1996).

RESULTS

Sexual dimorphism

We found extensive sexual dimorphism in our sample of *A. aquaticus*. PCA assessed on standardized amplitudes illustrated these overall morphological dimorphisms (including morphological differences between sexes due to the effects of body size dimorphism). In all cases, male and female groups were clearly separated for all studied characters (Fig. 2) on the first principal axis (PC1), which summarized an important amount of the morphological variability. The separation is particularly strong for paraeopods 4 and 5. For pleotelson, the two groups overlapped partly on PC1. No striking differentiation between males and females appeared on PC2 for any character (Fig. 2).

The MANCOVAs revealed that the shapes of the three characters were allometrically related to body size since effects of body size were always significant (Table 2). In addition, allometric relationships of males were different from those of females, the significant interaction terms demonstrating differences in allometric slopes between sexes (Table 2). Thus, the larger male body size compared to female size (*t*-test: $t = -18.28$, 216 d.f., $P < 0.0001$) was not exclusively responsible for the observed morphological dimorphisms, and male and female shapes differed even when body size was held constant.

Representations of the mean shapes revealed that the most striking morphological dimorphism concerned the carpos of the fourth pair of paraeopods (Fig. 3). For this character, differences in curvatures produced a more convex anterior margin for males than for females (Fig. 3A). Similarly, the concavity of the median posterior region was more pronounced in males (Fig. 3A). The only region of the carpos that was not dimorphic was the proximal posterior area that allows the insertion of the carpos in the meros (Fig. 3A).

On the carpos of the fifth pair of paraeopods, only the proximal anterior and distal posterior areas were dimorphic (Fig. 3B). These patterns of dimorphism were, to a much lesser extent, the same as the ones described above for the fourth pair of paraeopods (Fig. 3B).

Morphological dimorphism displayed by the pleotelson was weak. The posterior region of females' pleotelson seemed to be slightly more concave than the pleotelson of males (Fig. 3C).

Table 2. Results of the MANCOVA performed on the standardized amplitudes of the six retained harmonics for each character (Df Num. and Df Den. indicate the degree of freedom of the numerator and denominator, respectively)

	Paraeopod 4				Paraeopod 5				Pleotelson			
Effect	Df Num.	Df Den.	Pillai's trace	*F*	Df Num.	Df Den.	Pillai's trace	*F*	Df Num.	Df Den.	Pillai's trace	*F*
Model	18	504	1.400	24.519***	18	474	1.069	14.577***	18	435	1.124	14.471***
Sex	6	166	0.147	4.756***	6	156	0.288	10.511***	6	143	0.221	6.779***
Body size	6	166	0.491	26.695***	6	156	0.677	54.568***	6	143	0.701	55.957***
Sex*Body size	6	166	0.144	4.669***	6	156	0.191	6.135***	6	143	0.218	6.638***

***$P < 0.001$.

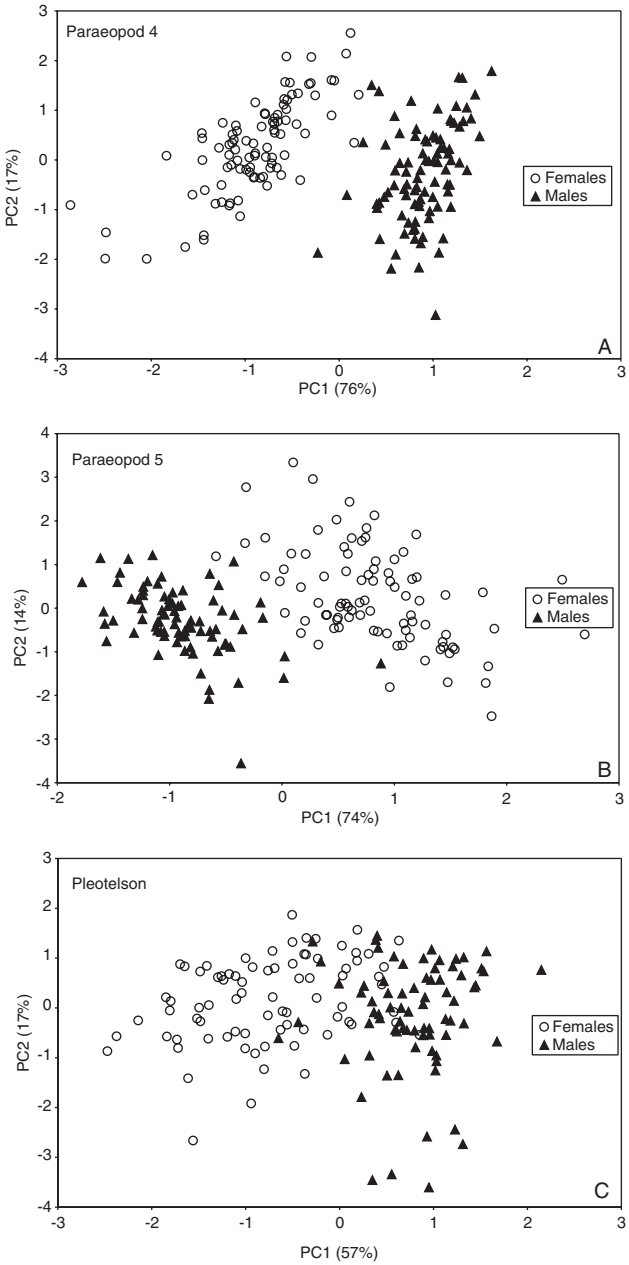

Figure 2. Plot of the scores of males and females for the principal component analyses performed on the standardized amplitudes of the six retained harmonics for paraeopod 4 (A), paraeopod 5 (B) and pleotelson (C).

Comparing paired and unpaired males

Paired males were significantly larger than unpaired ones (*t*-test: $t = -2.776$, 101 d.f., $P < 0.01$), supporting previous observations (Steel, 1961; Ridley & Thompson, 1979). The MANCOVAs performed on the standardized amplitudes of the six retained harmonics indicated variations in shapes between the two cat-

Table 3. Results of the MANCOVA performed on the standardized amplitudes of the retained harmonics for each character (Df Num. and Df Den. indicate the degree of freedom of the numerator and denominator, respectively)

	Paraeopod 4				Paraeopod 5				Pleotelson			
Effect	Df Num.	Df Den.	Pillai's trace	*F*	Df Num.	Df Den.	Pillai's trace	*F*	Df Num.	Df Den.	Pillai's trace	*F*
Model	18	243	0.726	4.311***	18	216	0.814	4.466***	18	198	0.944	5.050***
Pairing status	6	79	0.185	2.986*	6	70	0.069	0.867	6	64	0.200	2.661*
Body size	6	79	0.534	15.077***	6	70	0.615	18.650***	6	64	0.638	18.817***
Pairing status*Body size	6	79	0.171	2.716*	6	70	0.069	0.860	6	64	0.180	2.3488*

***$P < 0.001$; *$P < 0.05$.

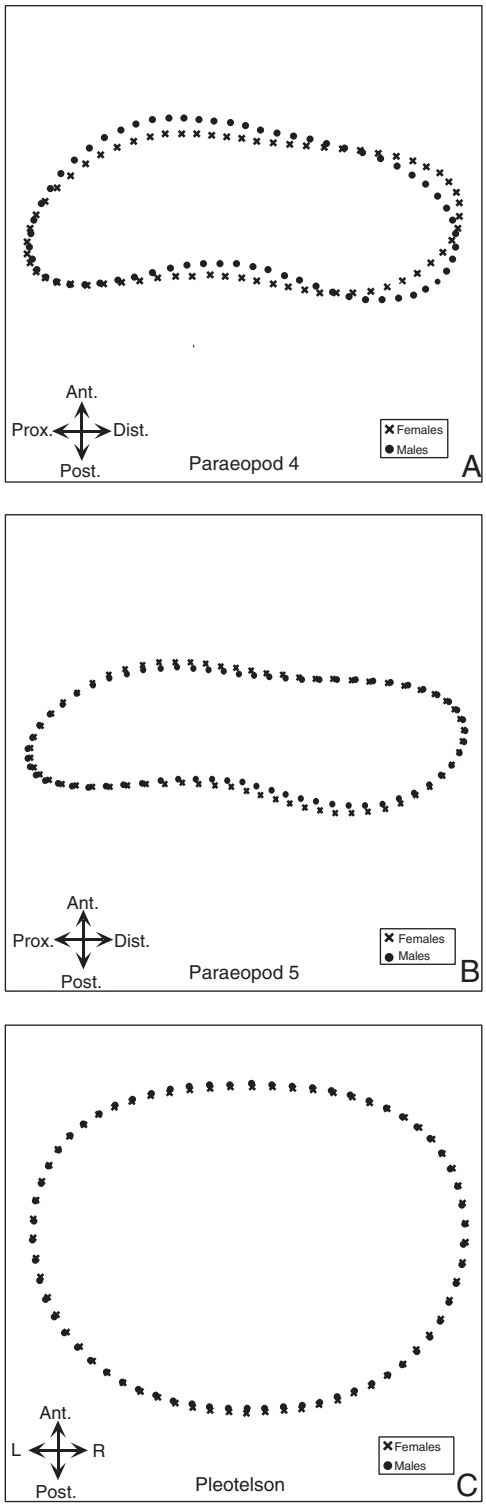

Figure 3. Average shape (consensus) of males and females calculated from all the reconstructions with six harmonics for paraeopod 4 (A), paraeopod 5 (B) and pleotelson (C). To improve the clarity of the figures, only 64 points of these consensus are represented instead of the 256 used for calculations.

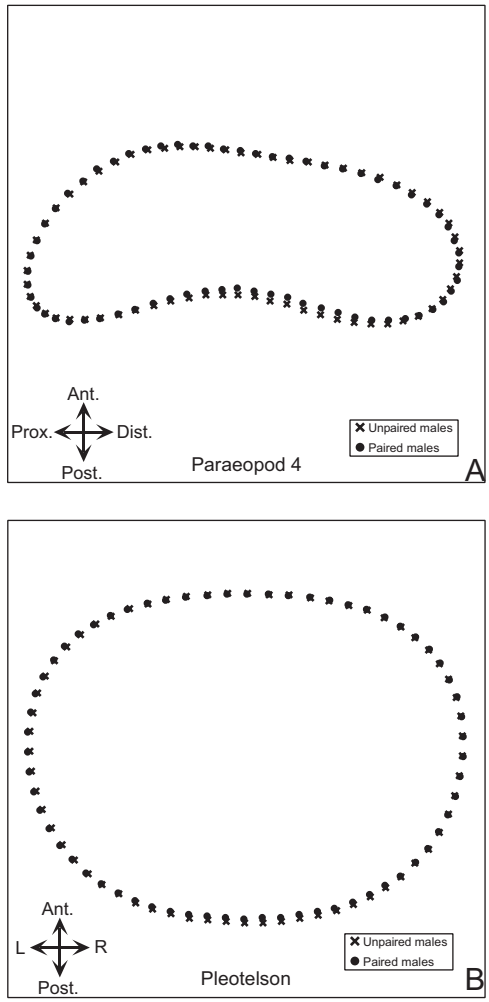

Figure 4. Average shape (consensus) of paired and unpaired males calculated from all the reconstructions with six harmonics for paraeopod 4 (A) and pleotelson (B). To improve the clarity of the figure, only 64 points of these consensus are represented instead of the 256 used for calculations.

egories of males (Table 3). For paraeopod 4 and pleotelson, allometric relationships between shapes and body size were significantly different between paired and unpaired males (Table 3). In contrast, for the fifth pair of paraeopods, allometric relationships were identical for the two categories of males since neither the allometric slopes nor the intercepts (pairing status effect) differed between paired and unpaired males for this character (Table 3) and, for intercepts, even when the interaction term was removed from the model. There was no divergence in the shape of this character when body size was held constant between paired and unpaired males.

Unsurprisingly, consensus representations indicated that morphological differences between the two

Table 4. Correlation coefficients between the shape of paraeopod 4, paraeopod 5 and pleotelson for paired and unpaired males. The residuals obtained from regressing PC1 scores on body size for both paired and unpaired males were used as shape variable (values in parentheses correspond to samples sizes)

Pairing status	Paraeopod 4 Paraeopod 5	Paraeopod 4 Pleoteson	Paraeopod 5 Pleotelson
Paired Males	–0.01 (42)	0.49* (33)	–0.11 (30)
Unpaired Males	0.05 (33)	0.08 (34)	–0.17 (31)

*$P < 0.05$ after Bonferroni correction for risk level on the table.

categories of males were weaker than those observed for sexual dimorphism (Figs 3,4). However, it is worth noting that differences in the shapes of the paraeopod 4 and the pleotelson between paired and unpaired males only concern regions that were described as sexually dimorphic in the previous section. Thus, the concavity of the median posterior region of the carpos of the fourth pair of paraeopods was more pronounced in paired males (Fig. 4A). In the same way, unpaired males seemed to have a more concave posterior margin of the pleotelson than did the paired males (Fig. 4B).

With regard to the covariation of the characters, when body size was held constant, the only significant correlation between the shapes of characters concerned paraeopods 4 and the pleotelson of paired males. All the correlations calculated for the other combinations of characters were not significant for both categories of males (Table 4).

DISCUSSION

Two previous studies (Vandel, 1926; Balesdent, 1964) reported strong sexual dimorphism in *A. aquaticus* for the first and fourth pair of paraeopods. However, both studies relied exclusively on a qualitative approach. Here, using a quantitative approach, we confirm earlier observations for pareopods 4 and demonstrate that sexual dimorphism in *A. aquaticus* concerns at least two others characters: the pleotelson and the paraeopods 5. Beyond their efficiency for intraspecific studies (Lestrel, Sarnat & McNabb, 1989; Lestrel *et al*., 1993; Loy, 1996; Roth & Mercer, 2000), Fourier methods now appear to be powerful tools for unravelling subtle morphometric differences. However, it is worth noting that, at this level of comparison, the measurement error, estimated as the proportion of the total variance attributable to intra-individual differences, increases. This was probably the case for the propodos of the paraopods 1 and for the head, for which measurement error inhibited our ability to quantify shape variation.

Shape differences between male and female *A. aquaticus* were established for all the three characters retained, two of them, the pleotelson and the paraeopods 5, having never been described as dimorphic structures. This result suggests that the divergence between sexes affects the shape of numerous characters and raises the question of their adaptive significance. Until now, male–male competition has been regarded as the major determinant for the evolution of sexual dimorphism in *A. aquaticus*. Indeed, sexually dimorphic characters such as paraeopods 1 and 4 are used by males to grasp females during mate guarding (Vandel, 1926; Balesdent, 1964). In addition, hypogean Asellidae species, in which copulation is not preceded by mate guarding, do not display such sexual divergences (Henry, 1976). Our study provides new arguments that support an influence of precopulatory mate guarding in shaping male morphology in *A. aquaticus*. Indeed, the significant differences found between paired and unpaired males for the shape of paraeopods 4 and pleotelson suggest that sexual selection, through males' pairing advantage, could be partly responsible for the maintenance of the sexual dimorphism within these two characters. Moreover, shape differences between the two categories of males are restricted to regions of the characters that are sexually dimorphic, with paired males showing the most extreme divergences from females. Since unpaired males can displace smaller males to take over females (Ridley & Thompson, 1979), the reproductive success of males may partly depend on the ability to withstand such attempts. Overcoming female resistance to male guarding attempts may also influence a male's pairing success. Female resistance has been documented in many mate guarding species as a result of intersexual conflicts in guarding duration (for review in amphipod and isopod crustaceans see Jormalainen, 1998). However, the role of female behaviour in pair formation in

A. aquaticus remains controversial. Female resistance was observed by Ridley & Thompson (1979) but was considered as negligible in other studies (Manning, 1975; Jormalainen & Merilaita, 1995). In such a context, the greater curvature of the fourth pair of paraeopods may benefit males by enhancing their grasping ability. How the differences in pleotelson morphology might confer a reproductive advantage is unclear since no implication of this character in mate guarding has ever been reported. Therefore, direct observations of sexual interactions are then required to test if pleotelson consistently plays a role in pair formation or whether its dimorphism arose as the result of a correlational response to selection for the shape of paraeopods 4 or body size (Lande & Arnold, 1983; Endler, 1986; Bell, 1997). A strong underlying genetic correlation of the shapes between the fourth pair of paraeopods and pleotelson might be excluded since no association between the shape of these characters was demonstrated for unpaired males. However, sexual selection probably favours a combination of these character states since such a relationship was detected for paired males.

Interestingly, sexual shape dimorphism was also demonstrated for a character that is not different between paired and unpaired males: the paraeopod 5. This result could indicate that mate guarding might not be the only force that entails sexual dimorphism in *A. aquaticus*. Morphological divergences between the two sexes might be induced by differences between males and females in reproductive roles. For instance, since males actively seek for mates during the breeding season whereas females do not (Vandel, 1926; Balesdent, 1964), males could display specific characteristics that enhance their walking performance. We might expect that such characteristics concern paraeopods because of their obvious role in locomotion. Further investigation of the link between female encountering rate, walking performance and the shape of paraeopods 5 should help to clarify this issue.

Until now, in the vast majority of invertebrate species including *A. aquaticus*, attention has mainly been paid to relationships between body size and mating success (Ridley & Thompson, 1979; Ward, 1988; Jormalainen, Tuomi & Merilaita, 1992; Carroll & Salamon, 1995; Savalli & Fox, 1998). However, because of correlations between quantitative traits, a mating advantage of large males may arise even if the actual target of sexual selection is not the overall body size *per se* but actually the size or shape of a particular morphological trait. Given allometric relationships between shape and body size, multivariate analyses of selection would then be required to determine which characters undergo direct sexual selection (Lande & Arnold, 1983). Thus, the combined use of a Fourier method and multivariate analyses of selection may provide powerful tools to investigate how morphology affects reproductive success, and hence to broaden our understanding of sexual dimorphisms, particularly in relation to the geometry of structures.

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8

Genomic Coadaptation, Outbreeding Depression, and Developmental Instability

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Since the publication of Dobzhansky's *Genetics and the Origin of Species*, the notion of genomic coadaptation has played a prominent role in the study of the genetics of evolution. The genome is thought of as a collection of harmoniously collaborating genes, which are pooled and maintained by selective processes over the evolutionary histories of populations. Genomic coadaptation corresponds to the overall genic balance resulting from this selection. In fact, different levels of interactions can be distinguished (table 8.1): a first levelconcerns interactions among genes between nonhomologous chromosomes, a second level involves interactions within chromosomes (the internal balance), and a third level results from interactions among genes between homologous chromosomes (the relational balance). For diploid or polyploid organisms, it can then be assumed that, when favored by natural selection, any combination of alleles at a particular locus (at a heterozygous or homozygous state) can be viewed as a kind of relational coadaptation (see Woolf and Markow, chapter 7, this volume). In summary, and in more quantitative genetics terms, coadaptation refers to selected nonadditive effects between (epistasis) and within (dominance and overdominance) loci.

An idiosyncrasy of the study of coadaptation is that this balance among genes can only be substantiated when disrupted. In other words, one cannot appraise how efficient the coadaptation is but only how efficient it was. Such a breakdown of coadaptation occurs when the genetic constitution of the organism is suddenly modified as, for instance, in cases of genic introgression from differentiated genomes, mutational events or intense directional selection (Levin 1970; Clarke et al. 1992; Clarke 1993). As a consequence, a part of the nonallelic (epistatic) and/or allelic (dominance and overdominance) relationships among genes will be affected and the fitness of the organism is theoretically impaired (Clarke 1993). The negative effects of the breakdown of genomic coadaptation on fitness, so-called outbreeding depression, has essentially been studied in terms of reduction of fertility and survival (Clarke and McKenzie 1987, 1992; Barton and Hewitt 1989; Arnold and Hodges 1995; Barton and Shpak 2000). However, in the past 30 years, several studies have attempted to approach the consequences of the disruption in coadapted gene complexes in terms of developmental instability (DI). Most of them have assessed the amount of fluctuating asymmetry (FA) in morphological characters in natural or laboratory hybrids between subspecies or species. Whereas the aims of some studies were to better understand the genetic basis of developmental stability, other studies have tried to infer the degree of incompatibility of the genetic systems of hybridizing taxa by estimating the DI of the hybrids.

In this chapter, we attempt to provide a critical review of the evidence linking genomic coadapta-

Table 8.1 Summary of Interactions and Mechanisms Related to the Different Genetic Conditions Influencing Developmental Instability (DI) in Organisms.

	Genetic conditions influencing DI					
	Genomic coadaptation					Single gene effects
				Heterozygosity		
Interactions	Interchromosomal balance: interactions among loci between non-homologous chromosomes	Internal balance: interactions among loci within chromosomes	Relational balance 1: interactions among loci between homologous chromosomes	Relational balance 2: interactions among alleles within loci	—	—
Mechanisms	Selection of favorable epistatic interactions	Selection of favorable epistatic interactions	Selection of favorable epistatic interactions	Selection of favorable combination of alleles (in a homozygous or heterozygous state[1])	Masking of deleterious recessive alleles (dominance) and greater contribution of heterozygotes than homozygotes to the phenotype (overdominance)	Depending on the nature of the gene

[1]Leads to coadapted heterozygosity when selected in a polygenic system (see Woolf and Markow, chapter 7, this volume).

tion with DI. First, we survey existing knowledge concerning the genetic basis of developmental stability. It is now believed that both genomic coadaptation and heterozygosity play a significant role in determining the level of developmental stability of organisms. However, studies that unambiguously show the impact of one or the other factor are scarce. Second, we focus on DI in hybrids between differentiated genotypes. By reviewing the existing literature we show that the number of cases where intersubspecific or interspecific hybrids show lower DI than parental groups is much higher than generally believed. Third, we analyze to what extent the study of DI can improve our understanding of genomic coadaptation. Therefore, the role of mutations (including chromosomal rearrangements) and recombinations is presented and discussed. Finally, we examine to what extent the study of DI in hybrids can test the effectiveness of using DI as a marker of fitness.

Genetic Conditions Affecting DI

The genetic basis of developmental stability is still debated among evolutionary biologists and geneticists. Single genes have been shown to have noticeable effects on DI but examples are fairly limited (Klingenberg, chapter 2, this volume; McKenzie, chapter 9, this volume). Rather, genomic coadaptation and heterozygosity are proposed to be two main causal genetic conditions associated with sustaining developmental stability in organisms (table 8.1). These two features are differently credited by authors to play a role in developmental stability and there is now agreement that both are likely involved (Graham 1992; Clarke 1993; Auffray et al. 1999a).

DI and Genomic Coadaptation

Genic balance is hypothesized to enhance an organism's developmental stability. Most of the evidence in favor of such a mechanism comes from studies reporting increased FA in interspecific or intersubspecific hybrids. It is thought that the amount of outbreeding depression in hybrids will depend on the degree of genetic divergence between hybridizing taxa, as well as, for natural hybridization events, the age of the first hybridization occurrence (Templeton 1986; Clarke 1993; Knowlton and Jackson 1993). This is mirrored by the developmental stability of morphological structures (Graham 1992; Clarke 1993). For example, Graham and Felley reported that natural hybrids between two species of sunfish (*Enneacanthus gloriosus* and *E. obesus*) showed a significant increase in FA levels for two out of the seven morphological traits studied. Moreover, the concordance in FA levels of the seven traits within the samples studied indicated an overall increase of DI in hybrids as compared to parental groups. Such a result was not surprising as the two hybridizing *Enneacanthus* have been shown to exhibit considerable genetic divergence (Roger's similarity index: $S = 0.55$) and the introgression is thought to result from humans' construction of impoundments and hence to be rather recent (Graham and Felley 1985). More recently, it has been reported that developmental stability of tooth characters was impaired in wild hybrids between two chromosomal races of the house mouse (Chatti et al. 1999). In contrast to the preceding example, this incompatibility between regulatory gene systems seems to occur despite a low divergence between structural genes of both chromosomal races, attested by the extremely low Nei's genetic distance ($D_{nei} = 0.07$) (Chatti et al. 1999).

Developmental incompatibilities between differentiated groups have been more clearly documented through experimental hybridization. A number of artificial crosses have been conducted, especially in plants (Manley and Ledig 1979; Mosseler 1990; Hochwender and Fritz 1999; Siikamäki 1999; Waldmann 1999), *Drosophila* spp. (Orr 1990; Markow and Ricker 1991; Civetta and Singh 1998), and fish (Leary et al. 1985; Ferguson 1986; Wilkins et al. 1995). Numerous studies have failed to detect any alteration of developmental stability in natural, as well as in experimental, hybrids between genetically differentiated groups (see, for example, Jackson 1973; Ferguson et al. 1988; Blows and Sokolowski 1995; Hutchison and Cheverud 1995; Hatfield 19997; Perfectti and Camacho 1999), even when outbreeding depression was clearly apparent from reduced fertility or survival (Gupta 1978; Alibert et al. 1994, 1997; Baranov and Zakharov 1997; Dosselman et al. 1998; Gharrett et al. 1999; Lu and Bernatchez 1999). Hence, the relative importance of genic balance in regard to heterozygosity as the genetic basis for developmental stability is still unclear.

