Cucumispora dikerogammari n. gen. (Fungi: Microsporidia) infecting the invasive amphipod *Dikerogammarus villosus*: a potential emerging disease in European rivers

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SUMMARY

Dikerogammarus villosus is an invasive amphipod that recently colonized the main rivers of Central and Western Europe. Two frequent microsporidian parasites were previously detected in this species, but their taxonomic status was unclear. Here we present ultrastructural and molecular data indicating that these two parasites are in fact a single microsporidian species. This parasite shares numerous characteristics of *Nosema* spp. It forms elongate spores (cucumiform), developing in direct contact with host cell cytoplasm; all developmental stages are diplokaryotic and the life cycle is monomorphic with disporoblastic sporogony. Initially this parasite was described as *Nosema dikerogammari* Ovcharenko and Kurandina 1987. However, phylogenetic analysis based on the complete sequence of SSU rDNA places the parasite outside the genus *Nosema* and it is therefore ascribed to a new genus *Cucumispora*. The key features characteristic to this genus are: presence of a very well-developed, umbrella-shape anchoring disk covering the anterior part of polaroplast; arrangement of isofilar polar filament into 6–8 coils convoluted with different angles, voluminous diplokaryon, thin spore wall and relatively small posterior vacuole containing posterosome. The parasite infects most host tissues but mainly muscles. It showed high rates of horizontal trophic transmission and lower rates of vertical transmission.

Key words: Amphipoda, Microsporidia, biological invasion, *Nosema dikerogammari*, *Cucumispora* gen. sp., *Dikerogammarus villosus*, SSU rDNA, phylogeny.

INTRODUCTION

Microsporidia are obligate intracellular parasites of animals and protists. They are particularly common parasites of amphipod crustaceans (Terry *et al*. 2004), in which they are transmitted both horizontally and vertically (MacNeil *et al*. 2003; Haine *et al*. 2004) and in which they cause pathologies ranging from extensive muscle degeneration (Slothouber-Galbreath *et al*. 2004) to sex-ratio distortion (Haine *et al*. 2007; Mautner *et al*. 2007). These parasites were discovered in amphipod hosts for the first time in Germany (Pfeiffer, 1895) and more than 20 species were described till the mid-20th century (Spraque, 1977). However, their taxonomic and phylogenetic status has recently been reappraised following a series of studies using ultrastructural and molecular techniques (Terry *et al*. 1999, 2003, 2004; Slothouber-Galbreath *et al*. 2004; Franzen *et al*. 2006).

The Ponto-Caspian amphipod *Dikerogammarus villosus* has recently colonized most large rivers of Western and Central Europe (Bij de Vaate *et al*. 2002; Bollache *et al*. 2004; Grabowski *et al*. 2007; Wattier *et al*. 2007). This amphipod can be considered an invasive species since the establishment of viable populations rapidly followed spread by man. Moreover, *D. villosus* is a predatory species which tends to outnumber native macrobenthic crustaceans in colonized areas (Dick and Platvoet, 2000; Bollache *et al*. 2004). Two microsporidia, *Nosema dikerogammari* Ovcharenko and Kurandina, 1987 and *Microsporidium* sp. *D* (Wattier *et al*. 2007) were described in both native and invasive populations of *D. villosus*, often at high prevalence.

Nosema dikerogammari was first described based on light microscopy, infecting abdominal musculature of *D. villosus* in the Dnieper Estuary, within its native range (Ovcharenko and Kurandina, 1987). It was placed within the genus *Nosema* due to its diplokaryotic development and disporoblastic

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sporogony. Ultrastructural data obtained from *D. villosus* collected in the Kiev Reservoir on the River Dnieper, later confirmed typical characteristics of *Nosema* (Ovcharenko and Vita, 1996). In addition, *N. dikerogammari* was detected in the Danube Estuarine Lakes, within the native range of *D. villosus*, and in eastern Poland (Bug River), a part of the invaded range of *D. villosus* (Ovcharenko *et al.* 2009). In parallel, Wattier *et al.* (2007) surveyed the main water bodies of Western Europe (invaded range) and the lower course of the Danube (southern native range) using a PCR-based molecular screening. This study indicated that *D. villosus* was infected by 4 microsporidian species. Three of them were close to parasites already described in other amphipods species: *Nosema granulosis*, *Dictyocoela mulleri* and *D.* sp. *roeselum*. They were found in few host populations, at very low prevalence and always outside the native range of the host. The fourth parasite, found in most *D. villosus* populations, was a new species of microsporidian which was provisionally named *Microsporidium* sp. *D*. Phylogenetic analysis based on a 476 bp region of SSU rDNA indicated that this species was closely related to an undescribed *Microsporidium* sp. *JES2002G* infecting a gammarid from Western Europe (Terry *et al.* 2004) with a closer affinity to the genera *Dictyocoela* and *Pleistophora* than to *Nosema* to which it was definitely distantly related (Wattier *et al.* 2007). *Microsporidium* sp. *D* was found at high prevalence and showed the very same symptoms as *N. dikerogammari* parasites (Wattier *et al.* 2007).

These studies raise the question of whether *N. dikerogammari* and *Microsporidium* sp. *D* were 2 different species or merely a single microsporidian species described using 2 different techniques (light microscopy and DNA sequencing). This question is important because *Dikerogammarus villosus* is one of the most invasive freshwater species in Europe (Devin *et al.* 2003; Van Riel *et al.* 2006) and has carried its microsporidian parasites during its rapid expansion into the main water bodies of Europe (Wattier *et al.* 2007; Ovcharenko *et al.* 2009). Surveillance and early detection of parasites after ecological changes is important to detect potential emerging disease (Woolhouse, 2002). For this reason, it is crucial to ascertain the number and diversity of microsporidian species introduced into European watersheds through the invasion of *D. villosus*.

