

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. © 2006 The Linnean Society of London, *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 develop-

ment, 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 non-functional 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 (Bonadonna, 1967; Darnaud, Lecumbergy & 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

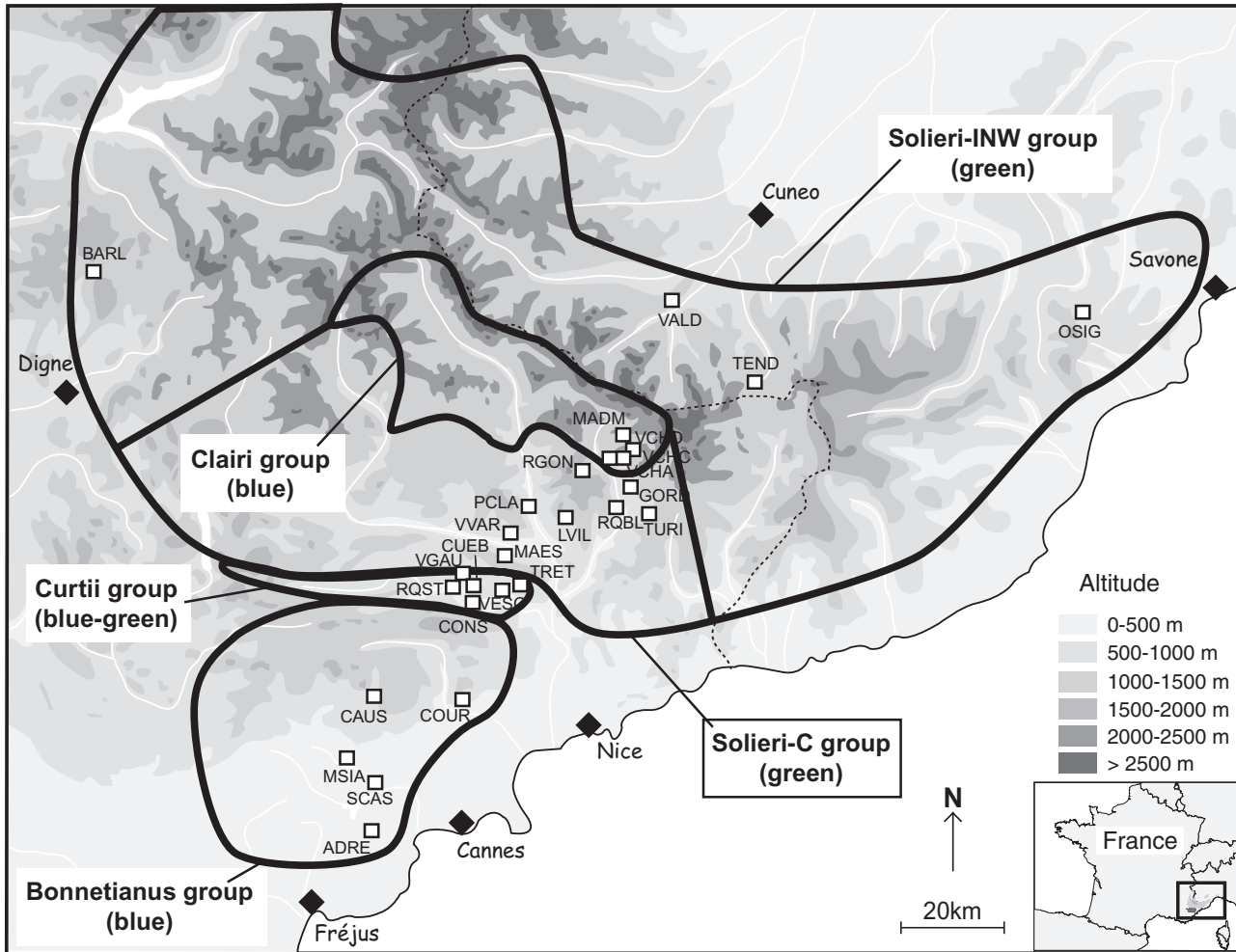


Figure 1. Distribution area of *Carabus solieri*, sampling locations (white squares), and limits of the groups of populations defined according to the colour of individuals (indicated in parentheses).

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 ($N = 9\text{--}30$ 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 tibiae 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 ($R_i - L_i$)] and absolute asymmetries ($|R_i - L_i|$). 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 $[(R_i + L_i)/2]$. 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 \times 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:

$$FA14 = \sum_j \frac{|FA_{ij}|}{|FA_j|}$$

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 \times 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

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	<i>F</i>	<i>P</i>
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.* $P < 0.05$.

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.

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 \pm 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 \pm 0.029
WINGW	0.060 \pm 0.052
TIBMID	0.010 \pm 0.009
TIBHIND	0.008 \pm 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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