DI and Heterozygosity

Developmental stability is thought to be positively correlated with levels of heterozygosity within and among populations. Most evidence supporting this hypothesis shows an increase in FA, or an increase in the proportion of phenodeviants, as levels of allozymic heterozygosity in populations decrease (see, for example, Soulé 1979; Vrijenhoek and Lerman 1982; Mitton and Grant 1984; Palmer and Strobeck 1986) or populations become more inbred (see, for example, Robertson and Reeve 1952; Bader 1965; Leamy 1984, 1992; Alados et al. 1995; Gomendio et al. 2000). The ability of heterozygotes to buffer against developmental perturbations is thought to be due to allelic dominance, which masks the expression of deleterious recessive alleles, as well as overdominance, that is, heterozygote superiority per se, which is thought to increase the efficiency of some biochemical and physiological functions in producing a greater variety of biochemical products (Vrijenhoek and Lerman 1982; Leary et al. 1984; Mitton and Grant 1984; Mitton 1993; table 8.1).

The positive association between developmental stability and heterozygosity, however, is now increasingly debated, because the relationship is only supported by correlational evidence and accurate mechanisms remain to be demonstrated (Markow 1995; Woolf and Markow, chapter 7, this volume). Moreover, numerous studies again failed to detect any relationship between developmental stability and heterozygosity (see, for review, Britten 1996; Vøllestad et al. 1999). In addition, Clarke (1993) emphasized that several studies reporting results supporting the heterozygosity hypothesis were theoretically and practically flawed. For example, the number of electrophoretic loci considered to assess the level of heterozygosity is often too limited. The power of a few heterozygosity indicators to reflect the organism-wide genetic variability is questionable (Clarke 1993; Mitton 1993; Vøllestad et al. 1999). However, heterozygosity at certain loci rather than at a genome-wide scale, may more reliably account for stable development of organisms (Mitton 1978; Leary et al. 1993; Leamy et al. 1997).

According to some authors (Patterson and Patton 1990; Clarke 1993), some studies supporting the heterozygosity hypothesis are ambiguously founded upon the demonstration of increased FA in more homozygous populations (e.g., Soulé 1979; Vrijenhoek and Lerman 1982). In such cases, one cannnot exclude confounding effects related to the evolutionary history of populations that may independently influence FA, such as an undetected breakdown in coadaptation and/or differences in environmental conditions experienced during the development of individuals (Patterson and Patton 1990; Clarke 1993). In this respect, within-population studies reporting correlation between individual levels of FA and individual estimates of heterozygosity provide the most convincing evidence that the maintenance of developmental stability is dependent on heterozygosity, at least for the loci studied (Biémont 1983; Leary et al. 1983, 1984, 1992). However, even in these cases it remains difficult to evaluate the respective effects of dominance and overdominance. Moreover, Woolf and Markow (chapter 7, this volume) point out that the results obtained on trout (i.e., those presented in the papers of Leary et al. cited above) are difficult to interpret because of the tetraploidy of the ancestor of the Salmonidae and hence the possibility of the presence of several loci coding for the same enzyme.

Finally, it has also been claimed that the positive relationship reported between inbreeding and FA does not unambiguously demonstrate the role of heterozygosity (Clarke 1993). Indeed, if inbreeding is accompanied by a rapid decrease in heterozygosity, it may also alter epistatic relationships among coadapted gene complexes (Clarke et al. 1992) and it is then difficult to know whether the observed decrease in developmental stability is due to either effect. Using haplo-diploid systems, inbreeding should theoretically increase homozygosity (in females) without affecting coadaptation among genes within chromosomes because of the haploidy of males. Clarke et al. (1992) used honeybees, *Apis mellifera*, to test this hypothesis. They found no significant relationship between the level of inbreeding and the level of FA in both males and females and concluded that genic balance had a stronger effect on developmental stability than heterozygosity. However, even though such results are appealing, generalization to diploid organisms remains difficult. For instance, the absence of increased FA with inbreeding could simply reflect the fact that the haploidy of males has allowed the elimination of deleterious recessive alleles by selection (Clarke et al. 1992; Markow 1995; see also Woolf and Markow, chapter 7, this volume).

The Balance Between Genomic Coadaptation and Heterozygosity

Heterozygosity and genomic coadaptation are interrelated and it is difficult to modify one without affecting the other (Clarke 1993; Auffray et al. 1999a). For instance, as pointed out above, distinguishing between the two genetic conditions can be problematic since interactions between alleles at a particular locus also constitutes a form of relational coadaptation (table 8.1). Even though studies reporting unambiguous results are rare, there is a general consensus that developmental stability in populations is a result of a balance between the effects of both genetic conditions and the outcome of past and present selection pressures on the populations compared (Vrijenhoek and Lerman 1982; Graham 1992; Clarke 1993). Such a balance is well illustrated in the case of hybridization between differentiated genotypes since hybrid genomes undergo both an increase in heterozygosity and a mixing of two different coadapted gene complexes (Ferguson 1986; Graham 1992).

DI in Hybrids

What Is Expected and What Is Found?

In hybrids, we might expect DI to increase as a result of the disruption of coadaptation; alternatively we might expect DI to decrease because of the increased heterozygosity, depending on the degree of divergence in the gene systems controlling development in the hybridizing taxa. Following Vrijenhoek and Lerman (1982), it is well known that, on a scale representing the levels of heterozygosity from those found in inbred populations to those found in hybrids between differentiated taxa, developmental stability is expected to be increasingly enhanced before decreasing in highly outbred groups. Therefore, if the two hybridizing genomes are divergent to the extent that the gene systems controlling development become incompatible, hybrid groups then suffer from outbreeding depression. As the concept of outbreeding depression is inherently attached to the mechanisms of postzygotic isolation, it is generally considered to occur when crosses involve subspecies or species. A survey (Graham 1992) that focused on developmental stability in naturally occurring hybrid zones partly supported this prediction as it reported that, of the 15 studies reviewed, eight found increased DI in hybrid populations, seven noted no significant differences between hybrid and parental populations, while none showed decreased DI in hybrids.

Since the review of Graham (1992) few studies have attempted to evaluate FA in natural hybrid zones (see table 8.2). Two were conducted on house mice: one concerned the European house mouse hybrid zone between *Mus musculus domesticus* and *M. m. musculus* (Alibert et al. 1994) and the other focused on wild hybrids between chromosomal races of *M. m. domesticus* (Chatti et al. 1999). Similarly, Dosselman et al. (1998) performed an analysis of FA in a hybrid zone between chromosomal races of the *Sceloporus grammicus* complex. Another study (Smith et al. 1997) investigated wing shape FA in the subspecies of the honey bee, *Apis mellifera mellifera* and *A. m. carnica*, and in their hybrids. However, in this case, samples of hybrids did not correspond to actual natural hybrids but to a hybrid originated strain (the "Nigra" strain) selected by breeders for several decades. Finally, Freeman et al. (1995) compared DI levels of a number of morphometric and biochemical traits across the big sagebrush (*Artemisia tridentata*) hybrid zone. Interestingly, three (Alibert et al. 1994; Freeman et al. 1995; Dosselman et al. 1998) of these five recent studies reported a decrease of FA levels in hybrids, that is, a heterotic effect on developmental stability (table 8.2). In addition, the examination of earlier published works shows that some of them could also be considered as supporting the hypothesis that hybrids have lower levels of FA. For example, the study conducted by Felley (1980) in the hybrid zone between two subspecies of bluegill *Lepomis macrochirus* (table 8.2) is generally considered as one of those failing to report any differences in FA between hybrids and parental taxa (Leary et al. 1985; Lamb et al. 1990; Markow and Ricker 1991; Graham 1992). In fact, this study reports that of nine morphometric traits studied, a significant difference in FA was found for only one trait, this latter being less asymmetric in hybrids than in the parental subspecies (Felley 1980, p. 26). Similarly, although generally cited as reporting no differences between hybrid and parents, the study of Lamb et al. (1990) shows significantly lower FA values in F_1 hybrids for one out of the eight osteometric trait studied (table 8.2). Heterotic effects in interspecific or intersubspecific hybrids have also been found in artificial crosses (table 8.2). For

Table 8.2 Reports of DI in Hybrids.

Only studies which explicitly tested the relationships between outbreeding and DI are considered. This corresponds to studies focusing on DI in hybrids between differentiated populations, lines, or taxa (all studies designed to assess the potential effect of heterozygosity on DI in crossing inbred lines have therefore been excluded). Moreover, only studies having estimated DI using FA, within individual variation or the presence of developmental anomalies have been taken into account.

Taxa	Cross [a]	Traits [b]	Hybrid class [c]	Results [f]	Reference
Plants					
Annona cherimola × *A. squamosa*	Exp.	2 Q	*Fn*	0	Perfectti and Camacho (1999)
Betula pendula × *B. nana*; *B. pendula* × *B. pubescens*; *B. nana* × *B. pubescens*	Nat. (garden)	1 Q + Δ_{within} for 2 Q	F_1	↑ FA, ↑Δ_{within}	Wilsey et al. (1998)
Picea mariana × *P. rubens*	Exp.	A (embryo and seed)	F_1, BC (5)	↑ anomalies	Manley and Ledig (1979)
Salix (8 species)	Exp.	A (growth, leaves, stems)	F_1	↑ anomalies	Mosseler (1990)
Salix sericea × *S. eriocephala*	Exp.	1 Q [c]	F_1, F_2	↑ FA	Hochwender and Fritz (1999)
Artemisia tridentata tridentata × *A. t. vaseyana*	Nat. (HZ)	7 Q + Δ_{within} for 6 B	*Fn* (1 or 3)	↑ DI for 2 traits; ↓ DI for 2 traits	Freeman et al. (1995)
Eichhornia paniculata (differentiated populations)	Exp.	A (stamen position)	F_1	↓ anomalies	Barrett and Harder (1992)
Lychnis viscaria × *L. alpina*	Exp.	1 Q	F_1	↑ FA	Siikamäki (1999)
Silene diclinis (populations)	Exp.	4 Q	F_1	↑ FA for 2 traits	Waldmann (1999)
Liatris aspera × *L. spicata*, *L. aspera* × *L. cylindracea*, *L. spicata*× *L. cylindracea*	Exp.	Δ_{within} (1 Q)	F_1	↑ Δ	Levin (1970)
Brassica cretica (populations)	Exp.	1 Q	F_1	0	Rao et al. (2002)
Brassica cretica × *B. oleracea*	Exp.	1 Q	F_1	0	Rao et al. (2002)
Insects					
Drosophila melanogaster (lines)	Exp.	1 M [c,d]	F_1 to F_7	↓ FA	Blows and Sokolowski (1995)
Drosophila melanogaster complex (4 species)	Exp.	7 Q	F_1	↑ FA for 3 traits	Civetta and Singh (1998)
Drosophila melanogaster × *D. simulans*	Exp.	2 M + 1 Q [c]	F_1	↑ FA and anomalies (females only)	Markow and Ricker (1991)
Drosophila pseudoobscura × *D. persimilis*	Exp.	3 M [c,d]	F_1, BC	0	Gupta (1978)
Drosophila virilis × *D. lummei*	Exp.	A (eyes and wing)	F_1, BC	↑ anomalies in F_1; 0 in BC	Orr (1990)
Drosophila mercatorum (outbred × parthenogenetic strains)	Exp.	1 M + 2 Q	F_1, F_2, F_3	↑ FA in F_2 and F_3	Anderson et al. (2002)
Apis mellifera mellifera × *A. m. carnica*	Nat.	1 Qs + A (wing)	*Fn*	0 (FA); ↑ anomalies in females	Smith et al. (1997)
Solenopsis invicta × *S. richteri*	Nat. (HZ)	7 Q + A (wing)	*Fn*	↑ FA for 2 traits	Ross and Robertson (1990)

Table 8.2 Continued

Taxa	Cross [a]	Traits [b]	Hybrid class [c]	Results [f]	Reference
Amphibians and Reptiles					
Hyla cinerea × *H. gratiosa*	Nat. (HZ)	8 Q	*Fn* (5)	↓ FA for 1 trait	Lamb et al. (1990)
Litoria ewingi × *L. paraewingi*	Nat. (HZ) + Exp.	A (eyes)	F_1, *Fn* (N)	↑ anomalies	Watson (1972)
Bombina bombina × *B. variegata*	Nat. (HZ)	A (tooth row, vertebral column, spotting pattern)	*Fn*	↑ anomalies	Szymura and Barton (1986)
Sceloporus woodi × *S. undulatus*	Nat. (HZ)	4 M + 2 Q [e]	*Fn*	0 (FA)	Jackson (1973)
Sceloporus grammicus complex (chromosomal races)	Nat. (HZ)	7 M	F_1, BC (5)	↓ FA for 3 traits	Dosselman et al. (1998)
Fishes					
Salmo clarki bouvieri × *S. c. lewisi*	Exp.	4 M	F_1	↓ FA (for 1 cross)	Ferguson et al. (1988)
Salmo gairdneri (3 hatchery strains)	Exp.	4 M [c,d]	F_1	↓ FA (for 2 crosses)	Ferguson (1986)
Salmo gairdneri × *S. clarki bouvieri, S. c. lewisi, S. c. clarki*	Exp.	5 M [c]	F_1	↑ FA	Leary et al. (1985)
Salmo salar × *S. trutta*	Exp.	3 M + 4 Q	F_1	↑ FA for meristic (0 in triploidized hybrids)	Wilkins et al. (1995)
Oncorhynchus gorbuscha (2 broodlines)	Exp.	3 M [c,d]	F_1, F_2	↑ FA in males F_2	Gharrett and Smoker (1991)
Oncorhynchus gorbuscha (2 broodlines)	Exp.	6 M	F_1, F_2	0	Gharrett et al. (1999)
Salvelinus confluentus × *S. fontinalis*	Nat.	5 M [c]	F_1	↑ FA	Leary et al. (1985)
Gasterosteus aculeatus complex (limnetics × benthics)	Exp. (Nat.)	2 M + 2 Q	F_1, BC, F_2 (6)	0	Hatfield (1997)
Gasterosteus aculeatus (3 morphs)	Nat.	1 M [c]	*Fn*	↑ FA	Zakharov (1981)
Coregonus clupeaformis (ecotypes)	Exp.	4 M + 3 Q	F_1	0	Lu and Bernatchez (1999)
Poeciliopsis monacha × *P. lucida*	Nat.	8 M [c]	F_1	0	Vrijenhoek and Lerman (1982)
Lepomis macrochirus macrochirus × *L. m. purpurescens*	Nat. (HZ)	6 M + 3 Q [e]	*Fn* (N)	↓ FA for 1 trait	Felley (1980)
Lepomis cyanellus × *Micropterus salmoides*	Exp.	A	F_1	↑ anomalies	Whitt et al. (1977)
Enneacanthus obesus × *E. gloriosus*	Nat. (HZ)	4 M + 3 Q [e]	*Fn* (N)	↑ FA for 2 traits	Graham and Felley (1985)
Birds					
Colaptes auratus auratus × *C. a. cafer*	Nat. (HZ)	3 Q + 2 M	*Fn*	↑ FA for 1 trait	Graham (1992) and pers. comm.

Mammals					
Mus musculus domesticus × *M. m. musculus*	Exp.	1 Q 6 Q 1 Q + 1Q	F_1, BC, F_2	↓ FA	Auffray et al. (1996) Alibert et al. (1997) Debat et al. (2000)
Mus musculus domesticus × *M. m. musculus*	Nat. (HZ)	6 Q [c]	*Fn* (5)	↓ FA	Alibert et al. (1994)
Mus musculus domesticus (chromosomal races)	Exp.	6 Q	F_1, BC	0	Auffray et al. (2001)
Mus musculus domesticus (chromosomal races)	Nat. (HZ)	6 Q	*Fn*	↑ FA	Chatti et al. (1999)
Saguinus fuscicollis illigeri × *S. f. lagonotus*, *S. f. leucogenis*, *S.f. nigrifrons*	Exp.	16 Q	F_1	↓ FA	Hutchison and Cheverud (1995)
Bison bonasus × *Bos taurus*	Exp.	25 M	F_1, BC	0	Baranov and Zakharov (1997)

[a] Nature of crosses: Exp. = experimental cross; Exp. (Nat.) = experimental cross conducted in natural conditions; Nat. (garden) = natural cross but development in controlled garden; Nat. = natural cross; Nat. (HZ) = natural cross restricted to a hybrid zone.

[b] Number and nature of the characters studied: Q = quantitative, M = meristic, B = biochemical. A means abnormal variation in one or several traits (the nature of the traits is given in parenthesis) and Δ_{within} means that within-individual variation has been used to assess DI instead or in addition to FA.

[c] No estimation of measurement error.

[d] No preliminary tests (detection of DA, AS, size dependence).

[e] Hybrid classes or sample considered: the number in parentheses indicates the number of different introgression classes defined whereas (N) indicates the case where several hybrid samples were considered but without reference to their introgression level.

[f] Results reported: the number of traits which significantly differed in DI between hybrid and parental groups is specified and 0 indicates that no significant differences have been found. When no indications are done, this means that synthetic indices of DI have been used and that the levels of DI of each trait has not been distinguished.

instance, crosses between different subspecies of saddle-back tamarin (*Sanguinus fuscicollis*) revealed consistently lower levels of cranial FA in hybrids than in the parental taxa (Hutchison and Cheverud 1995).

Several inferences can be drawn from the preceding discussion. Contrary to the common belief (e.g., see Møller & Swaddle 1997), the number of studies reporting evidence of increased developmental stability in intersubspecific or interspecific hybrids is far from negligible. The results summarized in table 8.3 show that 11 studies found a decrease in DI in hybrids as compared to parental groups in at least one trait (but see below for a discussion about the problems related to such comparisons). Whereas four studies were crosses between lines, strains, or races, six studies were crosses between subspecies, and one involved species. In addition, even if the definition of the different taxonomic categories is somewhat variable among taxa, the results presented in table 8.3 show that it is difficult to establish a clear pattern concerning the relationship between the taxonomic status of the hybridizing groups and DI of hybrids. Although there is a global trend in the direction predicted by the model of Vrijenhoek and Lerman (1982), a number of studies report results not consistent with the predictions. This means that the links between divergence at protein loci (genetic distance) and divergence at regulatory loci (assessed by developmental stability) are not always straightforward. Such a phenomenon, already noted for hybrids between salmonids (Ferguson et al. 1988), seems, therefore, more widespread than previously thought.

Problems Related to the Study of DI in Hybrids

Studied Organisms Inspection of table 8.2 shows that the relationship between outbreeding depression and DI has been assessed in a wide variety of organisms. Whereas the multiplicity of statistical approaches used to detect DI is often—rightly—criticized, the problem of comparing results obtained within many diverse organisms is rarely pointed out. Genomic coadaptation concerns any organism and should theoretically be considered as a general mechanism. However, it is likely that the mechanisms that control development, and resistance to accidents and perturbations occurring during development, differ among organisms because the different lineages may have evolved different strategies for the control of developmental homeostasis. As a consequence, organisms may not show the same sensitivity to genetic stresses. For instance, some authors have pointed out that poikilotherms show a stronger relationship between heterozygosity and DI than homeotherms (Vøllestad et al. 1999). This could be because they experience higher environmental variance during development than endotherms (Novak et al. 1993; Vøllestad et al. 1999). Such features, as well as the differences in metabolic pathways among organisms, may explain some of the different patterns observed between taxonomic groups. In table 8.2, it is clear that some taxa, particularly fishes, are over-represented. This is probably attributable to the fact that fish, at least among vertebrates, are most likely to hybridize

Table 8.3 Number of Studies Indicating Increased, Similar, or Decreased DI in Hybrids Relative to Their Parents

Studies were considered as indicating increased or decreased DI if they reported a significant difference between hybrid and parental groups in at least one trait, and as indicating similar level of DI if none of the traits studied showed a significant difference between hybrid and parental groups. In consequence, the study of Freeman et al. (1995) has been recorded twice because it reported increased DI for two traits and decreased DI for two other traits (see table 8.1). Crosses between species (or genus), subspecies and differentiated populations, races or lines are distinguished.

Level of crosses	Increased DI	Similar DI	Decreased DI	Total
Genus or species	17	6	1	24
Subspecies	3	0	6	9
Differentiated populations, races or lines	5	5	4	14
Total	25	11	11	47

and to several factors, such as good knowledge of the biological model or interest in aquaculture. It is also possible that fishes exhibit intrinsic properties due to a higher sensitivity to genetic stress.

It has been argued that plants are ideally suited to the study of the genetic basis of DI because they incorporate the possibility of studying clonal structures and because of the various kinds of morphological asymmetry they often demonstrate (Freeman et al. 1995, chapter 20, this volume; Møller and Shykoff 1999). Table 8.2 shows that studies conducted on plants remain a minority even though much of the work conducted in the last three years was on plants. In the case of hybrid zones, plants are especially interesting because they also allow researchers to conduct reciprocal transplant experiments, thus enabling researchers to distinguish between genetic and environmental effects. However, plants are also subjected to specific confounding effects, such as plasticity related to microenvironmental differences or somatic mutation, which can generate within individual variability that is unrelated to DI (Palmer 1996; Wilsey et al. 1998; Perfectti and Camacho 1999; Freeman et al., chapter 20, this volume).

Statistical Approaches A criticism often leveled against FA studies concerns the variety of statistical approaches used to measure and analyze FA. Several authors have continued to recommend a number of statistical procedures and tests for detecting the presence of directional asymmetry and antisymmetry, for avoiding bias due to measurement error and for allowing the quantitative comparison of the results (Palmer and Strobeck 1986; Palmer 1994; Swaddle et al. 1994). In the case of studies dealing with the relationships between outbreeding depression and DI, an additional source of bias concerns the possible heterogeneity of individual developmental instabilities within a sample (Swaddle et al. 1994).

Arnold and Hodges (1995) have highlighted the need, when trying to estimate any fitness parameter in hybrids, to define hybrid classes, that is, to consider hybrid individuals according to their level of genomic introgression. Indeed, it is likely that the different genotypic combinations of hybrid genotypes result in a range of fitness values. To take into account such variation, recombinant classes should be defined and considered independently, particularly in hybrid zones which are, by definition, composed of a wide variety of genotypes. These considerations are important for at least two reasons. First, when considering a single hybrid class it is possible that one kind of genotype was over-represented. If, for instance, such a class contained mainly slightly introgressed genotypes, one could then expect little or no differences in DI levels between hybrid and parental groups. This would lead to erroneous conclusions about the real effect of genomic recombination on hybrid developmental stability. The second reason is a statistical one. It is well documented that a mixture of subsamples having different levels of FA, results in a leptokurtic distribution of the individual asymmetry values (Graham 1992; Palmer and Strobeck 1992). Pooling all hybrids in a single hybrid class may lead to such a distribution and whenever possible, homogeneous hybrid classes are therefore preferential. To define precisely such classes, it has been argued that genetic markers should be preferred to morphological ones because they can provide a higher number of nonambiguous diagnostic markers than the morphological traits (Arnold and Hodges 1995).

Experimental versus Natural Conditions As already mentioned, studies focusing on the genetic basis of developmental stability can be difficult to interpret when carried out on natural populations because of the potential confounding of genetic and environmental effects. This point is of particular importance in stable hybrid zones, because they are thought to be maintained either by differences in environmental conditions across the zone (Moore 1977), or as the result of a balance between gene flow and counterselection of hybrids (Barton and Hewitt 1989). If differences in DI between hybrid and parental populations are found in a hybrid zone, it is thus crucial to determine whether they are due to exogenous (differences of habitats) or endogenous (genetic) factors. Furthermore, in cases where a hybrid sample exhibits a lower level of DI, one must also be sure that the result really reflects a heterotic effect and not simply the consequence of stronger counterselection on hybrids, which may have selected the best, developmentally fittest individuals (Alibert et al. 1997). If no clear patterns emerge from the study of direct fitness estimates, that is, parameters associated with survival or reproduction, it can be necessary to conduct a laboratory-based, environmentally controlled and homogeneous experiment.

Such an approach has been adopted in a series of studies focusing on the hybridization between the two European house mouse subspecies, *M. m. domesticus* and *M. m. musculus*. Alibert et al. (1994) reported lower levels of FA of tooth characters for populations in the center of the natural hybrid zone in Denmark, whereas previous genetic and parasitological studies revealed genomic incompatibilities between the two subspecies (Sage et al. 1986; Vanlerberghe et al. 1988; Moulia et al. 1991, 1993). Even though the authors postulated a heterotic effect on developmental stability of the morphological traits studied, an alternative hypothesis is that the decrease in FA is in fact the result of selection against more asymmetrical individuals. However, laboratory crosses between the two subspecies have revealed that F_1 hybrids, as well as backcrosses and F_2 hybrids, all showed decreased DI (Auffray et al. 1996; Alibert et al. 1997; Debat et al. 2000). In addition, comparisons of the FA levels obtained in the laboratory crosses with those found in wild populations of the hybrid zone revealed only slight differences between samples. This suggests that the breakdown in coadapted gene systems regulating development was not more pronounced in highly recombined genomes (wild hybrids). On the contrary, because heterosis occurs before, as well as after, meiotic recombination, the hypothesis of a heterotic effect was confirmed.