In this paper, we combine molecular phylogenetic and ultrastructural analysis to demonstrate that *Nosema dikerogammari* and *Microsporidium* sp. *D* are a single species, and cannot be related to the few *Nosema granulosis* parasites previously described in a few Western Europe populations. We further provide information on the parasite's tissue specificity and routes of transmission. On this basis, we propose to redescribe *N. dikerogammari* as *Cucumispora dikerogammari*, a member of the novel genus *Cucumispora* gen. nov.

MATERIALS AND METHODS

Sampling sites

Dikerogammarus villosus individuals were sampled from 3 sites: St Jean de Losne on the Saone River, France (N47°5′23·45″; E05°14′31·63″), Uraz on the Oder River, Western Poland (N51°14′54·79″; E16°50′47·61″) and Zegrze on the Zegrzynski Reservoir on the Bug River, Eastern Poland (N52°27′30·79″; E21°01′04·52″). All the sites were within the introduced range.

Light and electron microscopy

Light and electron microscopy were performed on individuals from the 2 Polish sites. The presence of spores in infected individuals was identified by dissection under the binocular microscope. Muscles of infected individuals were then stored in 100% ethanol for molecular analysis. Live spores and Giemsa-stained slides were examined under an Olympus BX50F4 light microscope. The software Analysis Pro 2.11 (Soft Imaging System GmbH, Germany) was used for measurements. For transmission electron microscopy, the infected muscle tissues were fixed in a 2·5% (v/v) glutaraldehyde in a 0·2 M sodium cacodylate buffer (pH 7·2) for 1–3 days. After washing and post-fixation in 2·0% (w/v) osmium tetroxide in cacodylate buffer for 1 h at 4 °C, the pieces were dehydrated and embedded in Epon-Araldite solution using a standard procedure (Vávra and Maddox, 1976). Blocks of embedded tissues were sectioned with an LKB III ultra-microtome. Semi-thin sections were stained with methylene blue. Ultrathin sections were then mounted on copper grids, double stained with uranyl acetate and lead citrate and examined with a JEM 100B electron microscope operated at 80 kV.

Molecular methods

DNA extraction. DNA was extracted from Polish and French infected *D. villosus*, including Polish individuals in which *Nosema dikerogammari* had beforehand been firmly identified by light and electron microscopy. Microsporidian DNA of French in dividuals was co-extracted with DNA from host muscle tissues. Around 4 mm^3 of the host muscle were homogenized in a 1·5 ml tube containing 200 μl of Queen's lysis buffer (Seutin *et al.* 1991) using a plastic pestle and incubated overnight at 55 °C with 5 μl of proteinase K (20 mg/ml). DNA was then extracted based on a standard phenol-chloroform method as described by Hillis *et al.* (1996). Air-dried DNA pellets were re-suspended in 100 μl of TE

Table 1. The list of primers used for amplification of microsporidian SSU rDNA, ITS and partial LSU rDNA

Primer	Sequence	Source
V1f	5′-CACCAGGTTGATTCTGCCTGAC-3′	Baker *et al*. (1994)
530r	5′-CCGCGGC(T/G)GCTGGCAC-3′	Vossbrinck *et al*. (1993)
MC2f	5′-TCCGGAGAGGGAGCCTGAGAGA-3′	This study
HG4f	5′-GCGGCTTAATTTGACTCAAC-3′	Gatehouse and Malone (1998)
530f	5′-GTGCCAGC(C/A)GCCGCGG-3′	Vossbrinck *et al*. (1993)
MC3f	5′-TTGTACACACCGCCCGTCGTTATC-3′	This study
964r	5′-CGCGTTGAGTCAAATTAAGCCGCACA-3′	Terry *et al*. (2003)
MC3r	5′-GATAACGACGGGCGGTGTGTACAA-3′	This study
1492r	5′-GGTTACCTTGTTACGACTT-3′	Weiss and Vossbrinck (1998)
580r	5′-GGTCCGTGTTTCAAGACGG-3′	Weiss *et al*. (1994)

buffer, pH 8·00. Microsporidian DNA of Polish individuals was co-extracted from host muscle tissue of the remaining individuals, in which *N. dikerogammeri* had been identified, using a Qiagen DNeasy Blood and Tissue kit. Tissue was lysed in 180 μl of ATL buffer and 20 μl of proteinase K for 3 h at 55 °C. DNA was then extracted as per the manufacturer's protocol.

Sequencing. DNA sequences of the complete microsporidian small subunit (SSU) rDNA, the internal transcriber spacer (ITS) and part of the large subunit rDNA (LSU) were obtained for 1 individual from each of the 3 sampled sites (St Jean de Losne, Uraz and Zegrze). In order to obtain overlapping fragments, the set of primers described in Table 1 was used. The product was amplified under the following PCR conditions: an initial denaturing step at 95 °C for 5 min was followed by 35 cycles of 95 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min. These cycles were followed by a final extension at 72 °C for 5 min. Direct sequencing was performed using BigDye technology either by Macrogen Inc., Korea or IBERS, Aberystwyth, UK. All sequencing was performed in both directions.