Studied Characters Characters differ in their levels of DI. Differences in the history of selection on characters, the type (meristic or metric) of the characters, but also mathematical properties of the FA estimates, are generally invoked to explain such variation (Palmer 1994; Fenster and Galloway 1997; Waldmann 1999; Anderson et al. 2002). Whether or not one should expect character-specific instead of a genome-wide control of developmental stability is still under discussion (Whitlock 1996; Clarke 1998a; Auffray et al. 1999a). For instance, it has been argued that there is a relationship between degree of developmental stability of a character and the extent to which this character influences the fitness of the organism (Palmer and Strobeck 1986; Clarke 1998a): the higher the functional significance, the greater the canalization and developmental stability of the trait.

In the context of differentiating populations or taxa, it is of a particular interest to consider species-specific (i.e., very divergent), as well as less divergent characters when assessing DI in hybrids. According to current speciation theories and a number of observational studies, traits linked to components of reproduction should be morphologically most divergent (see, for example, Civetta and Singh 1998). For instance, sibling species of the *Drosophila melanogaster* complex show a higher divergence in sexual traits compared with the divergence in nonsexual traits. As a consequence, higher DI of these traits was found in interspecific hybrids (Civetta and Singh 1998). In plants, floral traits have been shown to be developmentally more stable than vegetative traits (Evans and Marshall 1996; Sherry and Lord 1996; Freeman et al., chapter 20, this volume). Similarly, if higher divergence is expected for traits linked to mating components of reproduction, higher developmental incompatibilities should be found for floral traits in hybrids. This prediction was confirmed by Waldmann (1999) who reported increased DI for floral but not for leaf characters in hybrids between differentiated populations of *Silene diclinis* (but see Perfectti and Camacho 1999).

What Can DI Teach Us About the Nature of Genomic Coadaptation?

We have seen above how the notion of coadaptation (and heterozygosity) can explain variation in the levels of DI of organisms. It is also possible to approach such a relationship from the opposite perspective, that is, to see how DI can help us to enhance our knowledge of coadaptation.

Nature of Genes

Breakdown in genomic coadaptation is most often detected in introgressed genomes, but hybridization does not constitute a unique cause of disruption of coadapted gene complexes. The introduction of a single new allele into a genome can also lead to a break-up of genomic coadaptation among certain genes and may have deleterious effects on developmental stability. This has been demonstrated by a series of now classic studies of FA in resistant versus susceptible Australian sheep blowflies (*Lucilia cuprina*) to dieldrin and diazinon insecticides (Clarke and McKenzie 1987; McKenzie, chapter 9, this volume). Resistance to each of these insecticides is independently controlled by alleles at two unlinked loci. Prior to the use of insecticide, the substitution of the alleles which confer resistance

was shown to have deleterious effects on physiological processes and was accompanied by an increase of DI (McKenzie and Clarke 1988). These resistance alleles are selected against in a pesticide-free environment, and are therefore presumed to disrupt the coadaptation of the background genome (Clarke 1993). The increase in FA has been shown for both heterozygous and homozygous carriers of resistance alleles, so the alteration of developmental stability may not be ascribed to an underdominance effect; however, it is clearly related to negative epistasis between the introduced allele and the genetic background. Most interestingly, in diazinon-resistant populations, a modifier allele, mapped on another chromosome, was subsequently selected, restoring the FA to background levels, that is, those found in susceptible populations in the absence of insecticide (McKenzie, chapter 9, this volume). This clearly suggests that in such cases selection might rapidly act to re-establish appropriate coadapted gene systems.

Internal Balance

Disruption of genomic coadaptation is invoked to explain the incompatibility between hybridizing genomes. In terms of DI, breakdown of coadapted gene systems may occur in the F_1 generation or in subsequent ones, that is, backcrosses (BC), F_2 or F_n generations after recombination has taken place. DI appearing in F_1 hybrids would point to allelic underdominance or direct negative epistasis between genes of parental groups as the processes generating genomic incompatibility. In contrast, a significant increase of DI observed in F_2 (or BC) generations, as compared to the preceding one, would suggest that recombination is important in the disruption of coadapted gene complexes and that genic balance within chromosomes (internal balance) is involved in genomic coadaptation.

In the literature, when incompatibility between hybridizing parental groups is detected through an increase of DI in hybrids, this incompatibility is most often noticed in the F_1 generation (see table 8.2). This suggests that, although recombination could be important in the disruption of coadaptation, incompatibility between taxa often emerges from the simple juxtaposition of parental chromosomes, that is, prior to recombination. However, as most studies have focused on F_1 hybrids, it is thus difficult to appraise the effect of recombination on the disruption of coadapted gene complexes involved in developmental stability. In addition, among studies which could have addressed the effect of recombination, several demonstrated a decrease of DI in hybrids (table 8.2); these concern hybrids between lines of *Drosophila* (Blows and Sokolowski 1995), subspecies or strains of *Mus musculus* (Leamy 1984, 1992; Auffray et al. 1996; Alibert 1997; Debat et al. 2000) and chromosomal races of *Sceloporus grammicus* for some of the traits considered (Dosselman et al. 1998). In the latter species, the other traits did not provide any clear differences in FA between parental groups and subsequent generations. This was also the case for hybrids between *Drosophila* species (Gupta 1978) and between Bison and domestic cattle (Baranov and Zakharov 1997). In fact, there are only two studies indicating that recombination can disrupt coadapted gene systems involved in developmental stability. In the first of these studies, F_2 hybrid males between two broodlines of *Oncorhynchus gorbuscha* exhibited an increase in FA when compared to parental or F_1 groups (Gharrett and Smoker 1991). However, this pattern was not observed in a further study on the same hybrids (Gharrett et al. 1999). The second study concerns hybrids between two species of willow (*Salix*), in which F_2 hybrids seemed to exhibit higher levels of FA than F_1s even if the *P*-value testing the difference between these two generations was only "marginally significant" ($P = 0.06$; Hochwender and Fritz 1999). The only nonambiguous evidence that recombination between differentiated genomes could increase DI comes from a recent study of hybrids between a parthenogenetic and a sexually reproducing strain of *Drosophila mercatorum*. The authors reported that F_2 hybrids exhibited higher level of FA compared with the F_1 generation for all the three traits studied in females, and that not only F_2 but also F_3 hybrids were more asymmetrical for one trait in males (Andersen et al. 2002).

The poor support of the role of recombination in disrupting coadapted gene systems involved in developmental stability, suggests that genes interacting to control development are tightly linked, preserving their association despite recombination, or, conversely, widely dispersed among chromosomes. The latter assertion is supported by the result of a study which found that the few QTLs (quantitative trait loci) related to FA for certain traits were located on several chromosomes, with no relation to the location of the genes coding for

the traits under consideration (Leamy et al. 1997, 1998).

In conclusion, when it occurs, DI is clearly detected as early as the F_1 generation in crosses between differentiated groups, demonstrating the importance of genic interactions among chromosomes (relational and interchromosomal balances) in the control of developmental stability. However, the role of within-chromosome balance between genes (internal balance) has still to be clearly documented in diploid organisms.

Relational Balance

We have shown that the genic balance among chromosomes is involved in maintaining adequate levels of developmental stability. However, within this global genic interaction at a genome-wide scale, few studies addressed the precise role of nonallelic interactions between homologous chromosomes (relational genic balance 1 in table 8.1).

In haplo-diploid organisms, the simple existence of haploids suggests that, among the different levels of genic interaction presumably involved in genomic coadaptation, the relational balance may not have a major role. Although the pioneering study on the honey bee (*Apis mellifera*) concluded that haploid males exhibited higher levels of DI than did diploid females (Clarke et al. 1992), this result has not been supported by a survey of other haplo-diploid systems (Clarke 1997). The fact that haploid organisms do not exhibit higher levels of DI than diploid ones downgrades the role of both heterozygosity and relational balance as a genetic condition for developmental stability. However, these conclusions may specifically concern haploid or haplo-diploid organisms. First, it is classically admitted that one of the ways that heterozygosity controls developmental stability is by masking deleterious recessive alleles. In haplo-diploid systems, selection may have well eliminated these alleles, while they may persist in diploid systems (Clarke et al. 1992; Markow 1995). Second, although in haploid systems genic interaction between homologous chromosomes cannot be invoked in the control of developmental stability, such an interaction has been shown to play a significant role in diploid organisms. Experimental triploid hybrids between Atlantic salmon (*Salmo salar*) and European trout (*Salmo trutta*) exhibit similar FA levels in meristic traits compared to parental taxa, while regular diploid hybrids exhibited an increase in FA (Wilkins et al. 1995). Triploid hybrids carried one copy of the trout chromosomes and two identical copies of the salmon chromosomes. In these hybrids, restoration of the relational genic balance between coadapted homologous chromosomes of one of the parental groups, despite the fact that these chromosomes were identical, largely compensated for the deleterious effects of outbreeding between the two differentiated genomes.

Chromosomal Imbalance

It is well known that sudden modifications in the chromosomal organization of organisms can have strong effects on their developmental stability. For instance, aneuploidy corresponds to the loss or gain of one or several chromosomes. In such cases of chromosomal imbalance, any level of allelic or nonallelic interactions (internal, relational and interchromosomal balances) is presumably affected. In diploid organisms, such a deviation from the basic 2n chromosome number, is expected to result in an alteration of gene dosage, unless compensatory mechanisms exist as for most X-chromosome loci in man (Shapiro 1992). Trisomy of chromosome 21 in humans leads to major developmental perturbations including an increase of FA (Shapiro 1983, 1992; Reeves et al. 2001). It was shown that gene dosage was not compensated for loci on chromosome 21, neither in trisomy nor in monosomy. Similarly, the presence of an extra Y chromosome in the genome of *Drosophila melanogaster* has been shown to change the optimum temperature of development of the flies (Jokela and Portin 1991) and the loss of a microchromosome in hybrids between female *Drosophila virilis* and male *D. lummei* is believed to affect their developmental stability (Orr 1990).

In contrast to the major phenotypic manifestations arising from unbalanced chromosomal mutations, most of which are eliminated by selection, the substantial number of species exhibiting a certain amount of chromosomal diversity in nature suggests that balanced chromosomal rearrangements do not produce strong perturbations in the functioning of the genome. However, balanced chromosomal rearrangements could theoretically have an effect on genomic coadaptation. The structural reassociation of loci within and among chromosomes changes the immediate environment of genes. Translocations and inversions can disrupt chromosomal segments, separating closely located

loci, and/or, conversely, linking formerly separated loci. Additionally, by changing the number and the size of chromosomes, balanced translocations also change the rate of recombination (Davisson and Akeson 1993; Qumsiyeh 1994) and may increase the likelihood of breakdown of genomic coadaptation. Finally, it is thought that the transcriptional activity of genes, which may have a role in developmental stability, depends on the topology of chromosomes in the interphasic nucleus (Capanna and Redi 1994; Qumsiyeh 1995). This topology is modified by chromosomal rearrangements (Qumsiyeh 1995) and may indirectly affect developmental stability.

It is interesting that different homozygous chromosomal races occurring in nature present similar levels of FA, for example, in the house mouse, *Mus musculus* (Alibert et al. 1994; Chatti et al. 1999; Auffray et al. 2001), the mole rat, *Spalax ehrenbergi* (Auffray et al. 1999b), and for most traits studied in the iguanid lizard, *Sceloporus grammicus* (Dosselman et al. 1998), despite the fact that all these complexes of chromosomal races present extensive chromosomal differentiation. This suggests that balanced chromosomal rearrangements do not lead to detectable amounts of increased DI in homozygous populations or, alternatively, that selection has acted to restore background levels of FA. However, in contrast to this pattern, natural hybrids between chromosomal races diverging for nine Robertsonian fusions in the house mouse were shown to exhibit an increasing FA suggesting a breakdown in genomic coadaptation (Chatti et al. 1999). Conversely, house mouse hybrids for one Robertsonian fusion, as well as carriers of spontaneous whole arm reciprocal translocations do not exhibit a higher DI than the control groups (Auffray et al. 2001). Whether the DI in hybrids between highly divergent chromosomal races is due to structural or to genic heterozygosity remains uncertain. The accumulation of chromosomal rearrangements contributes to reduce gene flow between diverging races, and subsequently to expand their genic differentiation. The precise direct or indirect role of the accumulation of balanced chromosomal rearrangements in the emergence of incompatibility between taxa has still to be documented. By allowing the appraisal of this incompatibility, DI approaches may have an important role to play in this topic, as well as in any differentiation issue in evolutionary biology.

DI in Hybrids: Implications for the Study of Fitness

The field of DI has founded a part of its popularity on the hypothesis that FA, or any measure of DI, may provide a good measure of individual quality and fitness (Jones 1987; Møller 1997, 1999; Shykoff anad Møller 1999). However, the relationships between DI, stress, and fitness has become one of the most controversial and debated issues in evolutionary biology (Leung and Forbes 1997; Clarke 1998b, chapter 12, this volume; Nachman and Heller 1999). The question arising in the context of the present chapter is to what extent do the studies on hybrids or outbred groups enhance our knowledge of whether DI predicts reduced fitness. Graham (1992) has already stressed that in hybrids the relationship between DI and fitness is not straightforward. He pointed out that, because fitness depends not only on the survival of hybrids but also on their gamete and offspring viability, reproductive fitness and DI can be a generation out of phase. In fact, one could add that, in some cases, DI and reproductive fitness appear to be completely disconnected. For example, in the cases of the hybrids between chromosomal races of the lizard, *Sceloporus grammicus*, and those between the two subspecies of the house mouse in Europe, which were both found to have decreased DI (table 8.2), evidence of selection against hybrids was found in the hybrid zones. In the lizards, meiotic malassortment of one chromosome pair in male F_1 hybrids, and reduced litter size in female F_1 hybrids, indicated a decreased fitness (Dosselman et al. 1998). In the house mouse study, the coincidence in the location of the cline centers of various genetical markers, the reduced sex chromosome introgression, and the greater susceptibility of hybrid populations to intestinal worms strongly all suggest reduced fitness of the hybrids (see, for review, Boursot et al. 1993; Sage et al. 1993). In addition, it was found that a considerable proportion of the F_1 hybrids of both sexes were sterile (Alibert et al. 1997). Furthermore, sterile laboratory-reared house mouse hybrid individuals did not exhibit higher DI than fertile ones (Alibert et al. 1997). Although no data about the viability of these hybrid were collected, it is clear that there is *no direct link* between reproductive fitness and developmental stability in this case. Therefore, the results described above suggest that differences in

DI levels between samples cannot constitute a reliable indicator of differences in overall fitness. Whereas some hybrid functions clearly undergo outbreeding depression (e.g., loss of reproductive function), others seem to be unaffected or even show heterosis, as it is the case for developmental stability. Whether the apparent opposite effect that hybridization has on these different functions can be related to structural (number of genes implied in each function) or a functional (rate of evolution of genes and/or strength of interactions between them) effect is difficult to say.

Conclusions

The classical assumption is that the consequence of genome mixture on DI in hybrids depends on the time of divergence and, subsequently, on the genetic divergence between taxa, as well as on the time of first hybridization (Jackson 19973; Clarke 1993). Studying the radiation processes in the house mouse in Europe, we have found that most results obtained on DI in hybrids were fairly unpredictable. Hybrids between two subspecies, having diverged half a million years ago and presenting a significant genetic divergence (Boursot et al. 1993), exhibit lower levels of DI than parental subspecies (Alibert et al. 1994, 1997). Conversely, hybrids between two chromosomal races differentiated at most 5000 years ago suffer from outbreeding depression (Chatti et al. 1999). Finally, mice carrying a new chromosomal translocation, which is thought to be selected against in nature, do not exhibit an increase in DI (Auffray et al. 2001). The present review confirms that reliable predictions concerning the levels DI in hybrids are difficult to make unless factors, such as structural (e.g., chromosomal) versus genic divergence or the nature of traits studied, are taken into account. Hence we conclude that we are still in an exploratory phase, but it appears that the study of hybrids can help us to document the relevance of the presumed genetic conditions of developmental stability, and better understand the nature of genomic coadaptation, and its role in the speciation process.

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ARTICLE 6 :

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Hybridization, developmental stability, and functionality of morphological traits in the ground beetle *Carabus solieri* (Coleoptera, Carabidae)

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The assessment of developmental stability in hybrids can provide valuable information in the study of species formation because it allows an evaluation of the degree of incompatibility of genetic systems that control developmental processes. The present study assessed the impact of two hybridization events, assumed to have occurred at different times, on developmental instability in the ground beetle *Carabus solieri*. Developmental instability was estimated in 678 individuals from 27 populations from the fluctuating asymmetry (FA) levels of four morphological traits: the tibia length of middle and hind legs, which are functional structures, and the length and the proximal width of the hind wings, which are vestigial and thus nonfunctional structures. Significant variations of FA levels between populations were shown only for the wing width. For this trait, FA levels in hybrids were higher than in their parental entities for both hybridization events, indicating a significant divergence of the gene systems controlling development between the parental entities in the two hybridization cases. As expected, wing traits exhibited FA levels at least three times higher than leg trait. Finally, the potential interest of vestigial traits in the particular context of hybridization is discussed. *Biological Journal of the Linnean Society*, 2006, **89**, 151–158.

ADDITIONAL KEYWORDS: differentiation – fluctuating asymmetry – hybrid dysgenesis – speciation – vestigial traits.

INTRODUCTION

The impact of various environmental and genetical stresses on developmental instability is generally assessed through measurements of fluctuating asymmetry (FA), which is defined as the small random deviations from symmetry in otherwise bilaterally symmetrical structures (Palmer & Strobeck, 1986). This approach has been used in a wide array of fields, including evolution, ecology, behaviour, and development, and the study of developmental instability has become one of the most debated issues in evolutionary biology. Even though discordant results, methodological difficulties, and poor knowledge of actual mechanisms responsible for developmental stability make generalizations difficult (Clarke, 1998; Palmer, 2000; Clarke, 2003; Tomkins & Simmons, 2003), the usefulness of FA has been clearly demonstrated for all these topics (Polak, 2003). More specifically, the relationship between developmental instability and hybridization can provide valuable information in the study of speciation because it can be used to evaluate the degree of incompatibility of genetic systems controlling developmental processes (Graham, 1992; Alibert & Auffray,

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2003). Despite the fact that its genetic basis is not yet fully understood, developmental instability in hybrids is usually proposed to be affected by two main genetic factors acting in opposite directions. The increase in heterozygosity is expected to decrease developmental instability whereas the breakdown in genomic coadaptation should increase it. This situation is generally illustrated by a balance between both factors whose equilibrium is related to the genetic divergence between the hybridizing entities (Vrijenhoek & Lerman, 1982; Graham, 1992): the higher the divergence, the higher the chance for hybrids to be developmentally instable.

However, a recent review emphasized that the link between FA in hybrids and divergence in parental entities is not so simple (Alibert & Auffray, 2003). Among the potential sources of discordant results available across studies, the nature of traits is evoked. It is widely postulated that traits strongly subjected to natural selection are more buffered against both developmental noise and environmental/genetic influences (Debat & David, 2001). Nonetheless, the link between FA and natural selection remains unclear, partly because of the difficulty to define a priori the intensity of natural selection affecting a given trait. In this context, the comparison of functional and nonfunctional structures may offer a solution. Surprisingly, to our knowledge, only Crespi & Vanderkist (1997) have compared FA levels between functional traits and vestigial traits [i.e. traits that have been rendered nonfunctional or that have become selected against due to a shift in the environment (Fong, Kane & Culver, 1995)]. Their study in the thrips *Oncothrips tepperi* revealed a higher FA in the wings of soldiers, which are vestigial, than in the functional wings of dispersers, but no difference between soldiers and dispersers for FA of the fore femora, which are functional in both cases. These results were ascribed to relaxation of selection for functionality in vestigial traits.

In the present study, the impact of hybridization on developmental instability in the ground beetle *Carabus solieri* Dejean 1826 (Coleoptera, Carabidae) is evaluated. This species is a suitable model to study speciation. Despite a range restricted to the southern and Ligurian Alps, genetic, morphological, and colour variations are significant and exhibit a clear geographical structure (Bonadona, 1967; Darnaud, Lecumberry & Blanc, 1978; Rasplus *et al*., 2001; Garnier *et al*., 2004, 2005). This pattern probably results from limited dispersal ability (*C. solieri* being a flightless species), habitat fragmentation, and phylogeographical history. On the basis of recent studies using both molecular markers (mitochondrial DNA sequences and microsatellite markers, Rasplus *et al*., 2001; Garnier *et al*., 2004) and morphometric data (Garnier *et al*., 2005), a phylogeographical scenario is proposed postulating that *C. solieri* has differentiated into two subspecies following isolation in two refuges during last Pleistocene glaciations: one of green colour in Italy and one of blue colour in the South of France. After postglacial recolonization, the two subspecies would have met and hybridized. For convenience, groups of populations can be defined according to both colour and geographical location (Fig. 1). According to the phylogeographical scenario, the current Bonnetianus (blue) and Solieri-INW groups (green) are the descendant of the two original subspecies. The Clairi (blue) and Solieri-C (green) groups originate from hybridization and introgression between these subspecies. Finally, the Curtii group (blue–green), which occurs between the Bonnetianus and Solieri-C groups, originates from hybridization between them.

The model used in the present study is interesting for two reasons. First, it presents two hybridization events at different time and space scales. The first one, which occurred between the two original subspecies, can be considered a relatively old event, or at least as a past event because, currently, there are no genetic exchanges between Bonnetianus and Solieri-INW groups (i.e. the groups derived from the two original subspecies). On the other hand, the second hybridization event from which the Curtii group originated is a more contemporary event, and may still be in progress. Secondly, this species is brachypterous, which means that the hind wings are atrophied. The presence of this vestigial trait allows the study of developmental instability of clearly nonfunctional traits (i.e. hind wings) in addition to functional ones such as legs.

The first study objective was to compare FA levels between hybrids and parental entities for the two hybridization events. The hybrid or parental status of the different entities is determined from the genetic and morphometric characterization of numerous populations from all over the range (Rasplus *et al*., 2001; Garnier *et al*., 2004, 2005). This comparison allows an evaluation of the divergence between parental entities in terms of genomic coadaptation of the gene systems controlling developmental stability. Given that introgression appears to have occurred over a large area between the two original subspecies, an absence of strong incompatibilities between them is anticipated. Moreover, if new coadapted gene complexes have the time to be selected in cases where the hybrid zone is old enough (Graham & Felley, 1985; Graham, 1992), FA levels in the Solieri-C and Clairi groups (hybrids) are not expected to be higher than in their parental entities. Concerning the more recent hybridization event, the results of experimental crosses between individuals from Bonnetianus and Solieri-C groups suggest a partial reproductive isolation between these

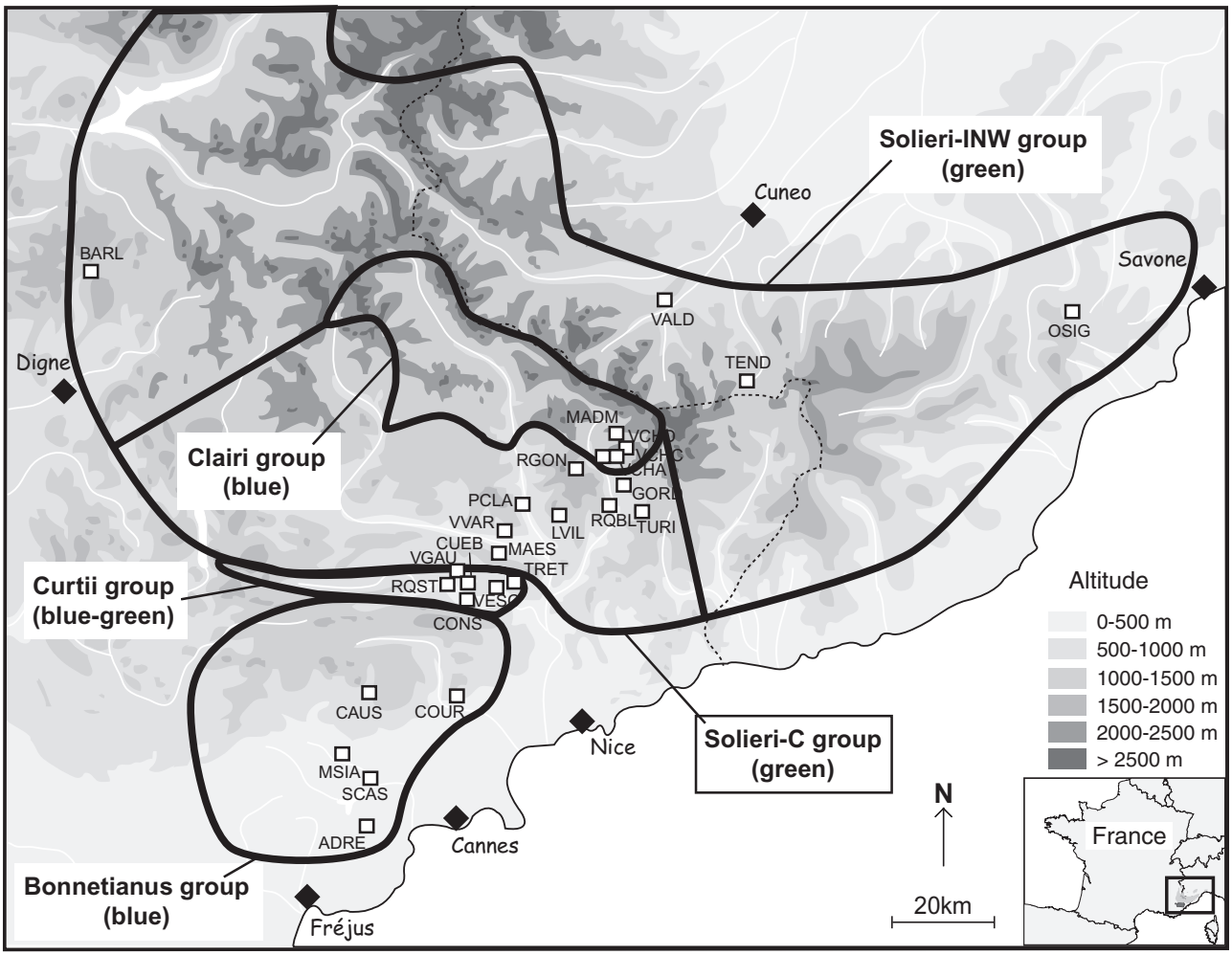

Figure 1. Distribution area of *Carabus solieri*, sampling locations (white squares), and limits of the groups of populations defined according to the colour of individuals (indicated in parentheses).

two entities (Puisségur, 1973; Malausa, Drescher & Armand, 1982). Because these entities also appear differentiated from molecular and morphologic markers (Rasplus *et al*., 2001; Garnier *et al*., 2004, 2005), higher FA levels are expected in the Curtii group (hybrid) than in the Bonnetianus plus Solieri-C groups (parental entities). The second main objective was to compare FA levels of functional and vestigial traits (i.e. legs and hind wings, respectively). If selective pressures on the control of developmental stability are released in nonfunctional traits, higher FA levels are predicted in vestigial wings than in legs.

MATERIAL AND METHODS

Sampling scheme and measured characters

A total of 678 individuals were collected using pitfall traps in 27 populations ($N = 9$–30 per population) along a South-West/North-East transect (Fig. 1). This transect was defined to cross the range of *C. solieri*, passing successively through the Bonnetianus, Curtii, Solieri-C, Clairi, and Solieri-INW group ranges. Note that two sampled sites (BARL and OSIG), which are not located in the vicinity of this transect, were included in the analyses to increase the sample size of the Solieri-INW group. The sampling occurred between 1997 and 2000. Nevertheless, all individuals from a given population were collected within the same year. Sex ratios were biased in favour of males for all samples, probably because males are more mobile and attracted by female pheromones. However, sex was not distinguished in the analyses because most of samples contained less than ten females. Moreover, it is unlikely that a difference in FA between sexes, if any, would introduce a bias in the results because the sex ratio is approximately constant across samples. All populations have been pre-

viously genetically and morphologically characterized (Garnier *et al.*, 2004, 2005).

Four bilateral morphological traits were measured to quantify FA: the length and the proximal width of the vestigial wing (WINGL and WINGW, respectively), and the tibia lengths of middle and hind legs (TIBMID and TIBHIND, respectively). These traits were selected because: (1) the difference of functional importance between vestigial wings and tibias is unambiguous and (2) these traits have a lower measurement error than other bilateral traits examined (P. Alibert, unpubl. data). Measurements were made by the same individual (M.C.) using a Nikon measuring microscope MM-60 to an accuracy of 0.001 mm.

Note that, for the trait TIBMID, only 20 populations were used because middle legs were missing for most individuals from seven populations, due to being used for other purposes.

Preliminary statistical analyses

A number of factors such as the presence of other forms of asymmetry, allometry or measurement error can lead to biased estimation of FA (Palmer, 1994; Graham *et al.*, 1998; Van Dongen, Lens & Molenberghs, 1999; Palmer & Strobeck, 2003). A series of preliminary tests were therefore performed for each trait and each sample on the distributions of signed asymmetries [right minus left values (Ri – Li)] and absolute asymmetries ($|Ri - Li|$). We first checked the presence of directional asymmetry and antisymmetry. Both directional asymmetry and antisymmetry occur when the two sides of a bilateral character consistently differ in size but, in the case of antisymmetry, the largest side varies randomly among individuals (Van Valen, 1962). The presence of directional asymmetry was assessed by testing for a departure from zero of the mean of the distributions of the signed asymmetries, whereas antisymmetry was tested using a normality test as well as kurtosis and skewness estimates (Palmer & Strobeck, 2003). Next, absolute asymmetry values were regressed on character size defined as [(Ri + Li)/2]. Finally, a subsample of 84 individuals (44 for the middle legs) chosen across all samples was used to evaluate measurement error. These specimens were measured twice and the between side variation was tested against the variation due to measurement error through a two-ways (side × individual) analysis of variance (ANOVA) with repeated measurements on each side (Palmer & Strobeck, 1986; Palmer, 1994). Because numerous identical tests were repeatedly performed, the sequential Bonferroni test (Rice, 1989) was systematically applied, for each trait, across samples to limit the occurrence of a type I error.

Measures and comparisons of FA

For each trait and each sample, the FA level was estimated by the mean of the distribution of absolute asymmetries (FA1; Palmer, 1994). Absolute asymmetries were used because they allowed, for each trait, to test differences in FA between samples through an ANOVA with planned comparisons (Sokal & Rohlf, 1995). For the ANOVA procedure, if an overall significant difference between samples was found, four contrasts were performed to compare hybrid with parental entities, as well as the parental entities with each other. The first contrast tested for a difference in FA levels between the combination of Clairi and Solieri-C groups (hybrid) and Bonnetianus plus Solieri-INW groups (parental entities). The second contrast compared FA levels of parental entities (i.e. between Bonnetianus and Solieri-INW groups). The third contrast opposed FA levels of the Curtii group (hybrid) to those of Bonnetianus plus Solieri-C groups (parental entities). Finally, a fourth contrast tested FA levels between Bonnetianus and Solieri-C groups.

Recently, it has been suggested that the combination of information from multiple traits could provide an accurate estimate of individual developmental instability (Leung, Forbes & Houle, 2000; Palmer & Strobeck, 2003). Under the hypothesis of the existence of an organism-wide level of developmental instability, the more traits combined in multivariate measures of FA, the better the probability of detecting differences in FA levels between samples. The present study used a multivariate index that sums over traits the individual absolute asymmetry of a trait divided by the average absolute asymmetry of this trait for the entire sample (FA14, Palmer & Strobeck, 2003; CFA2, Leung *et al.*, 2000). This index refers to individual FA, and is given by:

$$FA14 = \sum_j \frac{|FAij|}{|FAj|}$$

where i and j denote the individual and the trait, respectively. The advantage of this index is that it is independent of among-trait differences in mean FA (Leung *et al.*, 2000; Palmer & Strobeck, 2003). This index was calculated, on the one hand, across all four traits and, on the other hand, across both pairs of functional (TIBMID and TIBHIND) and nonfunctional (WINGL and WINGW) traits. Differences of FA levels estimated by multivariate indices were tested following the same ANOVA procedure as for single-trait indices (FA1).

RESULTS

Preliminary statistical analyses

Significant directional asymmetry was detected for only one distribution (trait TIBMID) out of the 101

distributions tested. Significant departure of signed asymmetry distributions from normality was also revealed in a single case (trait TIBHIND) by using normality tests, whereas kurtosis and skewness were significant for 12 and three distributions, respectively (all traits except WINGL). Because some distributions were significant for several tests, 14 distributions exhibited a significant departure from normality overall. However, because no distribution was platykurtic, the presence of strong antisymmetry can be ruled out. On the whole, it was assumed that the samples studied exhibited only true FA because of the low number of significant results and because no particular trait or sample were concerned by the presence of antisymmetry, directional asymmetry, or both. Besides, FA appeared to be independent from trait size because only one regression out of the 101 regressions tested was found to be significant (trait WINGL). Finally, the two-way (side × individual) mixed-model ANOVA performed on the subsample of individuals measured twice showed that the nondirectional asymmetry variances (i.e. the interaction variances in the model) were always significantly larger than the measurement error.

Comparisons of FA levels

Single-trait FA levels were significantly different among samples for the trait WINGW only (Table 1). When considering several traits simultaneously, ANOVAs revealed no significant differences in FA levels among samples, neither when indices were calculated from all four traits, nor when they were calculated from either the two wing or the two tibia traits (Table 1). Planned comparisons were thus performed only for WINGW. For this trait, the first contrast testing for a difference in FA between the combination of Clairi and Solieri-C groups (hybrid) and Bonnetianus plus Solieri-INW groups (parental entities) was highly significant ($F_{1,620} = 8.24$, $P < 0.01$), with hybrids being more asymmetric (FA1 = 0.085) than parental entities (FA1 = 0.066). The second contrast showed that FA levels were not different between the two parental entities (i.e. Bonnetianus and Solieri-INW groups) ($F_{1,620} = 2.03$, $P = 0.15$). The third contrast comparing FA levels between the Curtii group (hybrid) and Bonnetianus plus Solieri-C groups (parental entities) was also significant ($F_{1,620} = 4.46$, $P < 0.05$). Hybrids also were more asymmetric (FA1 = 0.085) than parental entities (FA1 = 0.073) in this case. Concerning the parental entities, the fourth contrast revealed that FA levels were significantly higher in the Solieri-C group (FA1 = 0.081) than in the Bonnetianus group (FA1 = 0.059; $F_{1,620} = 5.91$, $P < 0.05$).

Finally, the overall FA levels of the different traits were compared after each individual asymmetry value was divided by the average size of the trait over the entire sample. The mean of these size-standardized FA values is given for each character in Table 2. Overall, wing traits exhibit FA levels at least three-fold higher than tibia lengths (Table 2). The highest level of FA is observed for WINGW, which is the only trait exhibiting significant difference of FA levels among samples.

Table 1. Results of the tests for differences in fluctuating asymmetry (FA) levels of the four morphological traits among samples

	Traits	d.f. = 1, 2	*F*	*P*
FA1	WINGL	26, 620	1.07	0.36
	WINGW	26, 620	1.53	0.04*
	TIBMID	19, 457	1.15	0.30
	TIBHIND	26, 638	1.45	0.07
FA14	All	19, 425	1.54	0.07
	Wing	26, 620	1.22	0.21
	Tibia	19, 457	1.09	0.35

Analyses of variance were performed both on a single trait (FA1) and multivariate indices (FA14). WINGL, length of the vestigial wing; WINGW, width of the basis of the vestigial wing; TIBMID, tibia length of the middle leg; TIBHIND, tibia length of the hind leg; d.f., degrees of freedom for numerator (1) and denominator (2). Multivariate indices were calculated from all four traits (all) and from both pairs of traits of nonfunctional (wing) and pairs of traits of functional (tibia) morphological structures.*$P < 0.05$.

DISCUSSION

The present study shows significant variation of FA levels between *C. solieri* populations for one trait out of the four traits measured. More precisely, hybrid populations, at least for the width of the vestigial wings (WINGW), exhibit higher levels of developmental instability than their parental entities. From a theoretical perspective, hybrid developmental instability

Table 2. Mean ± standard deviation of the size-standardized fluctuating asymmetry (FA) index calculated over the entire sample for each trait

Traits	Size-standardized FA
WINGL	0.030 ± 0.029
WINGW	0.060 ± 0.052
TIBMID	0.010 ± 0.009
TIBHIND	0.008 ± 0.008

WINGL, length of the vestigial wing; WINGW, width of the basis of the vestigial wing; TIBMID, tibia length of the middle leg; TIBHIND, tibia length of the hind leg.

depends on a balance between the stabilizing effect due to increased heterozygosity and the disruptive effect caused by breakdown of genomic coadaptation (Vrijenhoek & Lerman, 1982; Graham, 1992). In such a context, the results of the present study indicate that, for one trait, the latter effect is predominant in both hybridization events considered, meaning that significant divergence of the gene systems controlling development has occurred between the parental entities.

Such a hybrid dysgenesis has been reported in a wide variety of organisms, including plants, insects, amphibians, birds, and mammals (Alibert & Auffray, 2003). However, the result obtained in the present study was somewhat surprising in the case of the combination of Solieri-C and Clairi groups. Although this hybridization is considered to be a relatively old event, or at least a past event, it appears that this hybrid group still expresses a breakdown of genomic coadaptation. Studies reporting similar levels of FA in hybrids and parental entities have led to the proposal that newly coadapted gene systems may have been selected where hybrid zones are old enough (Graham & Felley, 1985; Graham, 1992). The results of the present study, at least for the trait WINGW, do not support this idea even if we can not precisely date this 'old' hybridization event. Yet, any links between the age of a hybrid zone and the developmental instability of hybrids remain difficult to assess, in particular because of the paucity of precise data and the number of additional factors that have to be taken into account, such as the level of differentiation of parental entities or the nature of the traits studied (Alibert *et al*., 1994; Alibert & Auffray, 2003). The higher level of FA in hybrids suggests a substantial divergence in genetic systems controlling developmental stability in the two original subspecies. It adds to the genetic (assessed by molecular markers) and morphological (assessed by colour and morphometric measurements) divergence recently reported between these two entities (Rasplus *et al*., 2001; Garnier *et al*., 2004, 2005), which probably occurred in the course of their geographical isolation during the last glacial ice period (Rasplus *et al*., 2001). Note that the hybrid entity considered in the present study involves two population groups (i.e. Clairi and Solieri-C groups), which are distinguishable by the colour of individuals only, and not by molecular markers (Garnier *et al*., 2004) nor morphometric measurements (Garnier *et al*., 2005). The absence of difference in FA levels between these two groups (not shown) suggests that homogeneity within this hybrid entity also concerns developmental stability.

The second hybridization event can be regarded as much more recent, and may still be in progress. The Curtii group has been shown to be genetically and morphologically intermediate, and then considered as a hybrid between the Bonnetianus and Solieri-C groups (Garnier *et al*., 2004, 2005). The geographical zone occupied by this group corresponds to a barrier to gene flow (Garnier *et al*., 2004), which could be a physical barrier to migration or a secondary contact with partial reproductive isolation. Indeed, this zone depicts the limit of the expansion of the Italian subspecies and the results of experimental crosses between individuals from Bonnetianus and Solieri-C groups suggest a partial reproductive isolation between these two entities (Puisségur, 1973; Malausa *et al*., 1982).

If the higher FA levels in hybrids provide evidence for a hybrid dysgenesis of some systems involved in the control of developmental stability, the results of the present study are not sufficient to make strong inferences about the overall fitness of hybrids. It has been claimed that the link between developmental instability and fitness is not necessarily straightforward (Polak, 2003). For example, in the present study, despite the higher FA levels found in hybrids between Bonnetianus and Solieri-INW groups, introgression between the two original subspecies appears to have occurred over a large geographical zone, which could indicate an absence of strong fitness reduction in these hybrids. Unfortunately, no data are available with respect to experimental crosses between these two original subspecies. At present, more extensive studies are necessary to precisely estimate hybrid fitness and then to determine the dynamics of the narrow hybrid zone corresponding to the Curtii group.

Developmental instability can be influenced both by genetic and environmental stresses, and it could be argued that the variation of FA levels found in the present study mirror differences in environmental stresses experienced by some populations. However, the phylogeographical scenario described above provided a solid frame to assess the effects of genetic factors by allowing the a priori definition of parental and hybrid entities. Moreover, this species is ubiquitous (Darnaud *et al*., 1978; Rasplus *et al*., 2001) and there is no indication to suggest that environmental stress acts differentially on populations. For example, no correlation was found between FA1 and the altitude of sample sites ($r = 0.20, P = 0.36$). It appears reasonable to assume that the patterns observed are probably explained by the phylogeography of this species and, more specifically, by hybridization events and their genetic consequences, even if it is accepted that many other environmental factors should be examined.

It remains true, however, that a significant result was obtained for only one morphological trait out of the four traits measured. In addition, the absence of a correlation in FA levels both between tibia traits and between all four traits (S. Garnier, unpubl. data) could

explain why multivariate indices were not found different among populations. Numerous causes can be invoked to explain the absence of correlation of FA levels between different traits (Lens *et al*., 2002). Nevertheless, the consideration of other traits provides interesting insights. First, individual FA levels of the WINGL trait were correlated to individual FA levels of WINGW when considering the entire sample (Spearman correlation coefficient = 0.10, $P < 0.05$), but no correlation was found when considering the values of populations. Second, FA indices for WINGL showed the same trend of variation among groups of populations as those for WINGW (S. Garnier, unpubl. data). Hence, the absence of significant variation of FA levels in WINGL among populations could be due to insufficient statistical power. On the other hand, it could be argued that the correlation between patterns of FA variation of both wing traits is simply due to the fact that they are measured on the same morphological structure and then can be geometrically correlated. Indeed, signed asymmetries of WINGW and WINGL were negatively correlated ($r = -0.13, P < 0.001$) across the entire sample, suggesting that wing asymmetry mainly concerns shape rather than size.

The different patterns observed for wing and leg traits are probably related to their different functionality. Even though numerous studies have indicated a link between the functionality of a trait and its developmental instability (Palmer, 1994; Fenster & Galloway, 1997; Waldmann, 1999), the number of documented examples remains scarce. In this context, vestigial structures offer a solution. To our knowledge, the present study is only the second one to compare FA levels between vestigial and functional traits. The much higher levels of developmental instability observed in the vestigial morphological structure vs. functional ones could be due to both: (1) *in natura* elimination of the more asymmetrical individuals for functional important traits through natural selection and (2) relaxation of stabilizing selection on vestigial structures allowing a diminution of constraints on the stability of developmental pathways. Therefore, the lack of significant variation among populations in FA levels in functional traits could be related to a low baseline level of developmental instability in these traits. The present study also indicates that, even if multivariate FA indices can provide more powerful markers of stresses (Leung *et al*., 2000), the mixing of signals from traits experiencing different selective pressures can scramble the signal.

Finally, the difference of functionality between traits can be meaningful in the particular context of hybridization. The relaxation of stabilizing selection on vestigial structures could have allowed a higher accumulation of variation as a result of mutation and genetic drift than in strongly constrained structures (Fong *et al*., 1995). Therefore, gene systems coding for vestigial traits and/or controlling for their development may diverge more quickly in allopatry than those related to functional traits and, thus, may exhibit a greater hybrid dysgenesis. In addition, selection of higher developmental stability, if heritable (Fuller & Houle, 2003), may be stronger in functional traits after breakdown of genomic coadaptation in hybrids. As a result, vestigial traits could be more sensitive in detecting hybridization consequences in cases of weak divergence or ancient events. More generally, because vestigialization is not a rare evolutionary phenomena (Fong *et al*., 1995), vestigial traits provide good models to investigate several features of developmental instability, such as its genetic basis and its link with natural selection.

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ARTICLE 7 :

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PARASITIC INFECTION AND DEVELOPMENTAL STABILITY: FLUCTUATING ASYMMETRY IN *GAMMARUS PULEX* INFECTED WITH TWO ACANTHOCEPHALAN SPECIES

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ABSTRACT: Several studies have reported a negative association between developmental stability and parasitic infection. However, the host–parasite associations examined so far consist only of a limited number of parasite taxa, and developmental stability was appraised on definitive hosts. The present study examines the association between infection by 2 acanthocephalan parasites, *Pomphorhynchus laevis* and *Polymorphus minutus*, and the developmental stability of their common intermediate host *Gammarus pulex*. Developmental stability was estimated from the fluctuating asymmetry (FA) levels of 6 morphological traits. A positive association was found between FA and infection. Infected gammarids tended to be more asymmetrical than the noninfected ones for an index generated by combining FA scores from 2 characters out of the 6 studied, even though no significant relationships were found between FA levels and parasitic loads. The simultaneous presence of both acanthocephalan species in the same host seems to be associated with increased FA levels of gammarids, but this trend was not statistically significant. For the same characters, males exhibited higher levels of FA than females.

The fitness cost of parasitism is central to the study of parasite–host interactions. For example, parasites may reduce fertility and longevity or alter the development of their host. However, the estimation of these major components of fitness is not always obvious. During the last 2 decades, the study of developmental stability has attracted considerable attention in numerous fields of evolutionary biology, including population genetics, sexual selection, and the study of interspecific interactions such as host–parasite associations (Palmer, 1996; Møller and Swaddle, 1997). Much controversy has surrounded the debate on developmental stability, particularly among researchers working on its link with fitness (Leung and Forbes, 1997; Møller and Swaddle, 1997; Clarke, 1998; Nachman and Heller, 1999). Developmental stability refers to the suite of processes through which organisms reduce phenotypic variation resulting from developmental accidents (Zakharov, 1989; Palmer, 1994). In most studies, developmental stability was measured by evaluating the amount of fluctuating asymmetry (FA; Van Valen, 1962). The basic assumption justifying the use of FA as a valuable index of developmental instability is that the development of the 2 sides of a bilateral trait is presumed to be controlled by the same genome; hence, any deviation to perfect symmetry from this trait (FA) is thought to reflect the inability of organisms to buffer developmental accidents (the inefficiency of developmental stability mechanisms).

A decrease of developmental stability has been associated with numerous genetic and environmental stresses in a wide variety of organisms. Genetic stresses consist of the increase of homozygosity and the breakdown in genomic coadaptation (Vrijenhoek and Lerman, 1982; Graham, 1992; Clarke, 1993; Alibert et al., 1994, 1997), whereas environmental stresses include chemical pollution (Zakharov and Yakoblov, 1990), temperature extremes (Siegel and Doyle, 1975; Parsons, 1990), or food deprivation (Swaddle and Witter, 1994). In a recent review, Møller (1996) reported that both the prevalence and the intensity of infections with parasites are, in most cases, significantly associated with a decrease in developmental stability (but see Hoffmann et al., 1998; Quek et al., 1999). This seems true for a wide range of hosts as well as parasites, although various processes can explain this pattern. Parasites could directly act on the developmental stability of their host by imposing on them a metabolic cost during their ontogeny (Møller, 1992; Polak, 1993). In this case, it is predicted that host developmental instability should be directly related to parasite virulence, parasite load, or both (Polak, 1993; Agnew and Koella, 1997; Thomas et al., 1998). Alternatively, the more asymmetrical individuals could be those that are more susceptible to infection. FA would then simply reflect an inefficiency of the immune system to resist infection (Møller, 1996; Møller, 1999). Finally, it is also possible that developmentally unstable individuals are more frequently exposed to infection because they live in more marginal and stressful environments (Møller and Swaddle, 1997).

The present study examines the association between FA in *Gammarus pulex* (Crustacea, Amphipoda) and infection by 2 acanthocephalan parasites, *Pomphorhynchus laevis* and *Polymorphus minutus*, in natural populations. Acanthocephalan parasites have complex life cycles that require vertebrate definitive hosts and arthropod intermediate hosts (Nickol 1985, Dezfuli et al. 2000, 2001). Gammarids infected with *P. laevis*, *P. minutus*, or both constitute a particularly suitable model for the study of the influence of endoparasites on FA. First, it allows an appraisal of the degrees of association between host FA and different ecological demands imposed by 2 otherwise closely related parasite species. Both parasite species are infective to different final hosts; i.e., *P. laevis* develops in various species of fish, whereas *P. minutus* exploits birds (mainly ducks) as final hosts (Crompton and Nickol, 1985). The 2 parasite species induce contrasting behavioral and physiological alterations. In *P. minutus*–infected gammarids, geotaxis is reversed from positive to negative, whereas phototaxis becomes strongly positive in *P. laevis*–infected individuals (Brown and Thompson, 1986; Cézilly et al., 2000). Other effects of *P. laevis* in *G. pulex* include reduced glucose and O_2 consumption (Rumpus and Kennedy, 1974; Crompton and Nickol, 1985) and increased hemocyanin concentration (Bentley and Hurd, 1993, 1996). Both acanthocephalan parasites have a negative effect on female fecundity. Female *G. pulex* infected with *P. minutus* will not produce eggs (Ward 1986), whereas fecundity is only reduced by *P. laevis* (Poulton and Thompson, 1987). Moreover, in both

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TABLE I. Sample size for each of the 6 characters studied (males/females). Sample column indicates the species and the number of parasites infecting gammarids.