Phylogenetic analysis

The microsporidian sequences from *D. villosus* were edited and aligned by eye using BioEdit software (Hall, 1999). Alignment with published sequences of microsporidian parasites infecting *D. villosus* was also performed (GenBank Accession numbers: EF119217, EF119218, EF119219, EF119220, EF119221, EF119222, EF119223). Similarity of the new sequences to those already available in GenBank was determined using BLAST (Table 2). A set of 12 sequences was selected for use in the phylogenetic analysis. This included sequences which were close to parasites from *D. villosus*, sequences from species that morphologically resembled microsporidia from *D. villosus* (i.e. *Nosema, Pseudonosema cristatellae*, *Trichonosema pectinatellae*, *Bryonosema plumatellae*) and other sequences chosen to span the diversity of Microsporidia in amphipods. Finally, 3 fungi species were selected as outgroups following Vossbrinck and Debrunner-Vossbrinck (2005) (Table 2). Sequences were aligned with Clustal W (Thompson *et al*. 1994) in MEGA 4 software, using default settings (Tamura *et al*. 2007). Only fragments of the sequences that could be unambiguously aligned were used for analysis (1046 bp). Phylogenetic reconstructions included maximum likelihood (ML) processed with PHYML 2.4.4 software (Guindon and Gascuel, 2003) and Bayesian inference processed with MRBA YES v 3.1.2 software (Ronquist and Huelsenbeck, 2003). For ML and Bayesian analysis, the GTR plus gamma model was used. The model was selected with Modeltest software (Posada and Crandall, 1998). For the Bayesian approach, we ran the analysis for 500 000 generations, with 1 tree retained every 100 generations. Likelihood stationarity was achieved after 16 000 generations and we excluded this 'burn-in' period when constructing a consensus tree. Nodal support was assessed by 1000 bootstrap replicates ML analysis and posterior probabilities for the Bayesian method as estimated from the 3750 trees included after the burn-in.

Parasite detection and identification by PCR-RFLP. In order to determine if infection was preferentially associated with host sex and size, 140 *D. villosus* individuals from Zegrze were sexed under the light microscopy (Nikon SMZ 1500). The 4th coxal plate was measured using Lucia G 4.81 software. This measurement is known to be representative of total size (Bollache *et al*. 2000). Infection status was determined by *Microsporidium* sp. *D* diagnostic PCR-RFLP (Wattier *et al*. 2007). Microsporidian specific SSU rDNA primers V1f (Baker *et al*. 1994) and 530r (Vossbrinck *et al*. 1993) were used to amplify a ~480 bp long PCR fragment, which was then restricted with the enzyme *Vsp*I. The *Microsporidium* sp. *D* specific restriction profile shows 2 bands of ~400 bp and ~80 bp in length (Wattier *et al*. 2007). PCR, restriction and electrophoresis conditions were as described by Wattier *et al*. (2007). In female hosts DNA was extracted separately for

Table 2. Sequence similarities of the studied parasite with other microsporidia based on SSU rDNA

(D-FR, D-PL – percentage of identity with studied microsporidian parasites from France and Poland respectively. H, host taxonomic classification; G, Gammaridea; F, Fish; I, Insect; B, Bryozoa. Habitat, FW, Fresh Water; BW, Brackish Water; TSW, Specific zone at the upper limit the sea shore (terrestrial habitat regularly impacted by sea water); T, Terrestrial; FW-T: larvae, Fresh Water but adult, Terrestrial. Clade refers to the clade identified in the phylogenetic tree presented in Fig. 5. Out, outgroup.)

	Parasite species	Host	H	Country	Habitat	D-FR	D-PL	Query coverage	GC%	Clade	GenBank Acc. no.
	Microsporidium FR	*Dikerogammarus villosus*	**G**	FR	FW	—	99	100	40·67	A1	GQ246188
	Microsporidium PL	*Dikerogammarus villosus*	**G**	PL	FW	99	—	100	40·90	A1	GQ258752
1	*Microsporidium* sp. *JES2002G*	*Gammarus chevreuxi*	**G**	UK	BW	91	91	100	39·97	A1	AJ438962
2	*Kabatana takedai*	*Oncorhyncus masou*	F	N Pac.	SW	77	77	97	46·61	A2	AF356222
3	*Spraguea lophii*	*Lophius americanus*	F	USA	SW	79	79	99	49·26	A2	AF033197
4	*Microgemma* sp.	*Taurulus bubalis*	F	UK	SW	79	79	99	49·41	A2	AJ252952
5	*Dictyocoela muelleri*	*Gammarus roeselii*	**G**	FR	FW	80	80	87	48·16	A3	AJ438956
6	*Dictyocoela duebenum*	*Gammarus duebeni duebeni*	**G**	UK	BW	81	81	87	48·22	A3	AF397404
7	*Dictyocoela berillonum*	*Echinogammarus berilloni*	**G**	FR	FW	80	80	87	48·75	A3	AJ438957
8	*Dictyocoela cavimanum*	*Orchestia cavimana*	**G**	UK	TSW	79	79	87	50·11	A3	AJ438960
9	*Dictyocoela deshayesum*	*Talorchestia deshayesei*	**G**	FR	TSW	79	79	87	51·51	A3	AJ438961
10	*Pleistophora* sp. *3*	*Taurulus bubalis*	F	N	SW	79	79	87	52·10	A4	AF044390
11	*Pleistophora typicalis*	*Myoxocephalus scorpius*	F	NO?	SW	78	78	87	52·76	A4	AF044387
12	*Pleistophora mulleri*	*Gammarus duebeni celticus*	**G**	IR	FW	78	78	87	52·82	A4	AJ438985
13	*Pleistophora hippoglossoideos*	*Hippoglossoides platessoide*	F	NO?	SW	78	78	87	52·70	A4	AJ252953
14	*Nosema granulosis*	*Gammarus duebeni*	**G**	UK, Scot	SW	70	70	76	33·22	B	AJ011833
15	*Nosema furnacalis*	*Ostrinia furnacalis*	I	USA	T	70	70	78	33·93	B	U26532
16	*Nosema bombycis*	*Bombyx mori*	I	China	T	67	67	86	34·09	B	EU864525
17	*Nosema tyriae*	*Tyria jacobaeae*	I	UK	T	67	67	86	34·39	B	AJ012606
18	*Nosema apis*	*Apis mellifera*	I	USA	T	65	65	86	38·65	B	U26534
19	*Nosema necatrix*	*Apis cerana*	I	USA	T	66	66	86	37·05	B	U11051
20	*Vairimorpha necatrix*	*Pseudaletia unipuncta*	I	UK	T	66	66	86	37·05	B	DQ996241
21	*Microsporidium* sp. *JES2002I*	*Gammarus pulex*	**G**	UK	FW	69	69	68	44·19	B	AJ438964
22	*Microsporidium* sp. *JES2002H*	*Talorchestia deshayesei*	**G**	FR	SWT	64	64	54	36·62	—	AJ438963
23	*Visvesvaria algerae*	*Culicidae*	I	USA?	FW-T	67	67	79	46·49	C	AF024656
24	*Anncaliia algerae*	*Anopheles stephensi*	I	?	FW-T	66	67	86	46·73	C	AF069063
25	*Thelohania solenopsae*	*Solenopsis invicta*	I	USA	T	69	69	79	41·14	C	AF134205
26	*Janacekia debaisieuxi*	*Simulium* sp.	I	UK	FW-T	70	70	87	38·96	D	AJ252950
27	*Trichonosema pectinatellae*	*Pectinatella magnifica*	B	USA	FW	70	70	80	47·54	D	AF484695
28	*Pseudonosema cristatellae*	*Cristatella mucedo*	B	Europe	FW	71	70	73	45·82	D	AF484694
29	*Bryonosema plumatellae*	*Plumatella nitens*	B	USA	FW	70	70	87	48·51	D	AF484692
30	*Heterococcus pleurococcoides*	Free-living		Antarctica	T	87	87	19	48·21	Out	AJ579335
31	*Basidiobolus ranarum*	Free-living		?	T	87	87	23	46·79	Out	D29946
32	*Conidiobolus coronatus*	Free-living		Tropics	T	70	70	41	44·18	Out	AF296753