	Character					
	Metric				Meristic	
Sample	Antenna	Carpus	Basis	Merus	Spines	Articles
1 *laevis*	30/31	30/31	30/31	29/31	30/31	30/31
1 *minutus*	31/31	31/31	31/31	31/31	31/31	31/31
>1 *laevis*	32/31	32/28	32/30	32/27	32/29	32/29
>1 *minutus*	24/20	22/20	24/20	24/19	24/20	24/20
laevis + *minutus*	16/10	16/10	16/11	14/11	16/11	14/11
Uninfected	31/36	31/36	31/36	31/36	31/36	31/36
Total	164/159	162/156	164/159	161/155	164/163	162/158

parasite species, it has been shown that infected males gammarids could be less competitive than uninfected males with regard to access to receptive females (Bollache et al., 2001). Such alterations may represent pathogenic effects or could be adaptations to enhance trophic transmission to the final host (Combes, 1991; Lafferty, 1999). Second, if most studies have considered the relationship between parasitism and developmental stability in focusing on FA in the final host, very few have assessed FA in intermediate hosts. The case of the infection of gammarids by parasites such as acanthocephalans was interesting because the size, relative to the host size, of these endoparasites is far from negligible (about ⅛ that of the gammarid size). Third, growth in amphipods is markedly discontinuous (Sutcliffe et al., 1981), with several molts occurring during an individual's lifetime. So far, most studies relating FA to parasitic infection have considered organisms with continuous growth or with a definitive size reached at sexual maturity, as in most insects. Intermolt interval in *G. pulex* ranges from 16 days to 3 mo depending on temperature (Hynes, 1955). Such discontinuous growth implies that the effect of infection (if it does exist) on the developmental stability of a gammarid during its lifetime can theoretically be assessed, assuming the parasite was present during the last molt of the host. Hynes (1955) observed that, following infection, the development of *P. minutus* within *G. pulex* requires a time delay of about 3 wk before the parasite can reach the cystacanth stage infective to the final host.

In the present work, the following questions were addressed. (1) Is FA in *G. pulex* related to infection by acanthocephalan parasites? (2) Are the 2 parasite species associated with different FA levels of hosts? (3) Is the magnitude of FA related to the intensity of infection; i.e., are individuals with more parasites more asymmetrical? (4) Are individuals infected with both parasite species more asymmetrical than individuals infected by only one?

MATERIALS AND METHODS

Samples and measured characters

Uninfected and naturally infected *G. pulex* harboring cystacanths of *P. laevis* and *P. minutus* were collected from a site on the river Tille in Burgundy (eastern France) between February and July 1999 using the kick-sampling method described by Hynes (1954). An independent sample of 3,249 gammarids was collected from the same site in March 1999 to estimate the prevalence of the 2 parasite species. All individuals were fixed in 70% alcohol before being sexed and examined for parasites. The proportion of *P. laevis*–infected males and *P. minutus*–infected males was 9.8% and 3.4%, respectively. In females, the same proportions were 9.2% and 2.8%, respectively.

Gammarids used for the FA analysis were brought back to the laboratory alive and then sexed and measured. All measurements and dissection were made using a Nikon SMZ-10A stereoscopic microscope and a VT0 232 video-measure system from Linkam Scientific Instruments Ltd (Elvetec-Vénissieux, France). Left and right antennae and pereopods (walking legs) were removed and mounted on a microscope slide before being measured. We used body height at the level of the fourth coxal plate basis as an independent measure of body size (see Brun, 1971). This character is highly correlated with the total length of the gammarid (Brun, 1971, Bollache et al., 2000). Following removal of the appendages, each gammarid was dissected alive under a microscope to determine the number of cystacanths of each acanthocephalan species. Each species was identified using the key proposed by Brauer (1911) and Brown et al. (1986).

Samples used for morphometric analyses are presented in Table I. For each sex, 6 different samples were considered—1 *laevis*: individuals harboring a single *P. laevis* cystacanth; 1 *minutus*: individuals harboring a single *P. minutus* cystacanth; >1 *laevis* and >1 *minutus*: individuals harboring more than 1 parasite of each species, respectively; *laevis* + *minutus*: individuals harboring at least 1 cystacanth of each parasite species; Uninfected: all uninfected individuals.

Six bilateral morphological traits were measured for the quantification of FA. Four of them were metric characters: width of the last segment of the protopodite of the antenna (Antenna, ANT), distance between the 2 first spines of the carpus of the walking leg 3 (Carpus, CAR), largest width of the basis of the walking leg 3 (Basis, BAS), width of the merus of the walking leg 4 at the level of the second pair of spines (Merus, MER). The others were meristic characters: number of spines on the inner surface of the basis of the walking leg 5 (Spine, SPI) and the number of segments in the flagellum of the second antenna (Article, ART). These 6 morphological traits were selected for the following reasons. First, preliminary experiments have revealed that they were measured with a lower measurement error than the other bilateral traits we examined. Second, they allowed for the consideration of both metric and meristic characters. Third, it was hypothesized that they were different in terms of functional importance because 3 (CAR, BAS, and MER) of 6 characters seemed more directly involved in locomotion and thus under a higher stabilizing selection than the other 3.

Measurements were made using a Nikon measuring microscope MM-60 to an accuracy of 0.001 mm. All measurements were made by the same person (V.G.) and were blind in regard to the infection status of the gammarids. To take measurement error into account, all traits were measured (metric traits) or counted (meristic traits) twice. Because, for technical reasons, measurement error was estimated when antennae and legs were fixed, error arising from the mounting procedure was not considered. However, because the traits measured were relatively flat, one may assume that the variability in their orientation on the slide constituted a minor component of error and did not represent a significant source of bias.

The mean value of the 2 measurements were used for all analyses, except that used to assess measurement error.

Preliminary statistical analysis

Because the occurrence of different forms of asymmetry, allometry, or measurement error can considerably bias the estimation of FA, it is necessary to perform a series of preliminary tests on the distributions of either signed asymmetries (right minus left values, Ri − Li) or absolute asymmetries ($|Ri - Li|$) of the 4 morphometric traits (Palmer, 1994; Graham et al., 1998; Van Dongen et al., 1999).

The presence of the 2 other forms of biological asymmetry—directional asymmetry (DA) and antisymmetry (AS)—was checked. Both DA and AS occur when 1 side of a bilateral character is consistently larger than the other, but in the case of AS, the side that is larger varies randomly among individuals (Van Valen, 1962). Because these 2 forms of asymmetry are presumed to be genetically determined, they are generally considered uninformative (but see McKenzie and Clarke, 1988; Graham et al., 1993; Møller and Swaddle, 1997). Therefore, for each character and each sample, the presence of DA was assessed by testing for a departure from zero, the mean of the distributions of the signed asymmetries, whereas AS was detected using a normality test as well as kurtosis and skewness estimates (see Palmer, 1994).

The association between size and FA was tested using 3 different approaches. Linear regression analyses were used to assess the relationship between size of gammarids and FA as well as between character size and FA. In the former case, absolute asymmetry values were regressed on the length of the fourth coxal plate basis, whereas in the latter case they were regressed on character size defined as ([Ri + Li]/2). In addition, size dependence of FA among samples was tested by linear regression of log(var[Ri − Li]) on mean ([Ri + Li]/2).

Errors due to measurement were evaluated using a 2-way (side × individual) mixed-model analysis of variance (ANOVA) designed to test for the significance of between-side variance (nondirectional asymmetry variance) relative to variance due to measurement (Palmer and Strobeck, 1986; Palmer, 1994).

Differences in FA levels

For the 4 metric characters, FA levels of each sample were assessed using the means of the distributions of the absolute asymmetries (index FA1 in Palmer, 1994). Absolute asymmetries were used because, for each trait, they allowed the design of an ANOVA with planned comparisons (Sokal and Rohlf, 1995). This ANOVA procedure was conducted as follows. First, FA levels of uninfected and infected gammarids were compared, with infected gammarids grouped independently of the species or the number of the acanthocephalans they carried. Then, the effects of each acanthocephalan species were tested using a second contrast comparing uninfected to *P. laevis*–infected gammarids and using a third contrast comparing uninfected to *P. minutus*–infected gammarids. A fourth test examined the effect of each acanthocephalan by comparing the FA of *P. minutus*–infected individuals to that of *P. laevis*–infected individuals. The ANOVA also considered the effect of parasitic load by comparing, among infected gammarids (again grouped independently of the nature or the number of the acanthocephalans they carried), those carrying 1 parasite to those carrying more than 1 parasite. Finally, the influence of the simultaneous presence of both parasites was tested by comparing the FA level of the sample of gammarids simultaneously infected by both acanthocephalan species against the 4 other groups of gammarids infected by a single species of acanthocephalan.

As numerous identical tests were repeatedly conducted on the 4 metric traits, the sequential Bonferroni test (Rice, 1989) was systematically applied (across the series of $k = 4$ tests) in order to limit the occurrence of type I error.

Because we knew little about the expected distributions of the signed asymmetries of meristic characters, another statistical approach was required. According to Leary et al. (1983), for each sample, the mean of the sum of asymmetries per individual (MSA) was used as the FA index. Then, for each of the 6 comparisons detailed above for the ANOVA design, the significance level of the difference between MSA indices of the 2 samples (D) were analyzed using randomization tests. The principle of these tests is to generate the frequency distribution of *D*-values after a number of iterations (20,000 in this case) of random rearrangements of data of the 2 samples and to calculate the probability of obtaining a more extreme value of *D* than the initial value (Thomas and Poulin, 1997).

For all computations sexes were considered separately.

RESULTS

Preliminary tests

Normality tests detected significant departures from normality in 3 of the 48 signed asymmetry distributions, whereas kurtosis and skewness were significant for 4 and 6 distributions, respectively (Table II). Because the same distributions were often significant for several tests, overall, 7 distributions exhibited a significant departure from normality. However, the presence of strong antisymmetry can be excluded because no distribution was platykurtic. Significant DA was detected in only 1 of the 48 samples tested. Therefore, because of the low number of significant results and because no particular trait or sample were concerned by the presence of AS, DA, or both, it was concluded that the samples studied exhibited only true FA.

No relationship was found between size and FA because regression analyses did not reveal any significant relationship between character size and asymmetry and revealed only 1 significant relationship between the length of the fourth coxal plate basis and asymmetry (Table II). Besides, FA was also found to be independent from character size among samples.

The 2-way (side × individual) mixed-model ANOVA showed that, for all samples, the nondirectional asymmetry variances (the interaction variances in the model) were always significantly larger than the measurement error. The mean variance due to measurement error varied from 1.2% (character MER) to 4.9% (character ANT) of the nondirectional asymmetry variance. These values were sufficiently low to assume that they were not a source of bias in the analyses.

Differences in FA

Figure 1 depicts the FA levels of the 4 metric traits in the 6 samples for both sexes. Planned comparisons did not reveal any significant effect for the 4 characters in females (Table III). In males, the only significant difference concerned the trait CAR, for which gammarids infected by *P. laevis* appeared more asymmetrical than gammarids infected by *P. minutus* (Table III). Moreover, differences showing higher levels of FA for infected gammarids were found for the trait ANT in males for all comparisons involving infected and uninfected samples (infected vs. uninfected individuals, *P. laevis*–infected vs. uninfected individuals, and *P. minutus*–infected vs. uninfected individuals), but none reached the level of significance after Bonferroni correction. This trend was not confirmed for females.

The index computed using the 2 meristic characters (SPI and ART) exhibited more explicit results (Table III; Fig. 2). First, infected gammarids exhibited a higher level of FA than uninfected ones. This result was statistically significant for both sexes even if randomization tests revealed a lower *P*-value for males. Second, *P. minutus*–infected gammarids were significantly more asymmetrical than uninfected gammarids for both sexes, whereas the difference was significant only in males when *P. laevis*–infected gammarids were compared to the uninfected ones. Third, in females, infection with *P. minutus* was significantly associated to a higher level of asymmetry of hosts than infection with *P. laevis*. This was not the case in males. Fourth, gammarids infected with more than 1 acanthocephalan were not more asymmetrical than those infected with only 1 parasite. Fifth, the hosts that were simultaneously infected by

TABLE II. Detailed presentation for both sexes of the results of the preliminary tests for each of the 4 meristic traits and the 6 samples. Mean, D_{max}, skew, and kurtosis have been computed from the distributions of signed asymmetries (Ri − Li) whereas association between size and fluctuating asymmetry (FA) was tested using absolute asymmetry values |Ri − Li|. *P*-values were corrected with the sequential Bonferroni method applied within each series of tests, per sample, across the 4 traits.

		Mean (× 10^2)		D_{max}*		Skew		Kurtosis		Slope1†		Slope2‡	
Trait	Sample	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
Antenna	1 *laevis*	0.03	−0.03	0.07	0.10	−0.33	−0.10	−0.27	−0.72	0.14	0.15	0.01	0.004
	1 *minutus*	−0.2	0.4	0.16	0.09	0.02	0.07	0.76	−0.80	0.12	−0.17	0.02	−0.04
	>1 *laevis*	−0.02	−0.3	0.08	0.09	0.48	0.005	0.52	0.16	−0.04	0.05	0.03	−0.001
	>1 *minutus*	1.1	0.4	0.12	0.14	0.04	−0.40	−0.30	−0.25	0.03	0.18	−0.01	−0.01
	laevis + *minutus*	0.8	0.5	0.15	0.21	−0.36	0.12	1.18	−1.77	0.35	−0.51	0.01	0.08
	Uninfected	0.3	−0.03	0.11	0.11	−0.03	−0.52	0.62	0.40	−0.03	0.12	−0.01	0.01
Carpus	1 *laevis*	0.03	0.5	0.20§	0.17	1.52§	1.24‖	2.59‖	2.29‖	−0.06	−0.04	−0.01	0.002
	1 *minutus*	0.1	−0.2	0.09	0.14	−0.18	0.33	0.08	1.74	0.07	−0.05	0.005	−0.001
	>1 *laevis*	0.5	−0.03	0.12	0.15	1.43#	−0.87	5.25§	3.05§	−0.10	0.09	0.01	0.005
	>1 *minutus*	0.8	0.3	0.14	0.19	0.90	−0.28	0.91	0.33	0.08	0.07	0.02	0.003
	laevis + *minutus*	−0.09	0.9	0.16	0.14	−0.68	−0.99	0.28	2.07	0.11	0.18	0.01	0.02
	Uninfected	1.2	−0.04	0.10	0.09	0.01	−0.44	0.26	0.14	0.01	0.07	0.04‖	0.01
Basis	1 *laevis*	0.03	0.2	0.13	0.08	0.24	0.18	0.22	−0.44	−0.02	0.09	0.01	−0.005
	1 *minutus*	0.2	0.1	0.09	0.11	0.17	−1.16‖	−0.11	1.53	−0.03	−0.05	0.004	−0.002
	>1 *laevis*	−0.2	0.3	0.16	0.14	−0.90	0.24	2.01	0.85	−0.02	−0.08	0.01	−0.02
	>1 *minutus*	−1.1	−1.7‖	0.17	0.14	−0.77	−0.84	1.26	1.24	0.13	−0.02	0.02	0.01
	laevis + *minutus*	0.6	1.8	0.17	0.29‖	0.54	0.27	1.83	−0.99	0.10	0.11	0.03	−0.02
	Uninfected	0.9	−0.1	0.13	0.10	0.85	0.85	1.19	1.74	0.03	0.07	0.003	0.01
Merus	1 *laevis*	−0.2	−0.3	0.17	0.15	−1.61§	−0.43	3.41	1.38	0.07	0.04	0.01	0.01
	1 *minutus*	−0.2	−0.9	0.17	0.25#	0.31	−2.08#	2.03	4.18	−0.08	−0.04	0.01	0.01
	>1 *laevis*	0.3	−0.1	0.12	0.09	−0.95	−0.01	2.06	0.76	−0.04	−0.04	−0.01	−0.001
	>1 *minutus*	−0.03	−0.2	0.12	0.18	0.27	0.93	−0.24	2.17	−0.02	0.001	−0.01	−0.01
	laevis + *minutus*	−0.1	0.7	0.12	0.12	0.32	0.057	−0.36	−0.20	−0.01	0.11	−0.01	0.06
	Uninfected	0.3	0.1	0.14	0.07	−1.06	−0.15	4.49	−0.36	−0.02	0.001	−0.01	0.01

* Kolmogorov–Smirnov test statistic.
† Slope from regression of |Ri − Li| versus (Ri + Li)/2.
‡ Slope from regression of |Ri − Li| versus the length of the fourth coxal plate basis.
§ $P < 0.01$.
‖ $P < 0.05$.
# $P < 0.001$.

both species of parasites presented the highest levels of FA, but this trend was not statistically supported. Finally, males always exhibited higher levels of FA than females.

DISCUSSION

Parasitic infection of *G. pulex* by the 2 acanthocephalan species, *P. laevis* and *P. minutus*, was associated with a significant increase of the index generated by combining FA scores of the 2 meristic characters studied (SPI and ART). This pattern was found in both sexes and concerns the effect of both parasites. This trend was also observed for 1 metric character (ANT), but was not statistically supported after Bonferroni correction. Taken as a whole, these results are, therefore, consistent with the idea that parasitic infection is associated with a decrease in developmental stability (Møller, 1996). It is not obvious whether the 2 acanthocephalan species are associated with different levels of developmental stability in the gammarids. For metric traits, *P. laevis*–infected gammarids appeared more asymmetrical than the *P. minutus*–infected ones in males, whereas no difference was found in females. In contrast, FA for meristic traits was higher for female gammarids infected with *P. minutus* than those infected by *P. laevis*, whereas no such a trend was observed in males. Interestingly, it has also be shown that both acanthocephalan parasite species have different negative effect on female fecundity; i.e., *G. pulex* females infected with *P. minutus* were completely sterile (Ward, 1986), whereas fecundity is only reduced by *P. laevis* (Poulton and Thompson, 1987). However, the precise physiological pathways by which each parasite species exerts its influence on the host remain undocumented. Little is known about the metabolic costs associated with parasitic infection with acanthocephalans and how such costs can alter growth rate and developmental stability in gammarids, if they do so. Nevertheless, even though infection by *P. minutus* may be more costly to *G. pulex*, it does not appear to have a larger effect on FA than does infection by the relatively less costly *P. laevis*.

Interestingly, no detectable effect of parasite intensity was observed. This may suggest a priori that the acanthocephalans studied could not be directly responsible for the increase of FA of their host. Indeed, if the parasites were causally involved in the low levels of developmental stability of their hosts, one would expect a positive relationship between FA and parasite load, as reported for instance for the outermost tail feathers in male barn swallows infested by mites (Møller, 1992) or sternopleural bristles in *Drosophila* suffering from infections with a nematode (Polak, 1993). However in the present study, it should be noted that because of the insufficient number of hosts infected by more than 1 parasite in nature, we considered only 2 modalities of infestation (''1 parasite'' and ''more than 1 parasite'') and not a wide range of parasite loads. Moreover, the majority of gammarids infected by more than 1 parasite were, in fact, infected by 2 acanthocephalans only and quite rarely by 3, 4, or more. It is therefore possible that a causal relationship exists between FA and parasites, but it could not be detected. However, Cézilly et al. (2000) recently found that the number of acanthocephalan parasites present in a host had no significant effect on either reaction to light or vertical distribution of *G. pulex*. Rumpus and Kennedy (1974) similarly observed that the number of *P. laevis* parasites present in *G. pulex* did not affect the magnitude of the reduction of the host respiratory rate. Thus, the effects of both *P. laevis* and *P. minutus* may be dependent only on their presence, not on their intensity. This, combined with the moderate differences in FA between samples that concern only 2 morphological traits out of 6 stud-

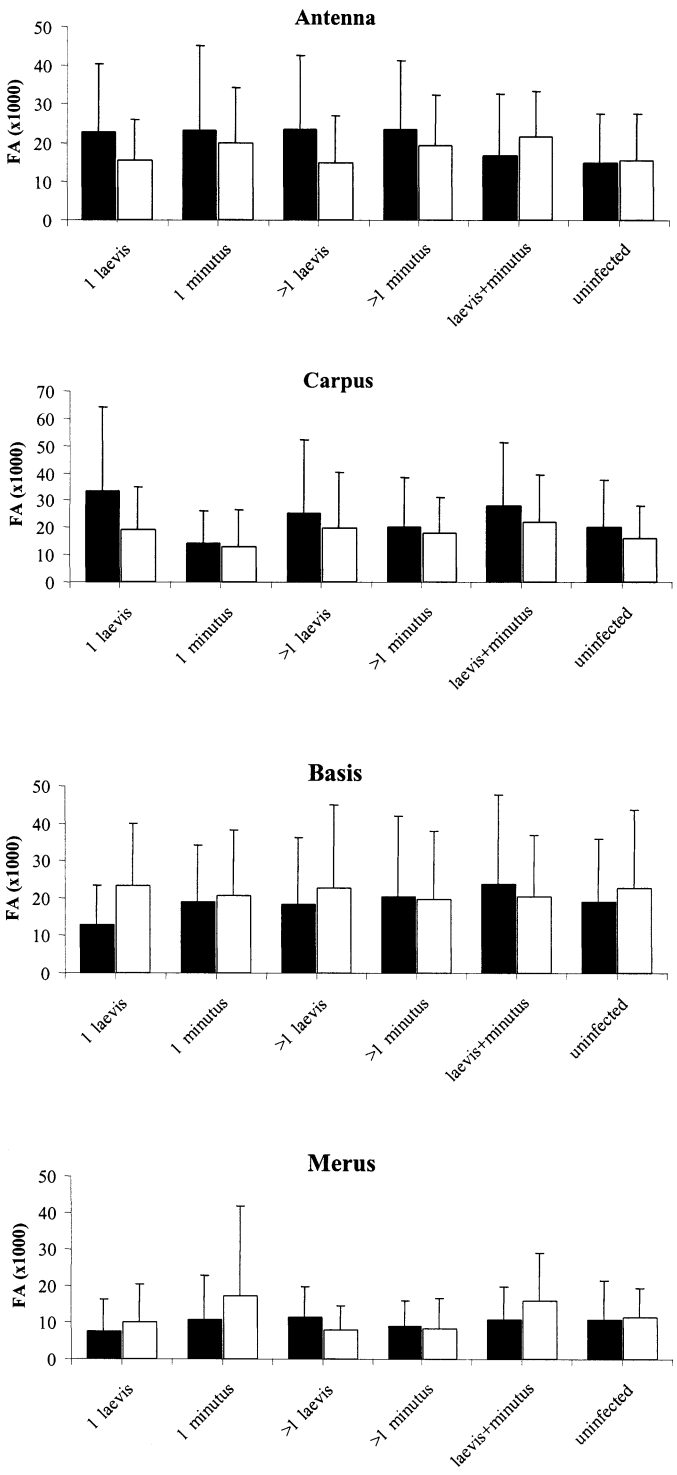

FIGURE 1. FA levels (+SD) in the 6 samples for the 4 metric traits. Black bars represent FA for males, and open bars represent FA for females.

TABLE III. Results for both sexes of the ANOVA with planned comparisons on fluctuating asymmetry (FA) values for each of the 4 metric traits and of the randomization tests on the index computed with the 2 meristic traits. For the ANOVA, mean squares $\times 10^5$ (MS), degrees of freedom (df), and probability values after Bonferroni correction are presented. The value in parentheses for the error MS corresponds to the degrees of freedom for females. For the percentages of values higher than *D* (randomization tests), the actual difference between the two samples after the randomization procedure are given.

		ANOVA (metric traits)								Randomization tests (meristic traits)	
		Males				Females					
Source of variation	df	Antenna	Carpus	Basis	Merus	Antenna	Carpus	Basis	Merus	Males	Females
Samples	5	42.04	131.8	30.07	5.93	37.96	94.73	24.93	21.17		
Uninfected vs. infected	1	129.9	41.80	0.14	1.29	20.25	12.23	4.48	1.07	0.88*	2.38†
Uninfected vs. *P. laevi* infected	1	147.8	173.4	24.8	2.71	0.14	24.0	0.47	11.26	2.06†	18.4
Uninfected vs. *P. minutus* infected	1	145.8	15.7	0.74	1.13	35.4	1.05	13.2	4.54	1.81†	0.48*
P. minutus infected vs. *P. laevis* infected	1	0.06	406.6†	47.92	0.44	52.07	42.45	24.11	36.79	62.1	2.52†
One parasite vs. >1 parasite	1	0.53	5.24	37.98	3.38	1.26	21.81	1.58	76.82	9.70	23.1
One species vs. >1 species	1	58.84	31.47	49.86	1.65	15.48	17.19	1.68	25.68	8.51	15.3
Error	158 (152)	32.68	50.25	30.56	8.96	15.50	23.57	36.99	18.67		

* $P < 0.01$.
† $P < 0.05$.

ied, may suggest that the acanthocephalan species considered could have a direct, but relatively weak, effect on the FA of the gammarids. Therefore, the effect of parasites on their intermediate hosts must be moderate not to reduce their chances of being transmitted to the final hosts. Interestingly, even if not significant, the trend found for meristic traits showing higher levels of FA in the gammarids simultaneously infected by both acanthocephalan species could then provide evidence that double infection would constitute a real disadvantage for both parasites (see Cézilly et al., 2000). If this trend can be confirmed, it would explain why, as mentioned in the introduction, the 2 parasites do not induce the same degrees of physiological pathology; thus, the double infection should result in more important damage in the host.