muscle tissues and ovaries to detect if parasites preferentially infect ovaries, as previously described for vertically-transmitted microsporidia in other gammarid species (Haine *et al.* 2004; Slothouber-Galbreath *et al.* 2004). In females with embryos laid in the brood pouch, we also extracted DNA from the whole brood to test if parasites could be present in the clutch.

Transmission experiment

Seven infected individuals of *D. villosus* were collected from the Zegrzynski Reservoir on the Bug River in October 2008. Their infection status was confirmed by examination of muscles for the presence of spores with a light microscope. The infected tissues were stored in a refrigerator at $+4\,^{\circ}C$ until the moment of experimental infection. One hundred individuals of *D. villosus* were collected from the Bug River in November 2008. Animals were maintained in the laboratory for 120 days to exclude any naturally-infected individuals from experiments. The infection status was checked by examination under the light stereoscopic microscope. During that time, gammarids were fed with dry forage (Hikari Tropical Algae Wafers). Forty uninfected individuals were then selected for the experimental infection experiment, and starved for 72 h. Pieces of the body of the 7 infected individuals were then provided as the only source of food for 3 days. After this exposure period, animals were fed with dry fish food pellets for 60 days. All living gammarids were then killed and dissected under a light microscope for examination of microsporidian infection. Control treatment consisted of 40 uninfected animals which were fed with fish food instead of infected tissues during the 3 days of the exposure period.

RESULTS

Macroscopical and light microscopical observations

Some whitish stripes of infected abdominal musculature were macroscopically noticed during observations of infected hosts. Heavy infection causes white colouration of muscles, easily visible through the semi-transparent cuticle (Fig. 1A). In semi-thin sections, the infected fragments of muscle tissue were filled with numerous parasite spores and developmental stages (Fig. 1B). At the later stages of infection, microsporidia could also be found in adipose tissues, gonads and within haemocytes, but were not observed in the gut wall. The free spores in fresh smears were easily recognized as belonging to the phylum Microspora (Fig. 1C). The developmental stages identified in Giemsa-stained smears were diplokaryotic merozoites and sporonts, tetranucleate plasmodia and elongate sporoblasts (Fig. 1E–I). All developmental stages possessed nuclei in diplokaryotic arrangement. Diplokaryotic merozoites were rounded measuring approximately $5 \times 6\,\mu m$ in size (Fig. 1E). Multiplication was by binary division of diplokaryotic cells (Fig. 1F, H). Rounded diplokaryotic merogonial stages occurred in chains (Fig. 1D, I). The spores were elongate (cucumiform), sometime slightly curved. The size of live spores was $3{\cdot}82 \pm 0{\cdot}02 \times 2{\cdot}21 \pm 0{\cdot}01\,\mu m$ ($n = 531$) and size of Giemsa-stained spores was $3{\cdot}88 \pm 0{\cdot}02 \times 2{\cdot}03 \pm 0{\cdot}01\,\mu m$ ($n = 493$).

TEM observations

Description of the developmental stages. Parasites developed in direct contact with the sarcoplasm of muscle cells. The earliest observed stages were rounded merozoites (Fig. 2A). These possessed 2 nuclei in diplokaryotic arrangement, each nucleus measuring $1{\cdot}5$–$2{\cdot}2\,\mu m$ in diameter. In the region of apposition between the nuclei, the nuclear membranes were markedly electron dense (Fig. 2A–D). The cytoplasm of the early merogonial stages was homogeneously granular (Fig. 2A). Lamellar endoplasmic reticulum was seen in the cytoplasm of late merozoites (Fig. 2B). Most of the merogonial stages occurred in chains (Fig. 2C).

The beginning of sporogony was marked by the structural transformation of the cell wall (Fig. 2D). During late merogonial stages, thickening of the cell surface was accompanied by the appearance of electron-dense granules adhering to the plasmalemma, eventually covering the sporont surface to a depth of about 40 nm (Fig. 3A). The appearance of the centriolar plaque (cp) at the nuclear periphery coincided with the release of early sporonts (Fig. 2D). Few membrane profiles and vacuoles were visible inside of the cytoplasm of the sporont (Fig. 3A). Early diplokaryotic sporonts were rounded to broadly oval in shape (Fig. 2D) while late sporogonial stages were elongated (Fig. 3A). The central diplokaryon with electron-dense nucleoles occupied most of the volume of the sporont (Fig. 3A).