One cannot definitively exclude the hypothesis suggesting a greater susceptibility of asymmetrical gammarids to infection, however. Under this hypothesis, infected individuals would simply correspond to those unable to defend themselves against pathogen attacks because of their poor general condition. However in this case, one should not expect higher levels of FA for the double-infected individuals.

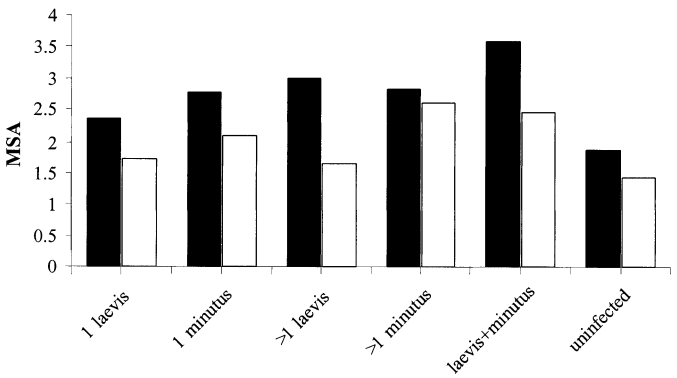

FIGURE 2. FA levels in the 6 samples computed using the 2 meristic characters (black bars represent FA for males, and open bars represent FA for females). MSA corresponds to FA levels computed using the mean of the sum of asymmetries per individual.

Another interesting result of the present work is that males, at least for the meristic characters, systematically exhibited higher levels of FA than females. According to current sexual selection theory, it is predicted that competition among males will be stronger if the operational sex ratio is skewed toward this sex. In such a context, it is likely that males must be closer to their physiological limits than females for mate acquisition (Polak and Markow, 1995). This is the case for the gammarids because females are sexually receptive only during the molt and there is, therefore, always strong competition among males for access to females (Bollache et al. 2000, 2001). The competitive interactions among males would then represent an energetic cost for males that could not be allocated to maintenance of developmental stability. It remains true, however, that this does not allow us to know if infected males are more asymmetrical because of the additional energetic cost induced by parasites or because of their low immune ability to defend themselves against pathogens (Møller, 1996).

Finally, it is unclear why only meristic, and not metric, traits showed differences in FA levels between samples. Palmer (1994) argued that, when asymmetrical individuals do not differ between sides by only 1, meristic characters can be more accurate for the study of developmental stability than metric ones because they are often measured without error and are usually independent of body size. This corresponds to the present study because differences in count between sides for both traits could reach 4 or 5. Moreover, for each side, all counts were systematically verified twice; thus, one can consider that measurement error was absent. Another nonexclusive explanation considered for the absence of congruence in FA levels between metric and meristic traits would be that the former express less FA because they are under a stronger stabilizing selection. It has often been reported that, because of their functional importance, bilateral characters do not express quantifiable variation in FA (wings in insects or wing feathers in birds, for example) (Møller, 1992;

Balmford et al., 1993; Polak, 1993; Thomas and Rowe, 1996). Even though 3 out of the 4 metric traits considered in the current study concerned pereopods and, hence, are involved in locomotion, it is actually difficult to say if higher FA of these traits would lead to higher cost of swimming for developmentally unstable individuals. Interestingly, the only metric character not related to locomotion (ANT) was also the one that exhibited a pattern of variation of FA levels between infected and uninfected samples similar to those of the meristic characters (but not statistically supported after Bonferroni correction).

This work suggests that there is a positive association between acanthocephalan infection and developmental instability in *G. pulex*. However, more work (in particular, experimental infection of hosts) is needed to definitively determine whether parasites are the cause or the consequence of the higher levels of FA found in infected hosts. Future studies must also investigate whether apparent differences between metric and meristic traits are related to the nature of the variation (continuous or discontinuous) of the trait or to its history of selection.

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Environmental and ontogenetic constraints on developmental stability in the spatangoid sea urchin *Echinocardium* (Echinoidea)

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Spatangoid irregular sea urchins are detritivorous benthic organisms particularly prone to variations of environment, and their mode of growth and plate morphology make them an appropriate model to assess the effects of environmental variations. Two populations of *Echinocardium flavescens* were sampled in two sites of the Norwegian coast characterized by contrasted environmental conditions. Different morphological descriptors (plate areas, interlandmarks distances, overall size, and shape of the posterior ambulacra) were used to appraise interindividual variations, and fluctuating asymmetry. The comparisons were carried out using classical fluctuating asymmetry (FA) methods, as well as Procrustean approaches. The population suspected to be less influenced by anthropic activities exhibits lower levels of FA for the size parameters (plate surfaces, interlandmarks distances, and centroid size) than the population located in a polluted area. Conversely, it shows higher FA values for the shape parameters (landmarks configuration). Interindividual variations appear to be correlated to FA. Variations are orientated according to the main growth axis of the ambulacra, and their intensity is stronger in the large posterior plates, which are also the youngest. These results are discussed with respect to architectural constraints involved in the sea urchin growth.

ADDITIONAL KEYWORDS: Developmental instability – fluctuating asymmetry – geometric morphometrics – morphological variations – sea urchins.

INTRODUCTION

Soft sea bottoms provide a wide range of environments for benthic organisms. Correspondingly, benthic organisms living and developing in those environments are particularly prone to variations of the sediment: granulometry, organic content, degree of pollution (Dafni, 1980; Telford & Mooi, 1996; Linton & Taghon, 2000; Zulkosky, Ferguson & McElroy, 2002). This is particularly true for spatangoids that are detritivorous, generally endobenthic sea urchins with a rather reduced mobility (Nichols, 1959; Buchanan, 1966; Kanazawa, 1992). In such organisms, we may expect an important influence of stressing environmental conditions on several aspects of their phenotype, including morphology. The shape and architecture of spatangoids tests should therefore provide an accurate record of substrate-related stresses experienced by the sea urchins during their postlarval development.

Modalities of the postlarval growth of sea urchins are now clearly understood in the framework of a new model: 'Extraxial-Axial Theory' (EAT) (David & Mooi, 1996; Mooi & David, 1997). The EAT distinguishes two principal regions in the skeleton of the developing sea urchin (axial vs. extraxial). The axial part grows by adding new elements according to a precise rule, the Ocular Plate Rule (OPR) (Mooi, David & Marchand, 1994). The OPR generates the precise order in which new plates appear, and is crucial in delineating homologies between the plates of the test. Most of the test of an adult is made of axial elements, organized into five growth zones. In regular sea urchins, the five growth zones are almost perfectly balanced in a radiating pattern. In irregular sea urchins, including spatangoids,

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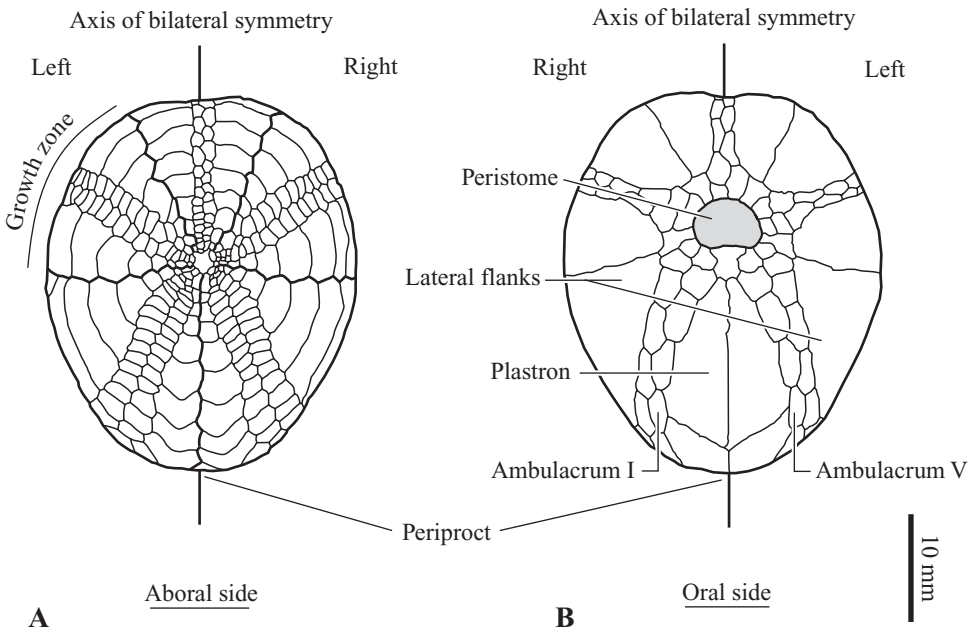

Figure 1. The test of *Echinocardium flavescens* displaying the aboral side (A) and the oral side (B) where posterior ambulacra (ambulacra I and V) were studied.

a bilateral symmetry appears secondarily during the growth as a change in the initial balance between the five growth zones. The balance is stabilized in such a way that the test clearly displays a left and a right part, as well as an anterior–posterior axis, defined by the positions of periproct and peristome (Fig. 1A, B). Moreover, the irregularity is expressed in the shape and size of the plates of the test, and is clearly visible in the array of these plates.

Stress can disturb the balance between growth zones (i.e. it can impair developmental stability of the tests). Developmental stability is defined as the suite of processes through which organisms reduce phenotypic variation resulting from developmental accidents (Zakharov, 1989; Palmer, 1994). It is generally measured by evaluating fluctuating asymmetry (FA) levels, which correspond to deviations from perfect symmetry of normally symmetrical structures (Van Valen, 1962). Environmental stressors such as chemical pollutions (Pankakoski, Koivisto & Hyvärinen, 1992; Zakharov, Valetsky & Yablokov, 1997), temperature (Siegel & Doyle, 1975; Parsons, 1990; Leary, Allendorf & Knudsen, 1992) or food deprivation (Swaddle, Cuthill & Witter, 1994) have been shown to be significantly associated with higher levels of FA. Thus, FA has been proposed as a valuable biological indicator of environmental quality (Clarke, 1993). However, the use of FA is not without difficulties. A growing debate concerns the methodological appraisal of FA [i.e. both the choice of morphological traits to consider (nature and number) and the statistical approach to use]. In such a context, developments in geometric morphometrics appear to be of a particular interest (Rohlf & Slice, 1990; Bookstein, 1991, 1996; Rolhf & Marcus, 1993). Such methods are appropriate to study interindividual variations, as well as developmental stability between left and right sides (Klingenberg & McIntyre, 1998; Auffray *et al*., 1996; Smith, Crespi & Bookstein, 1997; Auffray, Debat & Alibert, 1999). The test of sea urchins displays a plate architecture particularly well suited for investigations by geometric morphometrics. Irregular sea urchins display a secondary bilateral symmetry that is strongly expressed in the general shape of the test, as well as in plate architecture. In the present study, we aimed to appraise and compare FA levels of two different populations of the spatangoid sea urchin *Echinocardium flavescens* (Spatangoida, Loveniidae) that were sampled in two different sites of the Norwegian coast, differing in environmental conditions. The questions addressed were: (1) could variations of FA levels be determined by differences in environmental conditions and (2) could these variations be related to the growth processes of sea urchins? More specifically, is there a gradual change of FA levels along the ambulacra according to the age of the plates, and/or are some parts of the test more developmentally variable and/or unstable than others (i.e. less constrained)?

MATERIAL AND METHODS

Specimen collection

Echinocardium flavescens were collected in two localities of the Norwegian coasts in May 1997 (Fig. 2). The first sample was collected near the small town of Bodø (northern to the Polar circle) in a fjord largely free of anthropic pressure. The second was obatined from the Oslo fjord, 30 km south of Oslo (near Drøbak). Because of the important human activities occurring all around the fjord, particularly upstream of Oslo, it can be assumed that this site corresponds to a more stressful environment.

Thirty specimens were measured in each sample. Spines were gently brushed off the test after immersion in a sodium hypochlorite solution. Plate patterns were revealed by applying a solution of equal parts of absolute alcohol and glycerol, and drawings of the architecture of each specimen were made with a camera lucida.

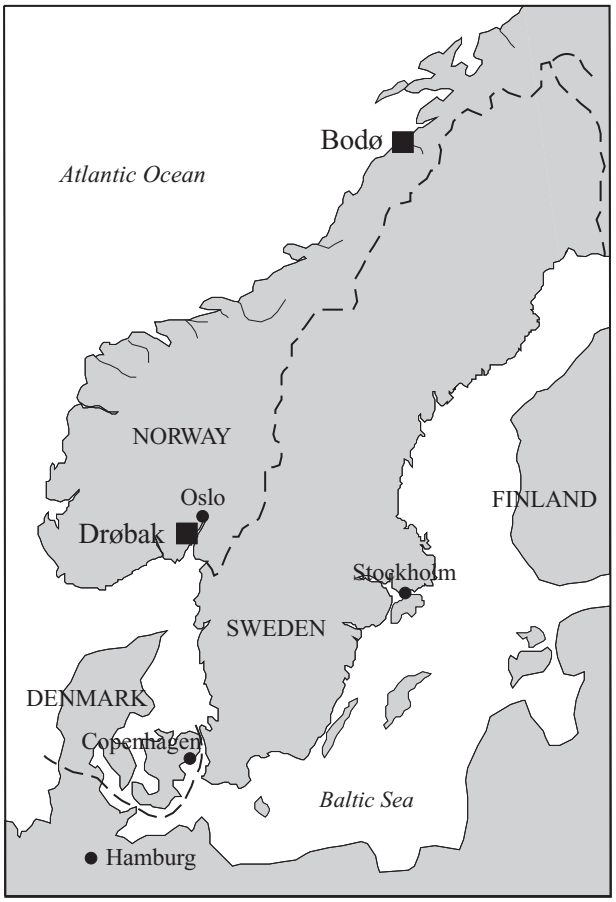

Figure 2. Geographical position of the two localities (black squares) where samples of *Echinocardium flavescens* were gathered.

Measurements and analyses

The study focused on the oral part of the posterior ambulacra (Fig. 1B) that constitute bilaterally symmetrical structures, the 'Oral-Posterior-Ambulacra' (OPA). They are inserted between two functional areas: the plastron and lateral interambulacra involved in locomotion and burrowing activities, respectively. Following Lovén's nomenclature (Lovén, 1874), the ambulacrum I on the right side is symmetrical to ambulacrum V on the left side (Fig. 1B).

The originality of the sea urchin biological model is that plate development occurs in two ways. Each individual plate of a sea urchin test displays its own size increments. Moreover, each plate is involved in the more general process of construction of its growth zone, controlled by the OPR, and its final shape is also constrained by the development of the surrounding plates. Hence, FA can be investigated for each plate independently, as well as for whole ambulacral structures, taking into account the architecture and all possible interactions between neighbouring plates.

Accordingly two descriptors were defined. First, nine individual plate surfaces were measured to assess FA between each homologous right and left plate independently (Fig. 3A). Second, 12 landmarks corresponding to triple junctions of plates (type 1 landmark *sensu* Bookstein, 1991) were defined on each ambulacrum (Fig. 3B). Left ambulacra drawings were mirrored for comparison with the right ones. From those descriptors, size and shape asymmetry could be envisaged separately. We used traditional morphometrics to analyse data relative to size: the nine plate surfaces and six independent interlandmarks distances, four measured transversally, and two meridionally (Fig. 3C). Procrustes superpositions of the 12 landmark configurations were performed to evaluate the whole OPA, shape asymmetry, and another aspect of size asymmetry based on centroid size. The centroid size is a proxy of size that corresponds to the square root of the sum of squared distances between each landmark to the geometric centre of the configuration (Slice *et al*., 1996).

Data acquisition (including the making of drawings with replacement of specimens) was carried out twice, independently, to estimate and take any measurement error into account.

Individual plate surface analyses

To detect size asymmetry on each of the nine plate surfaces, we performed a two-way (individual × side) mixed-model analysis of variance (ANOVA) (Palmer & Strobeck, 1986; Palmer, 1994). After a series of preliminary tests, this procedure allows assessment of the

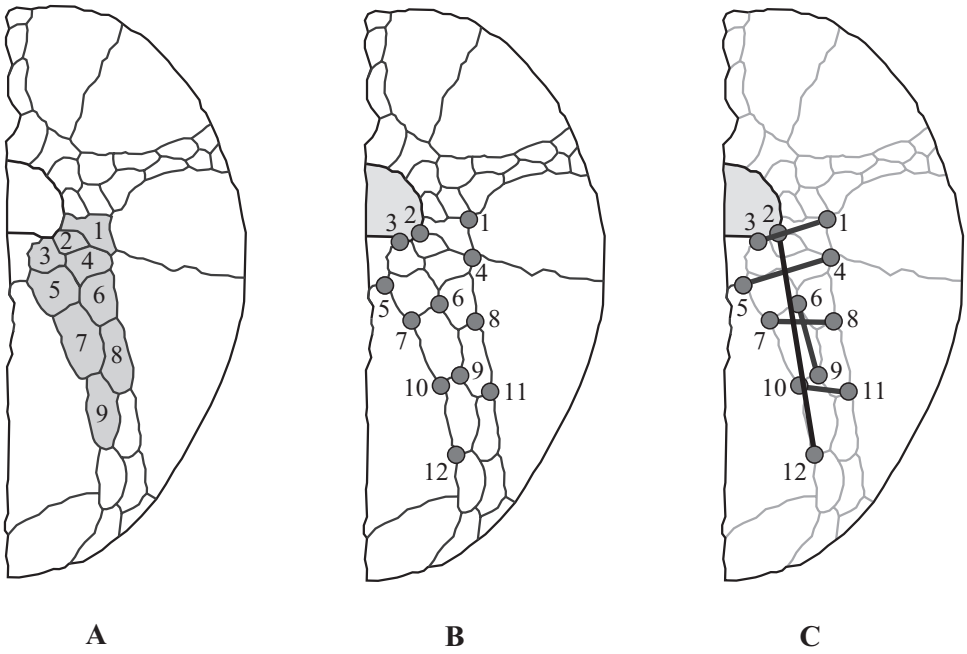

Figure 3. Location of the studied traits: the nine plates (A), the 12 landmarks (B) and the six distances (C) on a left half-test of *Echinocardium flavescens*.

significance of interindividual variations (individual effect), between side variations (side-effect, the so-called directional asymmetry or DA), non-directional asymmetry (i.e. FA in the absence of antisymmetry; interaction between individual and side-effects), and measurement error. For each variable and the two populations, we retained an index of interindividual variability (VAR) and an index of FA (FA10; calculated following Palmer, 1994) from the ANOVAs. Differences in VAR and FA10 between samples were assessed by *F*-tests.

Oral–posterior–ambulacra analyses

Size asymmetry of the six linear distances and of the centroid size were analysed according to the same ANOVA procedure as for individual plate surfaces. Shape asymmetry of the geometry of the 12 landmarks was quantified following the procedure of Klingenberg & McIntyre (1998). This procedure is an adaptation of the two-way mixed-model ANOVA (Palmer, 1994) to landmark geometry and Procrustes analyses. It was performed on Procrustes residuals to calculate the VAR and FA. A Procrustes fit corresponds to a geometric transformation that minimizes the sum of squared distances between corresponding landmarks of two configurations. It involves three basic steps: (1) a translation (decomposed according to x and y); (2) a standardized scaling; and (3) a rotation (Rohlf & Slice, 1990). After Procrustes superimposition, each sea urchin was described by 24 residuals that are the x and y coordinates of the vectors connecting, at each of the 12 landmarks, every single specimen to an average (consensus) configuration. In Bodø and Drøback samples, separately, ANOVAs were calaculated for each of the 24 residuals, and the sums of squares of every source of variation (individual, side, interaction, and error) were summed across the 24 variables to obtain the Procrustes sums of squares. These allow calculation of the equivalents of VAR and FA10 after division by the appropriate degrees of freedom (Klingenberg & McIntyre, 1998; Debat *et al*., 2000). Next, it was necessary to visualize back how VAR, asymmetry (FA10), and eventually measurement error were expressed on the different landmarks of the configurations. For each sample, a two-way multivariate ANOVA (individual × side) involving the 24 variables was performed, and two final variance-covariance matrices (VCV) were calculated: one related to the asymmetry calculated from the interaction between both effects, the other corresponding to the interindividual variation calculated from the individual effect. Then, principal components analyses (PCA) was carried out on VCV matrices for both sources of variation and for both samples. The contributions of the 24 variables to a given principal component can be depicted as vectors attached to each landmark. For each principal component of each analysis, this leads to a vector field allowing a visual interpretation that is connected with the ambulacral

plate architecture. In addition, we undertook comparisons of the two patterns, asymmetry and variation among specimens, as well as error, by testing the correlation between their respective VCV matrices within samples using permutation tests (the tests are suited so that the correspondence among x and *y* pairs of coordinates could be respected). Ten thousand permutations were realized. The above explanations concern comparisons within each sample, but correlation tests between VCV matrices were also carried out between samples for each source of variation (VAR, FA, and error). Further explanations of this technique are provided by Klingenberg & McIntyre (1998) and Debat *et al*. (2000).

RESULTS

Individual plate surfaces

The results of the ANOVAs regarding the variability and the asymmetry of the nine selected plates in both samples are rather homogeneous (Fig. 4). After Bonferroni's correction of probability levels for multiple tests within each sample (Rice, 1989), the nine interindividual variabilities appear significant in Bodø and in Drøbak. By contrast, no FA is significant. Table 1 summarizes the values of VAR and FA10 for the nine plates in the two localities. Note that the side-effect is never significant, indicating the absence of DA.

Oral–posterior–ambulacra size

Regarding the six interlandmarks distances, the results appear to be fully consistent in Drøbak, with interindividual variability, as well as FA effect, being significant for all traits (Fig. 5). The *Echinocardium* display a more contrasted pattern in Bodø. The VAR of one distance (between landmarks 10–11) is not significant, and two distances (between landmarks 7–8 and 6–9) are not significant for FA (VAR and FA10 values are listed in Table 2). Out of the four possible comparisons between localities, one is significant and indicates that FA is higher in Drøbak than in Bodø ($F = 5.9$, $P < 0.001$). Concerning DA, only one (between landmarks 2–12 in Bodø) shows a significant side-effect after Bonferroni's correction. The greatest value of the 2–12 distance is on the right OPA.

Regarding the centroid size of the landmark configurations, both localities express significant interindividual variability and significant fluctuating asymmetry (Table 3). DA is significant in Bodø only, with a larger centroid size for the right OPA. Variance ratios between Bodø and Drøbak reveal no differences between VAR indices, and a significantly higher FA10 in Drøbak.

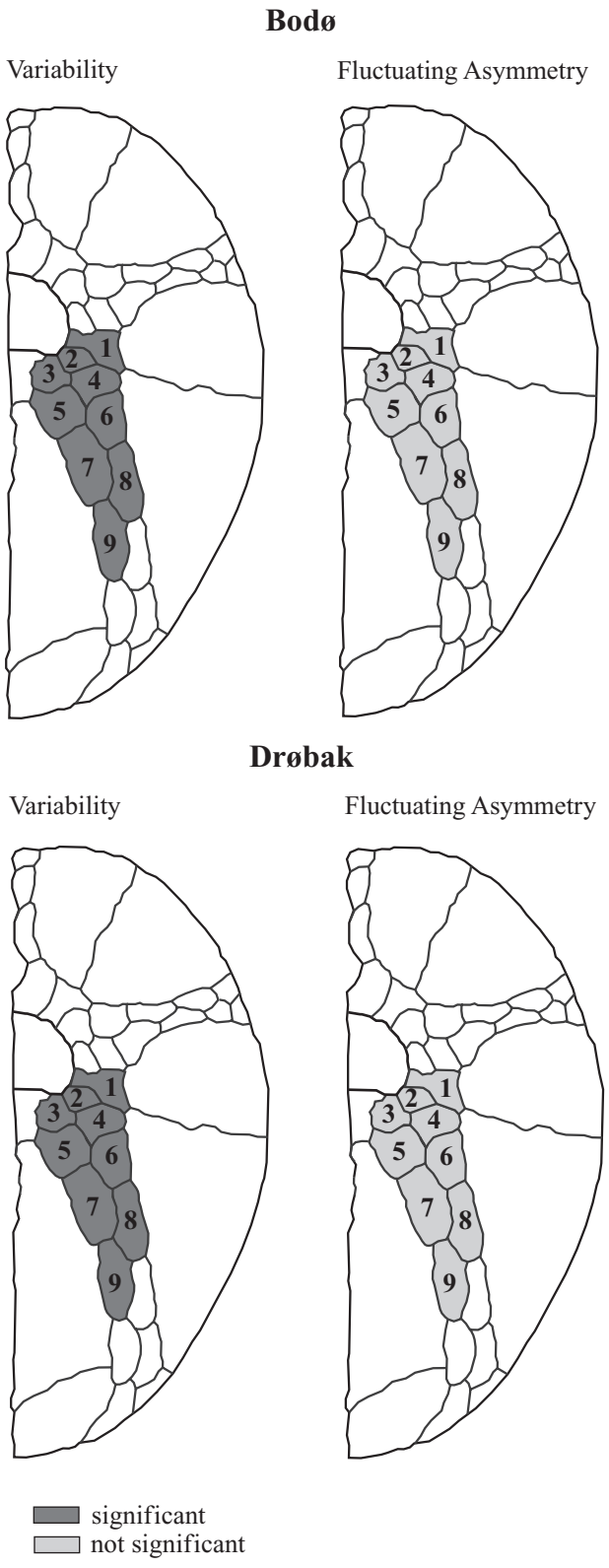

Figure 4. Results of Palmer's analysis of variance on plate surfaces for variability and fluctuating asymmetry.

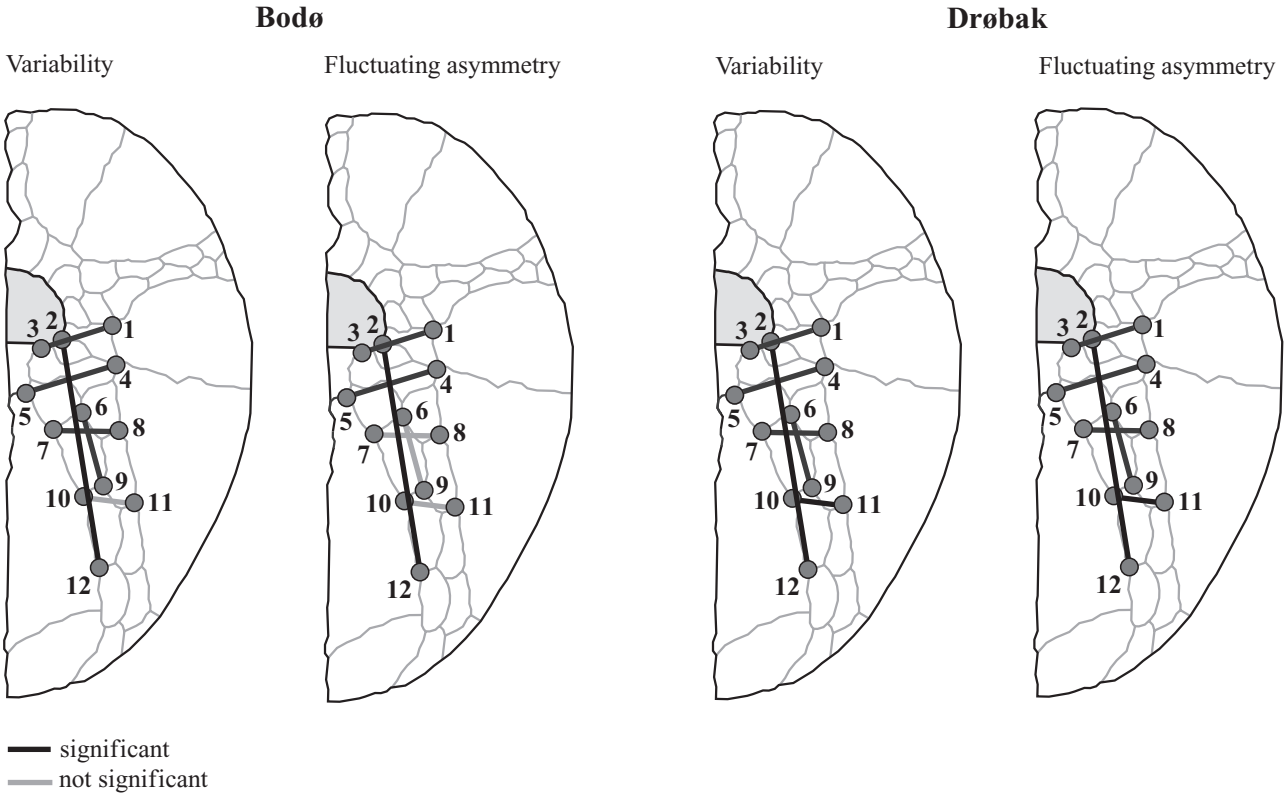

Figure 5. Results of Palmer's analysis of variance on the six distances for variability and fluctuating asymmetry.

ORAL-POSTERIOR-AMBULACRA SHAPE

The OPA shape, appraised by the geometry of landmarks, shows that there is significant shape variation between sea urchins in Drøbak, but not in Bodø (Table 3), whereas FA is significant in both localities and higher in Bodø. For centroid size, a significant DA is detected in Bodø only. If we consider the possible relationship between the patterns of expression of FA and interindividual variability, the comparisons of VCV matrices by permutation tests show a significant strong correlation at Bodø, and a weaker correlation at Drøbak (Table 4). These correlations are clearly illustrated in Figure 6, which shows the contribution of the 24 variables (x and y coordinates of residuals) on the first PCA as 12 vectors. In Bodø, vectors of FA and VAR are parallel for most of landmarks (Fig. 6A, B), whereas few vectors show parallel directions in Drøbak (Fig. 6C, D). There is no general pattern observed concerning measurement error (Fig. 6E, F); measurement error is only correlated with VAR in Bodø, and with FA in Drøbak (Table 4).

Similarly, it is also possible to compare the patterns of expression of FA and VAR between samples of the two localities. Visual comparisons of the vector fields between Bodø and Drøbak show a common pattern of size and orientation of the vectors for FA (Fig. 6B, D), but not for VAR (Fig. 6A, C). This observation is confirmed by permutation tests that reveal a highly significant correlation for FA ($r = 0.51$; $P < 0.0001$), but not for interindividual variability ($r = 0.17$; not significant) and measurement error ($r = 0.18$; not significant) (Table 5).

DISCUSSION

COULD VARIATIONS OF FA LEVELS BE DETERMINED BY DIFFERENCES IN ENVIRONMENTAL CONDITIONS?

All the OPA parameters exhibit significant levels of FA in Drøbak, whereas two distances are not significant in Bodø (Table 6). For parameters related to size (distances and centroid size), FA values are always higher in Drøbak than in Bodø. This appears to be consistent with the marked anthropic pressure present in the fjord of Drøbak and may be linked to the upstream proximity of Oslo, whereas the fjord of Bodø is far less affected by human activities. Conversely, shape asymmetry values are higher in Bodø than in Drøbak. Such a pattern may suggest two hypotheses: (1) either there

Table 1. Results of the plate surfaces analyses, showing interindividual, side and fluctuating asymmetry effects

	Plate 1		Plate 2		Plate 3		Plate 4		Plate 5		Plate 6		Plate 7		Plate 8		Plate 9	
	Bodø	Drøbak	Bodø	Drøbak	Bodø	Drøbak	Bodø	Drøbak	Bodø	Drøbak	Bodø	Drøbak	Bodø	Drøbak	Bodø	Drøbak	Bodø	Drøbak
Mean size (mm^2)	3.88	3.34	1.97	2.16	3.43	3.73	3.81	4.98	6.48	7.27	6,22	7.14	8.67	8.49	6.67	5.98	7.02	6.02
Size regression	0.041 ± 0.047	0.151 ± 0.045	0.265 ± 0.79	0.137 ± 0.054	0.265 ± 0.094	0.385 ± 0.095	0.328 ± 0.093	0.143 ± 0.069	0.25 ± 0.109	0.107 ± 0.049	8.49 ± 1.13	0.008 ± 0.038	0.075 ± 0.024	0.193 ± 0.073	0.195 ± 0.069	0.098 ± 0.05	0.114 ± 0.031	0.12 ± 0.06
F	0.77†	11.2**	11.3**	6.56**	7.9**	16.4***	12.3**	4.34*	5.3*	4.69*	56.6***	0.047†	9.5**	6.9*	7.98**	3.9†	13.6**	4.9*
Individual																		
d.f.	29	29	29	29	29	29	29	29	29	29	29	29	29	29	29	29	29	29
MS	2028.8	3388.9	2209.9	1833.2	2103.5	2445.7	2352.5	3606.7	3101.3	3619.3	3465	3017	3934.2	2957	2764.3	3575.8	3505.7	3250.3
F	1.95*	10.32***	3.1**	3.95**	4.99***	5.51***	5.07***	8.23***	12.69***	14.3***	7.29***	7.87***	9.37***	5.31***	5.29***	10.66***	10.69***	9.38***
Side																		
d.f.	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
MS	326.7	102.7	0.3	800.3	346.8	73.6	512.5	1209.7	1.2	53.3	177.6	407	681.6	78.4	1598.7	192.5	1360.1	95.4
F	0.31†	0.31†	0.001†	1.29†	0.82†	0.17†	1.1†	2.76†	0.005†	0.21†	0.37†	1.06†	1.62†	0.14†	3.06†	0.57†	4.15†	0.28†
Interaction																		
d.f.	29	29	29	29	29	29	29	29	29	29	29	29	29	29	29	29	29	29
MS	1042	328.4	712.5	620.9	421.8	443.7	464.4	453.3	244.3	253	475.4	383.1	419.8	556.8	522.5	335.4	328	346.6
F	1.63†	0.91†	1.49†	1.69†	1.04†	0.84†	1.09†	0.77†	0.6†	0.93†	0.99†	0.85†	0.65†	1.06†	0.83†	1.17†	1.44†	0.68†
Error																		
d.f.	60	60	60	60	60	60	60	60	60	60	60	60	60	60	60	60	60	60
MS	638.2	359.6	476.9	367.6	406.4	526.2	424.3	565.8	409.2	272.3	480.7	450.4	642.8	526.2	629.1	287.2	227.4	507.9
VAR	246.7	765.1	374.3	303	420.4	500.5	472	792.1	714.2	841.6	747.4	658.5	878.6	600	560.4	810.1	794.4	725.9
FA10	201.9	−15.6	117.8	126.6	7.7	−82.5	20.05	−63.7	−82.4	−9.6	−2.65	−33.6	−111.5	15.3	−53.3	24.1	50.3	−80.6

The regression for size is a linear regression of $|R - L|$ on $(R + L)/2$-values to test for the dependence between fluctuating asymmetry (FA) and size. MS, mean square; VAR, interindividual variability. The presentation follows the recommendations of Palmer (1994). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; †Not significant.

Table 2. Results of the distances analyses, showing interindividual, side and fluctuating asymmetry effects

	Distance 1–3		Distance 4–5		Distance 7–8		Distance 10–11		Distance 6–9		Distance 2–12	
	Bodø	Drøbak	Bodø	Drøbak	Bodø	Drøbak	Bodø	Drøbak	Bodø	Drøbak	Bodø	Drøbak
Mean size (mm)	4.7	3.85	5.68	4.93	3.52	4.16	2.46	3.19	4.75	4.4	14.24	13.62
Size regression	0.251 ± 0.78	0.036 ± 0.102	0.229 ± 0.093	0.167 ± 0.105	–0.063 ± 0.104	0.154 ± 0.049	–0.126 ± 0.221	0.223 ± 0.078	0.054 ± 0.038	0.135 ± 0.207	0.033 ± 0.043	0.185 ± 0.084
F	10.418**	4.863*	0.127†	6.066*	2.54†	0.37†	9.828**	0.326†	8.196**	2.044†	0.43†	0.565†
Individual												
d.f.	29	29	29	29	29	29	29	29	29	29	29	29
MS × 1000	996.623	551.466	998.829	766.98	600.029	700.137	372.904	687.294	1549.562	887.032	9553.137	6309.241
F	9.511***	6.952***	6.504***	7.205***	6.675***	15.529***	1.346†	9.825***	37.064***	8.443***	35.538***	18.225***
Side												
d.f.	1	1	1	1	1	1	1	1	1	1	1	1
MS × 1000	552.163	45.63	215.901	274.563	257.613	27	159.141	234.968	444.083	128.708	3534.941	237.63
F	5.269*	0.575†	1.406†	2.579†	2.866†	0.599†	0.574†	3.359†	0.622**	1.225†	13.373**	0.686†
Interaction												
d.f.	29	29	29	29	29	29	29	29	29	29	29	29
MS × 1000	104.786	79.32	153.582	106.451	89.894	45.086	277.044	69.954	41.807	105.056	268.817	346.18
F	2.262**	5.172***	2.434**	5.565***	1.445†	2.598***	2.110**	8.008***	1.684†	8.786***	3.605***	3.781***
Error												
d.f.	60	60	60	60	60	60	60	60	60	60	60	60
MS × 1000	46.322	15.335	63.107	19.13	62.22	17.355	131.299	8.736	24.833	11.956	74.566	91.558
VAR × 1000	223	118	211	165	128	164	24	154	377	196	2321	1491
FA10 × 1000	29	32	45	44	14	14	73	31	8	47	97	127

The regression for size is a linear regression of | R – L | on (R + L)/2-values to test for the dependence between fluctuating asymmetry (FA) and size. MS, mean square; VAR, interindividual variability. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; †Not significant.

Table 3. Results of the size and shape analyses, showing interindividual, side and FA effects

	Size		Shape	
	Bodø	Drøbak	Bodø	Drøbak
Mean centroid size	16.966	17.104	1	1
Size regression	0.110 ± 0.039	0.055 ± 0.035	–0.001 ± 0.001	0.001 ± 0.0004
F	7.885**	2.485†	0.282†	2.455†
Individual				
d.f.	29	29	580	580
MS × 1000	14348.34	10051.91	0.306	0.133
F	24.255***	25.651***	1.003†	3.964***
Side				
d.f.	1	1	20	20
MS × 1000	5971.94	429.6	0.636	0.044
F	10.095**	1.096†	2.086**	1.301†
Interaction				
d.f.	29	29	580	580
MS × 1000	591.56	391.88	0.305	0.034
F	11.077***	2.519**	16.495***	1.53***
Error				
d.f.	60	60	1200	1200
MS × 1000	534.01	155.59	0.018	0.022
VAR × 1000	3439.195	2415	0.00025	0.025
FA10 × 1000	28.775	168.145	0.143	0.006

The regression for size is a linear regression of |R – L| on (R + L)/2 centroid size values to test for the dependence between size asymmetry and centroid size. The regression for shape is a regression of |R – L| Procrustes residuals values on (R + L)/2 centroid size values to test for the dependence between shape asymmetry and size. MS, mean square; VAR, interindividual variability; FA, fluctuating asymmetry. †Not significant.

Table 4. Results of the permutation tests performed on variance-covariance (VCV) matrices: within sample analysis of the correlations between VCV matrices of individual, fluctuating asymmetry (FA) and measurement error effects

Samples	Effects	Correlation	*P*
Bodø	Individual/FA	–0.90	< 0.0001
	Individual/error	–0.36	0.01
	FA/error	0.36	0.0637
Drøbak	Individual/FA	–0.30	0.0393
	Individual/error	0.13	0.3241
	FA/error	–0.51	0.011

Table 5. Results of the permutation tests performed on variance-covariance (VCV) matrices: between sample analysis of the correlations between VCV matrices of individual, fluctuating asymmetry and measurement error effects

Effects	Sample	Correlation	*P*
Individual	Bodø/Drøbak	0.17	0.1227
Fluctuating asymmetry	Bodø/Drøbak	0.51	< 0.0001
Error	Bodø/Drøbak	0.18	0.3423

is no correlation between shape and size asymmetry whatever might be the mechanism explaining a stronger shape asymmetry in the less stressed environment or (2) shape asymmetry values in *E. flavescens* are not dependent upon environmental conditions, but may result from internal disruptions such as genetic (Auffray *et al*., 1996) or epigenetic stressors (Evans & Marshall, 1996).

ARE SOME PARTS OF THE TEST MORE DEVELOPMENTALLY VARIABLE AND/OR UNSTABLE THAN OTHERS (I.E. ARE SOME PARTS LESS CONSTRAINED)?

FA was detected to be statistically significant for distances, centroid size and shape asymmetry in both samples, whereas it is statistically absent for individual plate surfaces. Thus, significant FA values for the entire OPA could suggest an additive effect of incon-

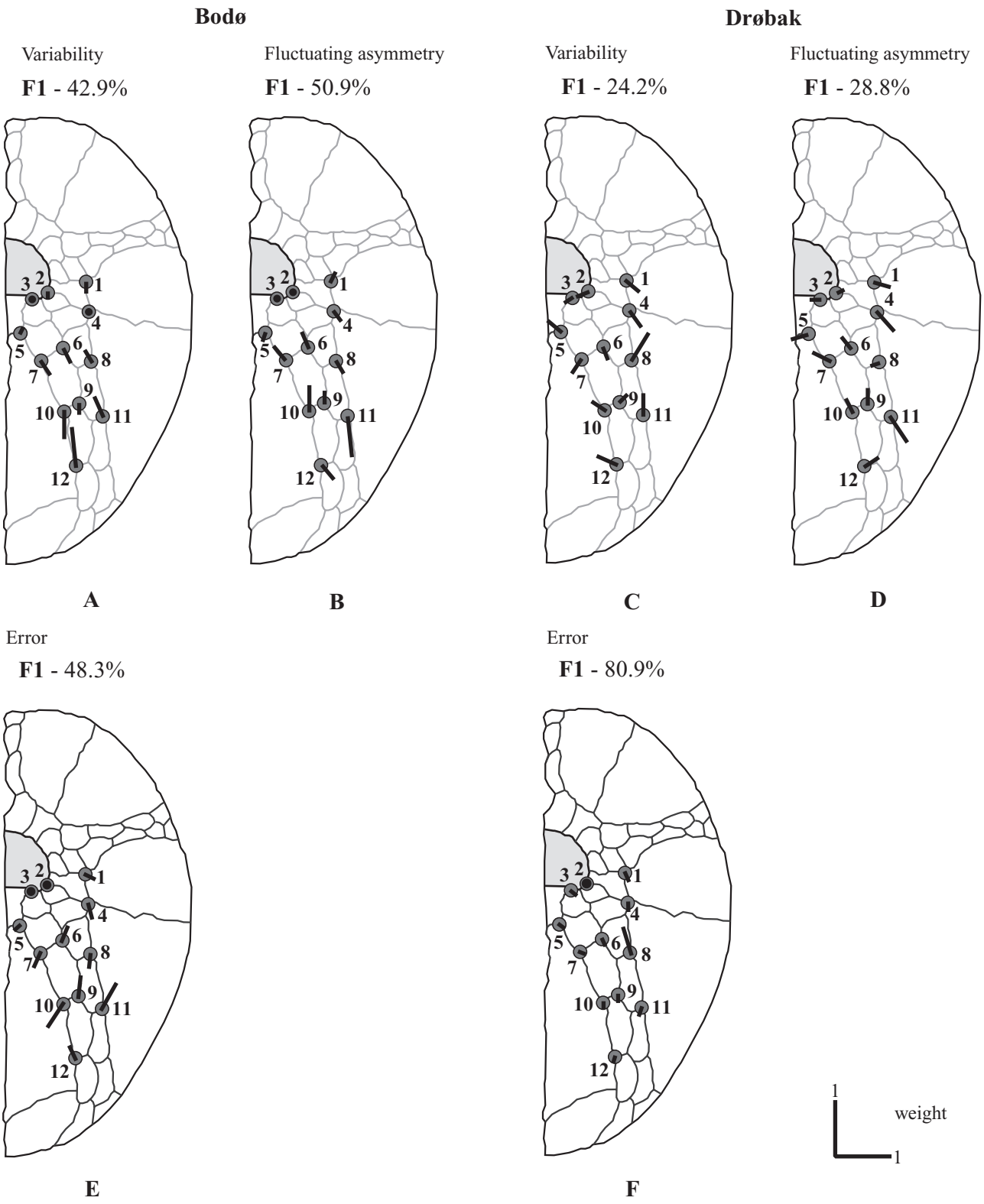

Figure 6. Factorial weights of Procrustes residuals on the first axis of the principal components analyses performed on variability, fluctuating asymmetry and error matrices in the two samples.

Table 6. Review of FA levels for the different descriptors and approaches

Descriptors	Bodø	Drøbak
Number of plate surfaces	0	0
Number of distances	4 (1–3, 4–5, 10–11, 2–12)	6
Size asymmetry	s	S
Shape asymmetry	S	s

s, lowest FA10 value; S, highest FA10 value.

spicuous asymmetries that are statistically non-significant at the plate level. The only two non-significant distances observed in Bodø correspond to short distances implying one or two plates. In addition, Table 2 shows higher FA10 and VAR values for distance 2–12 than for the other distances. This can be explained by the longer distance separating landmarks 2 and 12 compared to any of the other landmarks, in addition to distance 2–12 being positioned according to the growth axis of the OPA. This pattern is also consistent with the higher FA and VAR values presented by the anterior–posterior (y coordinates) than by the lateral–medial (x coordinates) directions of vectors in Figure 6. Accordingly, the length of the ambulacrum appears to be more sensitive to variability and developmental instability than the width. The elongated shape of posterior plates (as discussed above) certainly reinforces this hypothesis. FA expression could be particularly influenced by the antero-posterior stretching of ambulacral plates during growth. In other words, developmental instability could be more important when growth is locally more pronounced in a given direction.

Such results have to be considered with respect to the morphological descriptors used. Indeed, significant FA values were detected for distances and the Procrustes approach (i.e. for methods using landmarks as descriptors of plate boundaries). When the growth of a given plate is locally disrupted, the corresponding space is filled in by the adjacent plates in such a way that the overall OPA is not reduced in size. It works as a compensation phenomenon between plates that induces random fluctuations of plate boundaries and guarantees a certain robustness of the overall OPA shape. Local variations of plate boundaries may not necessarily be under any developmental control, but are only triggered by local and random disturbances of plate development. Hence, more than plate surfaces, plate boundaries appear to be the favoured place of FA expression, which means that methods using descriptors of plate boundaries (such as landmarks) may be more pertinent for assessing developmental instability in *E. flavescens*.

Is there a gradual change along the ambulacra according to the age of the plates?

Among the 12 vectors that depict the factorial weights of Procrustes residuals on the first axis of the PCA performed on the FA matrix (Fig. 6), a stronger weight (i.e. a greater length) characterizes the vectors of the posterior part of the OPA (vectors 6–12), in both localities. In other words, FA values increase from the anterior to the posterior part of the OPA. If FA results from the cumulative effect of numerous tiny developmental accidents during growth, a decrease of FA level from the anterior (first plates formed) to the posterior (younger plates) part of the OPA may be expected. The observed pattern contradicts the increase in developmental instability with growth, but it may be explained by the larger size and the more elongated shape of the posterior plates of the OPA. These posterior plates are slightly younger than the anterior ones; nevertheless, they grow more and undergo a stronger stretching and re-modelling of their shape during growth than the anterior plates. In other words, posterior plates are younger but undergo faster growth than older anterior ones, which may suggest that growth speed and growth increments are more auspicious than age with respect to cumulative departures from perfect symmetry.

FA and interindividual variations

The statistically significant correlations between FA covariance matrices of both samples, as well as between FA and VAR covariance matrices within each sample, suggest that, in *Echinocardium*, the expression of morphological variability is partly constrained and that variability shows similar patterns between specimens and between the sides of specimens. The idea of a constrained expression of FA is supported by the stronger FA values presented by anterior–posterior directions of vectors on the first PCA. This rather good correspondence between the patterns of FA and variation among individuals is in agreement with several previous studies (Leamy, 1993; Klingenberg & McIntyre, 1998; Klingenberg & Zaklan, 2000; Klingenberg *et al*., 2001). After Klingenberg & McIntyre (1998), the congruence between the patterns of variation of FA and interindividual variability could be an indication that the same developmental processes are involved; but see also Klingenberg (2004) for a review.

These initial results concerning the developmental stability of a spatangoid sea urchin demonstrate that their plate architecture is a suitable characteristic for the calculation of FA. The results open promising avenues of research for using such benthic animals to assess the influence of environmental alteration

(pollution) on marine fauna, as well as for studying the ontogeny of FA.

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ARTICLE 9 :

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On hidden heterogeneity in directional asymmetry – can systematic bias be avoided?

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fluctuating asymmetry;
methodology.

Abstract

Directional asymmetry (DA) biases the analysis of fluctuating asymmetry (FA) mainly because among-individual differences in the predisposition for DA are difficult to detect. However, we argue that systematic bias mainly results from predictable associations between signed right–left asymmetry and other factors, i.e. from systematic variation in DA. We here demonstrate methods to test and correct for this, by analysing bilateral asymmetry in size and shape of an irregular sea urchin. Notably, in this model system, DA depended significantly on body length and geographic origin, although mean signed asymmetry (mean DA) was not significant in the sample as a whole. In contrast to the systematic variation in DA, undetectable, random variability in the underlying DA mainly leads to reduced statistical power. Using computer simulations, we show that this loss of power is probably slight in most circumstances. We recommend future studies on FA to routinely test and correct for not only as yet for mean DA, but also for systematic variation in DA.

Introduction

Fluctuating asymmetry (FA) (Van Valen, 1962; Palmer & Strobeck, 2003) refers to small, random deviations from perfect morphological symmetry. For bilaterally symmetric traits showing 'ideal' FA (see below), individual asymmetries cannot be explained by genetic or environmental differences between the sides, but by imprecision of development (Palmer & Strobeck, 1986). Consequently, the unsigned asymmetry (|right-left|) is widely used to estimate the developmental instability (DI) of individuals or populations (Polak, 2003).