Each sporont gives rise by binary fission to 2 diplokaryotic sporoblasts. Sporoblasts and spores lying pairwise were frequently observed on slides and ultrathin sections (Fig. 3C). The cytoplasm of each sporoblast contained 2 nuclei (nu), cisternae of the endoplasmic reticulum, numerous ribosomes and the extrusion apparatus primordia (Fig. 3B, C). The wall of the future spore consists of an electron-transparent endospore about 30 nm thick and a uniform exospore (Fig. 3B). The anchoring disc was initiated as a rounded area, connected to the manubrial part of polar filament at the anterior pole of young spore (Fig. 4A). During spore development, the anchoring disc was transformed to an umbrella-like construction closely connected to the polar filament via a narrow pad-like structure covering the apical part of manubrium (Fig. 4B).

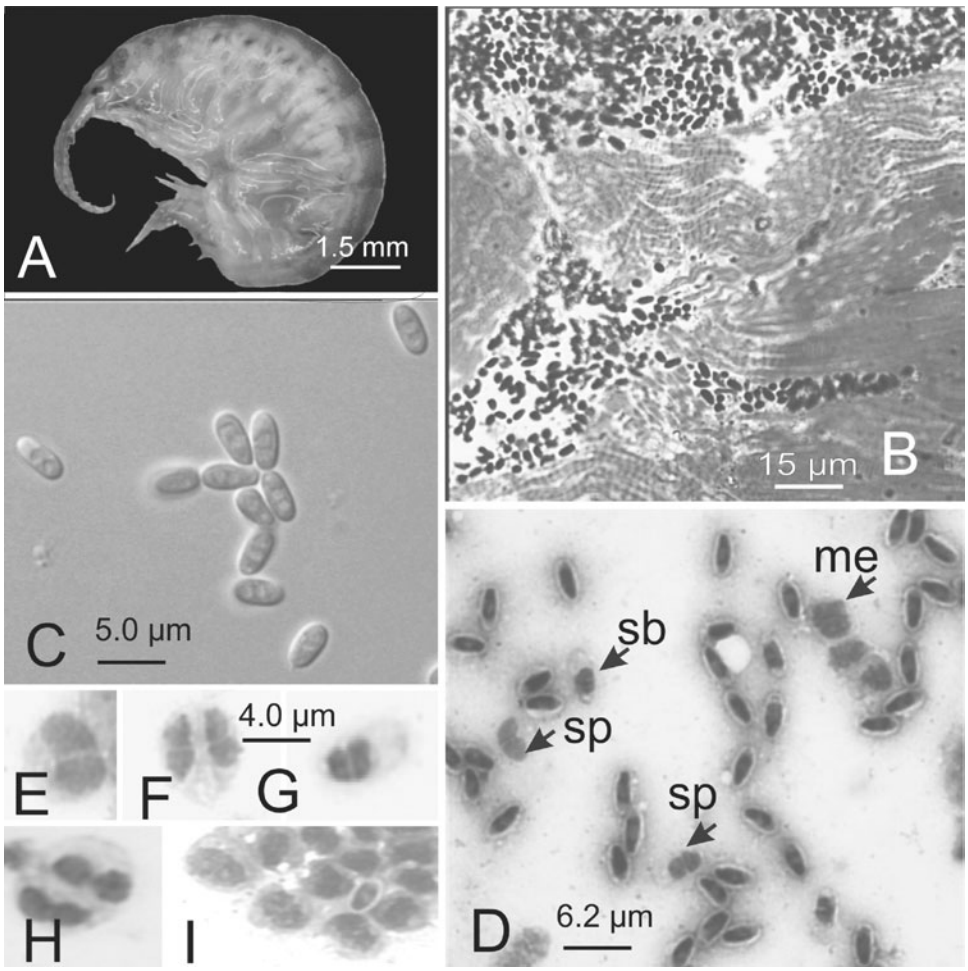

Fig. 1. (A–I) Light micrographs of the microsporidium *Cucumispora dikerogammari* n. gen., n. sp. (A) Heavily infected host (*Dikerogammarus villosus*). Pale-coloured musculature indicates microsporidian infection. (B) Semi-thin section of infected muscle blocks filled with numerous spores and developmental stages. (C) Live spores in fresh smears (Nomarski contrast). (D) Methanol-fixed and Giemsa-stained smears. Diplokaryotic meronts (me) in chain-like disposition, sporont (sp), sporoblasts (sb) and separate spores are visible. (E–I) Giemsa-stained developmental stages: meront (E), merogonial plasmodium with 2 diplokaryons (F), sporont (G), sporogonal plasmodium (H) and groups of meronts (I) are shown.

Spore ultrastructure. The mature spores were visible separately or in irregular groups in the muscle tissue. The episporal space was surrounded by a lucent area, outside of the developing sporophorous vacuole wall (Fig. 4C). The spore envelope was composed of the plasmalemma, a 60–80 nm thick electron-transparent endospore and a 20–25 nm wide electron-dense uniform exospore (Fig. 4D, E). The endospore was greatly thinned over the anchoring disc (Fig. 4F, G). In the anchoring disc, strata of varying electron density were seen (Fig. 4G). The anchoring disc was umbrella shaped, with a diameter of 850–900 nm; and a width of 15–30 nm, covering approximately 80% of the anterior polaroplast (Fig. 4F).

The relatively short polaroplast was composed of 2 distinct lamellar parts (Fig. 4F, G). In the anterior zone, the thinnest lamellae were tightly packed within a 100–120 nm wide umbrella-shaped structure measuring 900–1000 nm in diameter (Fig. 4F). The posterior polaroplast was composed of wide lamellae closely packed in a rhomboidal structure, and exhibited a tubular structure filled with an electron-dense substance in transverse section (Fig. 4F).

In longitudinal section, the anterior part of the polar filament (manubrium) measured about 120 nm in diameter and displayed a dense anterior core, surrounded by a structureless zone covered by the plasmalemma (Fig. 4G). The isofilar polar filament was arranged into 6–8 coils in a single layer close to the spore wall (Fig. 4D, E), with a diameter of approximately 100 nm. The angles of tilt of the 3 anterior coils were different from those of the 4–5 posterior coils.

The posterior vacuole was relatively small (not more than 1/6 of spore volume). The voluminous diplokaryon occupied the spore space between the polaroplast and the posterior vacuole and polar filament coils surrounded the nuclei (Fig. 4D). Each nucleus measured about 800 nm in diameter. The

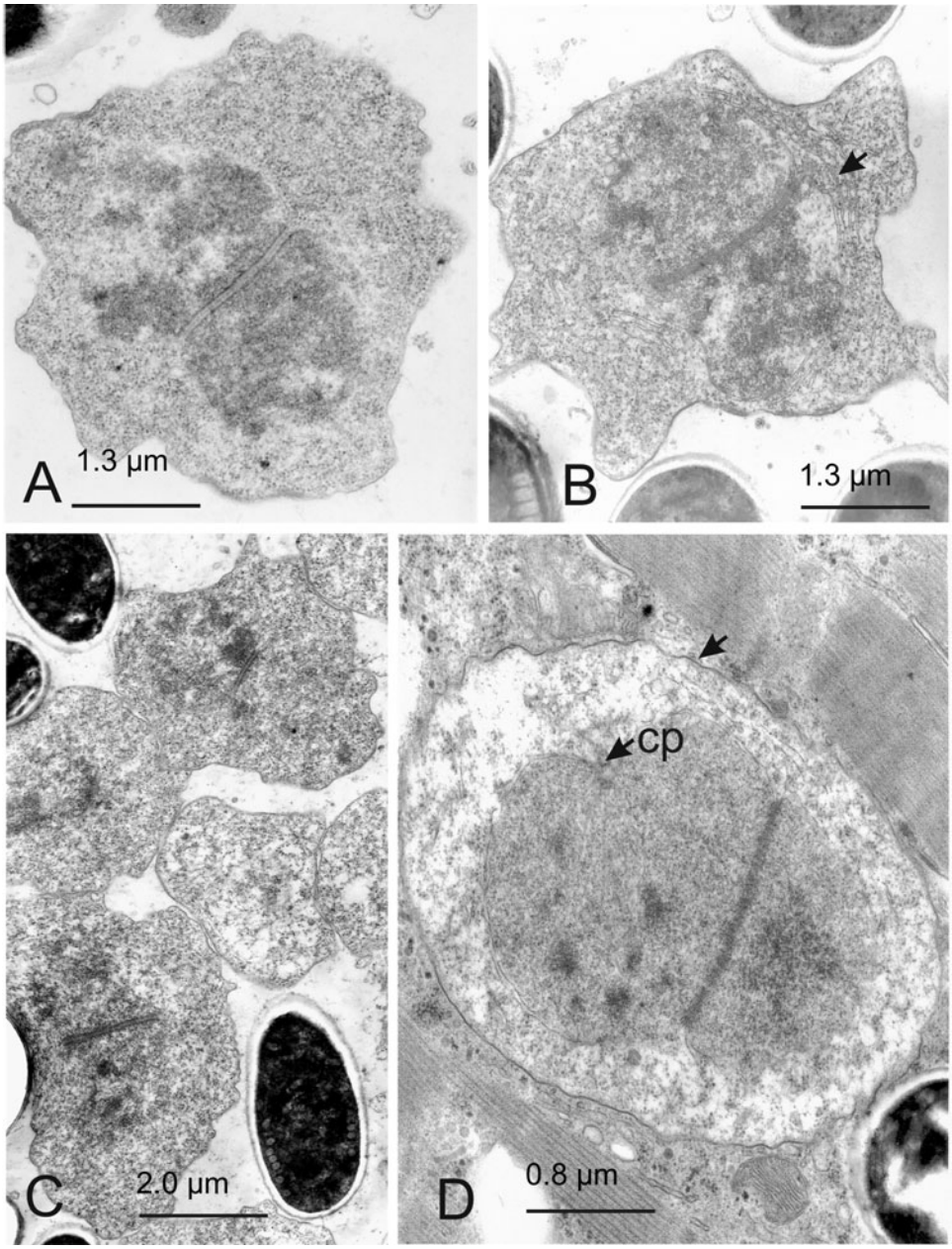

Fig. 2. (A–D) Transmission electron micrographs of early developmental stages. (A) Diplokaryotic meront of first generation with cytoplasm containing free ribosomes. (B) Meront of second generation with endoplasmic reticulum inside of cytoplasm (arrowed). (C) Group of meronts presents chain-like structure. (D) Ultrastructure of young sporont showing thickening of plasma membrane (arrowed). Epinuclear plate (ep) and dark clusters of chromatin indicate beginning of nuclear division.

cytoplasm was dense and rich in ribosomes, with traces of rough endoplasmic reticulum visible on ultrathin sections (Fig. 4D–F).