Fluctuating asymmetry can be separated from two other forms of bilateral asymmetry based on the distribution of signed asymmetry values in the population (Van Valen, 1962; Palmer, 1994). For a trait showing 'ideal' FA, the right–left differences are normally distributed around a mean of zero. *Directional asymmetry* (DA) is characterized by a normal distribution with a mean different from zero. Conspicuous examples of DA include the position of the mammalian heart and the anatomy of many species of flatfish. *Antisymmetry* (AS) is characterized by a platykurtic or bimodal distribution with a mean of zero. AS can be exemplified by the American lobster that has one large crusher claw and one slender cutter claw, and right- and left-biased individuals are equally frequent in the population. DA and AS are generally thought to have adaptive bases (Palmer & Strobeck, 1986). Because asymmetry is then the norm and not just a result of imprecise development, the unsigned asymmetry should not be used as an index of DI (Palmer & Strobeck, 1986; Palmer, 1994; Palmer & Strobeck, 2003; but see Graham *et al.*, 2003).

Statistical corrections for DA (Graham *et al.*, 1998; van Dongen *et al.*, 1999; Palmer & Strobeck, 2003) and AS (Graham *et al.*, 1998) have therefore been suggested. We will in this paper focus on DA, which is probably of more general occurrence than AS. Most DA corrections essentially consist in considering FA as deviations around the mean signed right–left asymmetry (*mean DA*) in the sample instead of as deviations around zero. For example, mean DA can be subtracted from the individual asymmetry values (Palmer & Strobeck, 2003), or corrected for by ANOVA or regression procedures, using the fixed side effect to quantify DA (Palmer & Strobeck, 1986; van Dongen *et al.*, 1999). However, these corrections provide unbiased estimates of DI only in the situation where the underlying DA is the same for all specimens. With the *underlying DA*, we refer to the

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'targeted' right–left asymmetry for a given genotype in a given environment (cf. the 'creode' of Zakharov, 1992). We thus consider the underlying DA as a biological property of an individual, not a population. But because observed asymmetries differ from the targeted because of DI, the underlying DA of a single individual is not directly measurable (except perhaps for clonal organisms). As a consequence, we can never exclude the possibility that individuals differ in the underlying DA. Because of this problem, it has been argued that traits displaying significant DA are best excluded from FA analyses (Palmer & Strobeck, 1992; Palmer, 1994; but see: Graham *et al.*, 1998; Palmer & Strobeck, 2003).

However, we argue that parts of the among-individual variation in the underlying DA can in fact be detected and corrected for statistically. If the underlying DA depends predictably on another factor, such as sex, body size or geographic origin, we can expect an association between the observed right–left asymmetry and this factor. We here demonstrate how such systematic variation in DA can be tested and corrected for within the methodological framework already developed for FA analyses. If not corrected for, systematic variation in DA can systematically bias FA estimates, and this can lead to false conclusions about the relationship between FA and these factors. On the other hand, variation in the underlying DA that is not associated with any other factor can in effect be considered as random. Such variation, like random measurement error, mainly reduces the precision with which FA estimates DI (but see Discussion). This reduces the power of a study, but is not likely to lead to falsely positive conclusions.

As model system, we study bilateral asymmetry of the test of *Abatus cordatus*. *A. cordatus* is an irregular sea urchin endemic to the subantarctic Kerguelen Islands (70°E; 49°S). Irregular sea urchins possess bilateral symmetry imposed on a pentaradial body plan, which allows the analysis of FA. The body plan also includes directionally asymmetric components, which make these animals good model organisms for the analysis of DA. Both shape and size data are considered. Specifically, we test and correct for systematic variation in DA as evidenced by the associations between signed asymmetry and geographic origin or body size. Using a simulation approach, we also explore the possible loss of precision from uncorrected variation in the underlying DA.

Methods

Model system and morphological measurements

We investigated a total of 420 *A. cordatus* from four locations 15–30 km apart in the Kerguelen Islands (Halage des Swains: 'POP_{HDS}' $n = 175$, Ile Haute: 'POP_{IH}' $n = 128$, Port-aux-Français: 'POP_{PAF}' $n = 91$, Ile Sûhm: 'POP_{IS}' $n = 26$). The samples were collected in the period 1989–2003 by scuba-diving and dredging and conserved in 70% ethanol. Spines were removed with a toothbrush after a 5-min immersion in 0.1–0.3% Cl-solution. The plate pattern was revealed by applying a 40 : 60 solution of ethanol : glycerol after drying.

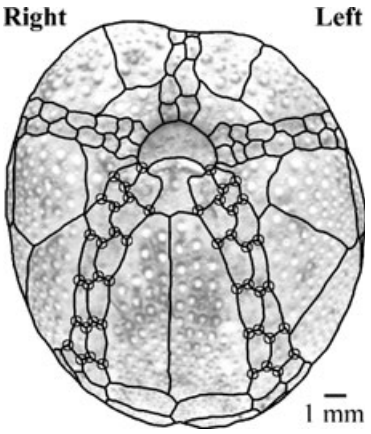

Fig. 1 Plate pattern of *A. cordatus*, oral view. Circles: landmarks used for asymmetry analyses.

In each of the two posterior ambulacral zones, 23 landmarks corresponding to boundaries between test plates were defined (Fig. 1). Two-dimensional landmark coordinates were scored to the nearest 0.001 mm using a Nikon MM-60 measuring microscope. To assess measurement error, two independent sets of measurements were made of all specimens. Total length of the test was measured to the nearest 0.01 mm with a digital calliper.

Procrustes superimposition

The data were analysed by generalized Procrustes superimpositions of right and mirror-reflected left halves of the landmark configurations as described in Klingenberg & McIntyre (1998). By this procedure, asymmetry in shape is separated from asymmetry in centroid size (c.s.: the square root of the sum of squared distances of a set of landmarks from their centroid, Slice *et al.*, 1996). While specialized procedures are needed for the subsequent analyses of the shape data (Klingenberg & McIntyre, 1998; Klingenberg *et al.*, 2001), the centroid size data can be analysed similarly to asymmetries of ordinary metric traits (e.g. van Dongen *et al.*, 1999; Palmer & Strobeck, 2003).

Modelling systematic variation in DA

Fluctuating asymmetry data are traditionally analysed by mixed-effects models in which DA is represented by a fixed side effect (Palmer & Strobeck, 1986; Klingenberg & McIntyre, 1998; van Dongen *et al.*, 1999). The fixed side effect then estimates the mean signed asymmetry (mean DA) in the sample. These models can be extended to include systematic variation in DA by adding fixed-effect

interactions between side and other variables. These terms represent predictable associations between signed asymmetry, i.e. DA, and other factors. Thus, the fixed-effect interaction between side and body size can be used to represent an allometric change in DA. The fixed-effect interaction between side and population represents population differences in DA. By including such interaction terms in FA models, systematic variation in DA can be specifically tested, and if found significant, statistically corrected for. This extension of the basic FA model was also demonstrated by van Dongen *et al.* (1999), yet the potential to correct for systematic variation in DA seems later largely to have been overlooked.

Size analyses

Centroid size data were analysed using a restricted maximum likelihood (REML) mixed regression approach (van Dongen *et al.*, 1999). By this method, side is coded as a numeric variable with the possible values −0.5 and 0.5, the fixed side effect represents DA and the random individual side effect represents FA (see van Dongen *et al.*, 1999, for details). Systematic variation in DA was included by adding fixed-effect interaction terms between side and body size (measured as test length) and/or population. All fixed effects were tested by *F*-tests (Pinheiro & Bates, 2000). Individual FA-estimates ('$FA_{C.S.}$') corrected for effects of DA and heterogeneous measurement error were provided by the random side estimates from the best model (van Dongen *et al.*, 1999). This procedure thus allowed simultaneously testing and correcting for DA, systematic variation in DA and heterogeneous measurement error.

Shape analyses

Shape data were analysed by Procrustes ANOVA (Klingenberg & McIntyre, 1998; Klingenberg *et al.*, 2001). Systematic variation in DA was included by adding interaction terms between side and body size (measured as centroid size) and/or population. In addition, allometric effects on shape were included by adding centroid size (both as a linear and a quadratic term) to the model (Klingenberg *et al.*, 2001). Terms were entered and tested sequentially by permutation tests using 10 000 iterations (Klingenberg & McIntyre, 1998; Klingenberg *et al.*, 2001). After choosing a model in which all terms were significant, the following procedure was used to calculate individual absolute asymmetry in shape (FA_{SHAPE}) corrected for effects of allometry and population-specific DA: (i) Right–left Procrustes-aligned landmark configurations were calculated after averaging across repeat measurements. (ii) These configurations, representing signed shape asymmetry, were used as response in a multivariate regression with the predictor variables population (representing population differences in DA) and right–left centroid size (representing allometry). (iii) FA_{SHAPE} of each individual was calculated as the square root of the sum of squared residuals from this model. DA in shape was visualized by plotting population-specific shape and DA using coefficients from a multivariate regression model with right and left Procrustes scores as response and the explanatory variables determined by the Procrustes ANOVA.

Simulating the loss of precision caused by undetected DA heterogeneity

The proposed methods correct for systematic variation in DA. However, there may also be among-individual variation in the underlying DA that is not associated with any measured factor, and is therefore not detectable. Such variation reduces the precision with which DI is estimated. To quantify this loss of precision, we estimated the correlation between FA and the underlying DI in response to: (i) the amount of DA heterogeneity, and (ii) the amount of DI heterogeneity. For a given level of DA heterogeneity and DI heterogeneity, we randomly generated 100 000 signed FA-values from normal distributions with individual-specific means and SDs, DA_i and DI_i, respectively. DA_i- and DI_i-values were randomly generated from a normal and a gamma distribution, respectively. The characteristics of these two distributions determined the level of DA heterogeneity and DI heterogeneity. It can be shown that the coefficient of variation (CV; SD over mean) of DI is $a^{-0.5}$, and the proportion of the total asymmetry variance originating from variation in DA is $\sigma^2 \, (a^2 \, s^2 + a \, s^2 + \sigma^2)^{-1}$, where a and s are the shape and scale parameters of the DI gamma distribution and σ^2 is the variance of the DA normal distribution. For each level of DA heterogeneity and DI heterogeneity, we calculated the Pearson's coefficient of correlation between DI and unsigned FA.

All statistical analyses were performed with the program R (R Development Core Team, 2003). A critical level of 5% is used in all tests.

Results

Preliminary analyses

Because of a positive scaling between unsigned asymmetry and trait size, centroid size was transformed by taking the natural logarithm of the square root of the original values. Asymmetry calculated from the transformed values was unrelated to trait size ($F_{1,415} = 0.06$, $P = 0.81$, analysis of covariance, with population as a categorical covariate). Both size and shape FA was more than an order of magnitude larger than measurement error and highly significant in each population (all $P < 0.001$, REML mixed regression or Procrustes ANOVA). Measurement error for centroid size depended negatively on the total length of the test ($\chi^2_1 = 79.3$, $P < 0.001$, likelihood ratio test) and it differed between

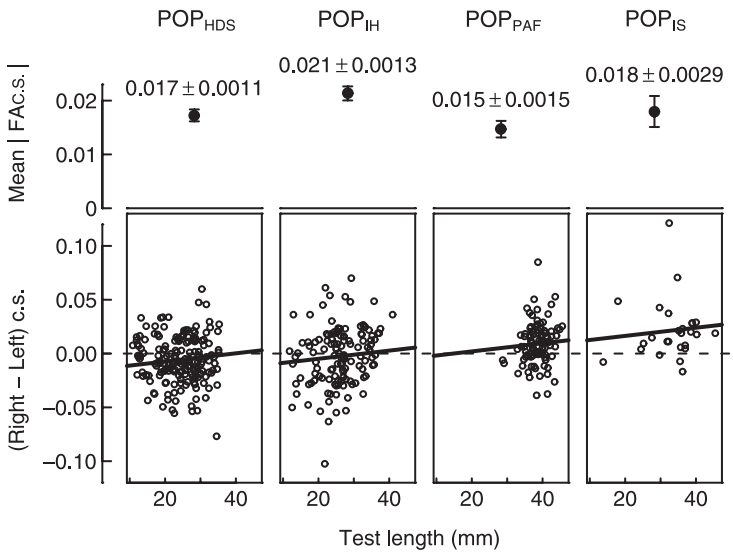

Fig. 2 Unsigned and signed asymmetry in centroid size (c.s.). The lower panels show right–left c.s. as a function of test length for each population. Bold lines show predictions from a global mixed REML regression model. These lines represent systematic variation in DA. The upper panels show mean ± SE for unsigned $FA_{C.S.}$. $FA_{C.S.}$ are individual random side effect estimates from the regression model and represent FA corrected for effects of DA and heterogeneous measurement error.

populations ($\chi^2_3 = 47.5$, $P < 0.001$, likelihood ratio test). In the subsequent analyses of centroid size asymmetry, models with heterogeneous error structure were therefore used. There was no indication of AS in any population (Klingenberg & McIntyre, 1998; Palmer & Strobeck, 2003). Two outlier centroid size asymmetry values were identified (Palmer & Strobeck, 2003) and it was controlled that the conclusions of the study were robust to the exclusion of these.

Directional asymmetry

Directional asymmetry in centroid size varied in both sign and magnitude among populations (Side × Pop: $F_{3,1249} = 16.7$, $P < 0.001$), and DA depended allometrically on test length (Side × Test length: $F_{1,1249} = 4.4$, $P < 0.05$) (Fig. 2). Overall mean DA was not significant, however (Side: $F_{1,1249} = 0.004$, $P > 0.5$). The population differences and the allometric changes in DA are partly confounded because test lengths differed between populations, but both effects contribute independently towards explaining DA variation. After accounting for measurement error, 12% of the variation in signed asymmetry could be explained by systematic variation in DA, while 88% was attributed to FA.

Mean DA in shape across all populations was highly significant, but there were also significant differences in DA between the populations (Table 1). Plots of population-specific DA suggested that the DA pattern was similar across populations; it was mainly the magnitudes that differed ($POP_{HDS} > POP_{IH} > POP_{PAF} > POP_{IS}$; this ranking was confirmed by Procrustes ANOVAS of each population separately). There were no significant allometric changes in shape DA (Table 1). As for centroid size DA, the population differences and the allometric changes in DA are partly confounded, but for shape DA only the population effect contributes independently towards explaining DA variation (Table 1). Population differences in shape DA accounted for 2.9% of the total variance in signed asymmetries across all landmarks.

Table 1 Procrustes ANOVA for asymmetry in shape.

Effect	d.f.	MS	*F*	*P*-value	Interpretation
C.s.	42	41031	129.9	<0.001	Allometric change in shape (linear)
C.s.^{2}	42	18792	59.5	<0.001	Allometric change in shape (quadratic)
Pop.	126	9738	30.8	<0.001	Population differences in shape
Ind.	17598	316	3.1	<0.001	Individual differences in shape
Side	42	7299	72.1	<0.001	Mean DA
Side × Pop.	126	417	4.1	<0.001	Population differences in DA
Side × C.s.	42	251	2.5	>0.1	Allometric change in DA
Side × Pop. × C.s.	126	5.1	0.1	>0.1	Pop. diff. in allometric change in DA
Side × Ind	17598	101	78.2	<0.001	FA
Residual	35280	1.3			Measurement error

All populations are analysed in one model. Effects are tested sequentially by permutation tests. The Side × Pop. effect is also significant if Side × C.s. is accounted for first ($F_{126,17598} = 3.4$, $P < 0.01$). Sums of squares are multiplied by 10^6. C.s., centroid size.

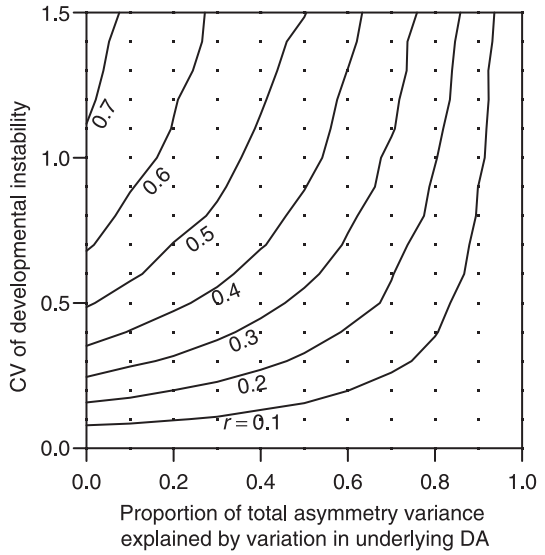

Fig. 3 Contour plot showing the estimated Pearson's coefficient of correlation (r) between unsigned FA and DI in response to heterogeneity in DA (x-axis) and heterogeneity in DI (y-axis). The correlations are estimated from randomly generated data sets of $n =$ 100 000. Data sets differ in the amounts of heterogeneity in DA and DI (points). Heterogeneity in DA is represented by among-individual differences in the statistical expectation of signed asymmetry. Heterogeneity in DI is represented by among-individual differences in the SD of signed asymmetry (measured as the coefficient of variation, CV).

Fluctuating asymmetry – with and without correction for systematic variation in DA

For comparison, we calculated FA both with and without correction for systematic variation in DA. The uncorrected estimates of mean centroid size FA (mean $|FA_{C.S.}|$) in each population were 2–34% higher than the corrected estimates (POP_{HDS}: +4%, POP_{IH}: +2%, POP_{PAF}: +12%, POP_{IS}: +34%). For shape (FA_{SHAPE}), the uncorrected estimates were 1–10% higher than the corrected ones (POP_{HDS}: +1%, POP_{IH}: +1%, POP_{PAF}: +5%, POP_{IS}: +10%). In our case, these differences did not alter the conclusions that centroid size FA varied significantly among populations ($F_{3,415} = 3.87$, $P < 0.01$, ANOVA; Fig. 2) while shape FA did not ($F_{3,416} = 0.97$, ANOVA, $P = 0.4$, permutation test). However, when using uncorrected centroid size FA estimates, none of the pairwise comparisons between populations reached significance, but when using corrected estimates, POP_{IH} was found to be significantly more asymmetric than POP_{PAF} (Tukey's 'Honest Significant Difference' method).

We also compared individual-level FA estimates. On average uncorrected individual unsigned $FA_{C.S.}$-estimates differed in absolute value by 0.0061 from corrected estimates, that is by 34% compared with mean unsigned $FA_{C.S.}$ (=0.018). The corresponding average difference for individual FA_{SHAPE}-estimates was 0.0031, which is 5.8% of mean FA_{SHAPE}.

Effect of DA heterogeneity on precision

The isoclines in Fig. 3 are relatively flat in the lower left part of the figure. This means that if the heterogeneity in DI is at a low to intermediate level, the introduction of some heterogeneity in the underlying DA does not lead to a drastic loss of precision. For example, if the coefficient of variation of DI is 0.2 (which is typical for many species according to estimates of Gangestad & Thornhill, 2003) and there is no heterogeneity in DA, the correlation between FA and DI is 0.25. If the heterogeneity in DA increases to a level where 20% of the total asymmetry variance is caused by among-individual differences in the underlying DA, the correlation between FA and DI is only reduced to 0.20. The curves are steeper at higher DA heterogeneity as well as at higher DI heterogeneity, but the relevance of this to real populations may be questionable.

Discussion

Directional asymmetry in *A. cordatus*

The results show that DA in *A. cordatus* depends on body size as well as on population. This suggests an ontogenetic change in DA, in addition to genetic and/or environmental effects on DA. Because populations differed genetically (Poulin & Féral, 1998) as well as environmentally (Poulin & Féral, 1995), it is not possible to separate the two effects in our study. Note that genetic differentiation in *A. cordatus* is comparatively high across short geographic distances because of the absence of planktonic stages in the life cycle.

A likely cause of DA in the tests of irregular sea urchins is asymmetry in the digestive system (Lawrence *et al.*, 1998). In spatangoids, the intestine, which forms the main part of the digestive system, comprises two circuits (loops) coiled in opposite direction. This may cause DA in the test either directly by bilaterally asymmetric pressure of the gut contents, or indirectly by genetic control to accommodate the more spacious intestine circuit on the right side. The observed association between size and DA, which has also been found in the irregular sea urchin *Mellita tenuis* (Lawrence *et al.*, 1998), may be because of an allometric effect of the intestine. Population differences in DA could also be related to differences in the digestive system. In addition, DA may be a consequence of asymmetric plate formation pattern, as plate formation at the left and right sides are not exactly mirrored by each other (see David *et al.*, 1995 for details). Note that the two posterior ambulacral zones retained for the present study are ontogenetically perfectly symmetric, and therefore homologous, but because the test is an

integral entity asymmetric plate formation pattern in other parts of the test may have contributed to the observed DA.

Heterogeneous DA in other organisms

Systematic variation in DA has been reported in a wide range of organisms (e.g. Pither & Taylor, 2000; Mazzi & Bakker, 2001; Kellner & Alford, 2003; Oleksyk *et al.*, 2004; Stige *et al.*, 2004). In some cases, variation in DA may be related to differential adaptive function of DA (Windig & Nylin, 1999). In other cases, environmental stress appears to cause a transition from 'ideal FA' to DA (Teather, 1996; Collin, 1997; Pither & Taylor, 2000; Kellner & Alford, 2003). But in many cases the reasons for the differences are not apparent, and would have been impossible to predict *a priori*. Notably, several studies in which the growth of single individuals was monitored have reported ontogenetic changes in DA (Teather, 1996; Collin, 1997; Pither & Taylor, 2000; Kellner & Alford, 2003).

We suspect that systematic variation in DA may be more common than evident in the literature, as it is typically not tested for. The fact that the heritability of signed asymmetry is usually estimated to be low, though occasionally significant (Coyne, 1987; Tuinstra *et al.*, 1990; Leamy *et al.*, 1997, 1998; Leamy, 1999; Roff & Réale, 2004; Santos *et al.*, 2005; Stige *et al.*, 2005), suggests that there is generally little additive genetic variation for DA. However, differences in DA could also be linked to age variation (as demonstrated in the present study), environmental variation or nonadditive genetic variation. Therefore, until more tests for systematic variation in signed asymmetry are made, we cannot know what the general occurrence of such variation is.

Why correct for systematic variation in DA?

The results on centroid size asymmetry in *A. cordatus* demonstrate the importance of testing for systematic variation in signed asymmetry. If all data had been pooled, no significant DA had been detected, as mean signed asymmetry was close to zero. Still, systematic differences in DA between populations and size classes existed.

If not corrected for, systematic variation in signed asymmetry can systematically bias FA estimates. In the present study, uncorrected vs. corrected population-level FA estimates differed by up to 34%. Although the final conclusions remained the same, this would obviously not always be the case. With different baseline levels of FA or DA, undetected population differences in DA could easily lead to false conclusions about differences in FA. Also, associations between DA and continuous factors could lead to systematic bias. For example, if a linear relationship between DA and body size goes undetected, FA of the smallest and largest individuals will tend to be over-estimated. By statistically correcting for systematic variation in signed asymmetry, such bias is avoided. One then controls that inferences about differences in unsigned asymmetry, i.e. FA, cannot be explained by differences in signed asymmetry, i.e. DA.

Statistical correction for systematic variation in DA leads to more precise estimation of DI, and therefore also to increased power to detect associations between FA and other factors. However, this is probably less important than the avoidance of systematic bias.

An important advantage of the approach presented here is that DA is analysed by one global model instead of separately for each subsample in a data set. If a study comprises many groups, the statistical power to detect DA in each group separately may be very low, and DA may easily be overlooked. In contrast, a global approach allows precise estimation of both mean DA and systematic variation in DA using information from all the data.

Limitations of the corrections

The proposed DA corrections are unlikely to remove absolutely all systematic bias that can result from DA. Especially, the amount of among-individual variation in underlying DA may differ between groups. This will have the same effect as heterogeneous measurement error, and is in practice impossible to detect or correct for. If mean DA or the systematic variation in DA is large, FA comparisons across groups should therefore be made with caution.

An unavoidable limitation is the fact that we cannot correct for among-individual variation in the underlying DA that is not correlated with any of the measured variables. Undetected DA variation reduces the precision with which DI is estimated by FA. The simulation analysis suggests that this loss of precision is probably not a very serious concern unless a considerable proportion of the total asymmetry variance is caused by differences in the underlying DA. For *A. cordatus*, we can conclude that if the unknown variation in the underlying DA is not of much larger magnitude than the systematic component of the signed asymmetry variance (12% and 2.9%), the loss of precision is small.

Conclusion

We suggest that future FA studies should test and, if necessary, correct for systematic variation in DA. We here demonstrate methods applicable for metric traits (represented by centroid size) and for shape data. Finally, we think that a strict separation between 'ideal FA' and DA should be abandoned – at least some very slight difference in developmental condition between sides may be the norm rather than the exception (Kraak, 1997) and whether or not DA is actually detected depends to a great deal on sample size and measurement precision. Consequently, instead of omitting from FA

studies all traits that show significant DA, information is gained if we include these traits, making the best possible statistical corrections for DA, but interpret the results with the limitations of these corrections in mind.

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