Phylogenetic analysis of SSU rDNA sequences

The amplified region of SSU rDNA was 1318 bp long. We identified 2 haplotypes differing by 4 nucleotides: 1 found in the Polish samples and 1 in the French sample (GeneBank Accession numbers GQ258752 and GQ246188, respectively). These sequences were aligned with partial sequences of SSU rDNA of *Microsporidium* sp. *D* infecting *D. villosus* (Wattier *et al*. 2007) available in GenBank and they showed 99·7% identity, which indicates the same parasite species. A BLAST search showed that our sequences have 91% identity with an undescribed parasite of *Gammarus chevreuxi*, named provisionally *Microsporidium* sp. *JES2002G* (Table 2). In contrast, our novel sequences showed a low level of identity (67%) with *Nosema bombycis*, the type species of the genus *Nosema*. The analysis of GC content confirmed this tendency (Table 2). The GC content is similar among 2 haplotypes of *Microsporidium* sp. *D*. and *Microsporidium* sp. *JES2002G* and differs with values observed in the case of *Nosema* spp.

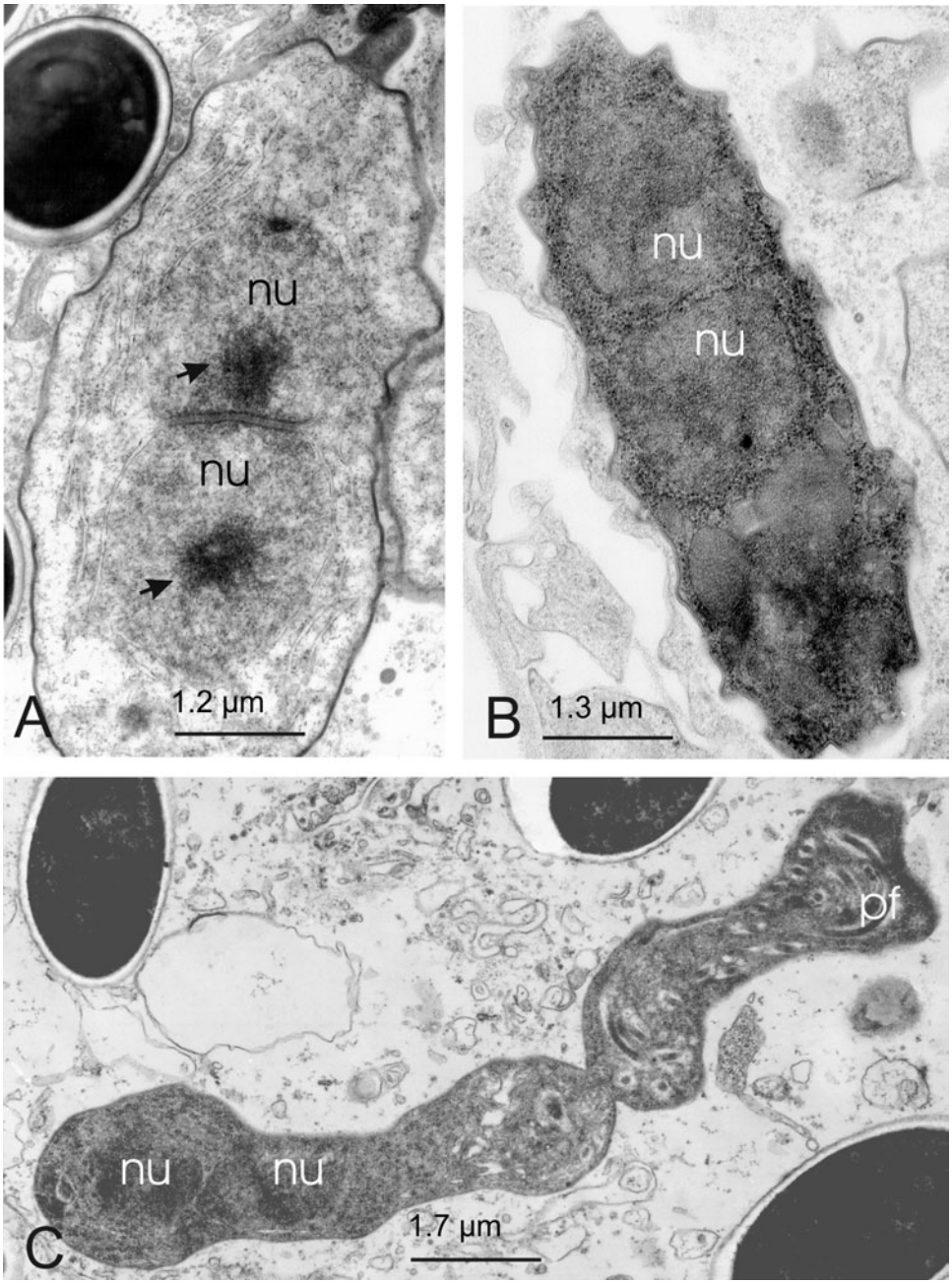

Fig. 3. (A–C) Ultrastructure of sporogonial stages. (A) Elongate sporont with nuclei (nu) and electron-dense nucleoli (arrowed). (B) Sporoblast with 2 nuclei (nu) and cytoplasm filled with numerous ribosomes and vacuolar structures indicating extrusion apparatus development. (C) Divided late sporoblast with developing polar filament coils (pf).

A phylogenetic tree constructed with Bayesian inference analysis grouped the studied microsporidia with *Microsporidium* sp. *JES2002G* within 1 clade (A1) (Fig. 5). Outside this clade, the closest relatives of the *D. villosus* parasites were *Kabatana takedai*, *Spraguea lophii*, *Microgemma* sp., and also the gammarid microsporidia *Dictyocoela* spp. and *Pleistophora* spp. in the clade A. Moreover, all of the microsporidian sequences previously ascribed to genus *Nosema* (*Anncaliia algarae*) or displaying *Nosema*-like morphology (*P. cristatellae*, *T. pectinatellae*, *B. plumatellae*) occurred in the separate clades C and D, very distant from the novel sequences of the *D. villosus* parasites. The maximum likelihood tree showed similar typology, with high support for the 2 new sequences being outside the *Nosema* clade (bootstrap value 92) and put the novel sequences together with *Microsporidium* sp. *JES2002G* on a separate branch (bootstrap value 100) within clade A outside clades A2, A3 and A4.

Prevalence of microsporidia in the host

Molecular screening for the parasite presence by PCR-RFLP showed that 50·0% of *D. villosus* individuals ($n=140$) were infected with *Microsporidium* sp. *D.* No other RFLP pattern except the one typical for *Microsporidium* sp. *D.* was observed. Among the

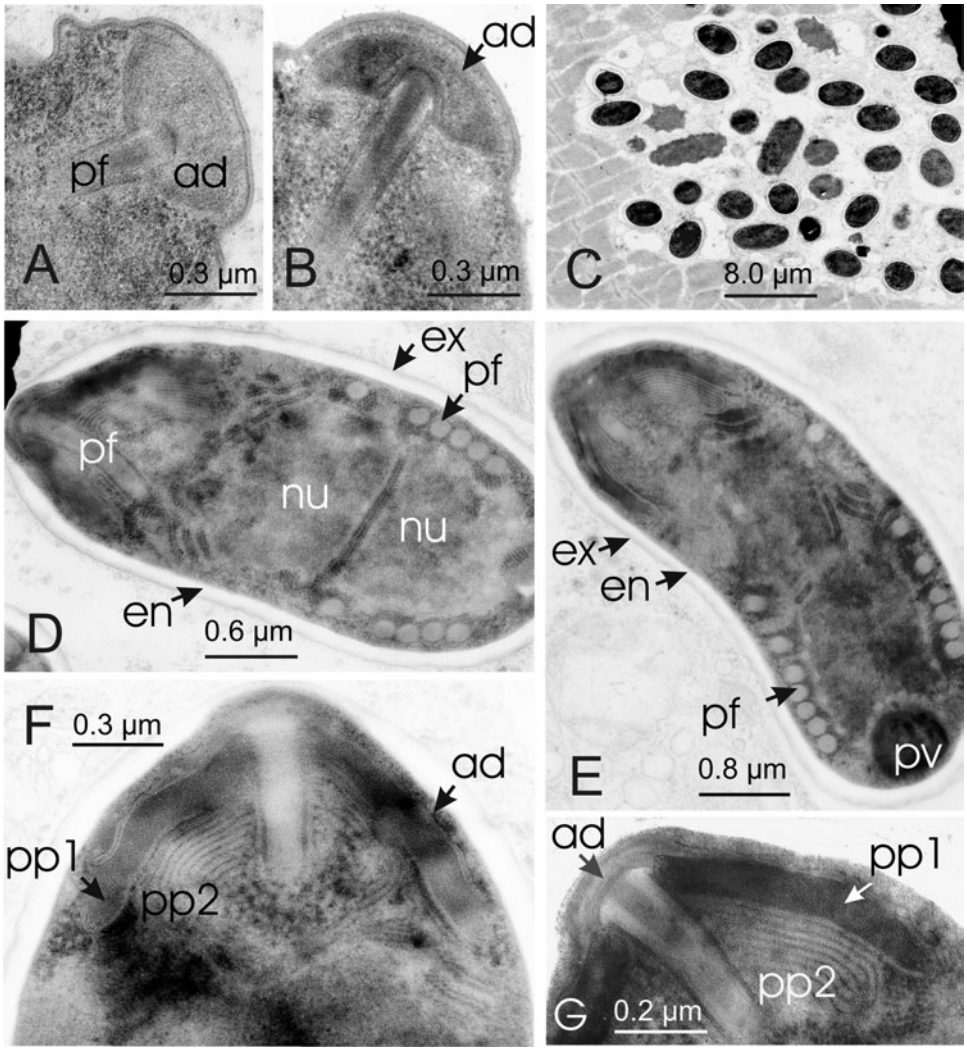

Fig. 4. (A–G) Ultrastructure of the spores. (A–B) Longitudinal section of developing anchoring apparatus (ad) and manubrial part of polar filament (pf). (C) Ultra-thin section of infected muscle block containing numerous late sporoblasts and electron-dense spores. (D–G) Ultrastructure of mature spores showing large nuclei (nu), polar filament (pf), bipartite polaroplast (pp1, pp2) and well-developed anchoring disc (ad).

72 individuals tested positive by the molecular screen, only 21 (29·2%) were symptomatic, probably meaning that symptoms appear late within the host. Taking all animals into account, i.e. adults ($n=127$) and juveniles ($n=13$), the probability of being infected increased with the size of the host (Logistic regression: $\chi^2=9{\cdot}76$, D.F. $=1$, $P=0{\cdot}002$) (Fig. 6). Among adult hosts, the probability of being infected was not significantly different between males (57·5%, $n=73$) and females (48·3%, $n=58$) (2-tail Fisher's Exact test, $P=0{\cdot}38$). Finally, among the 27 infected females where it was measured, 26 (92·6%) were infected in muscles or in both muscles and ovaries, while only 1 was infected in ovaries only. We detected infection in 6 clutches out of 14 breeding infected females.

Experimental transmission

Twenty nine *D. villosus* out of 40 individuals fed with infected tissue survived until the end of the experiment. All of them demonstrated clearly visible symptoms of infection. Light microscopic observations confirmed the presence of numerous spores and other developmental stages of microsporidians inside the somatic tissues of the host. None of the controls displayed any evidence of infection.

DISCUSSION

The taxonomy of *Nosema*-like species parasitizing amphipods has not been fully defined. Classification of *Nosema*-like microsporidia using morphological data is very difficult because the description of genus *Nosema* is practically devoid of diagnostic characters. This genus has been used to collect species with oval or elongate diplokaryotic spores and with development in direct contact with host cell cytoplasm (Sato *et al*. 1982; Larsson, 1999). However, recent studies using electron microscopic and molecular techniques have revealed that many microsporidian taxa initially included within the genus *Nosema* are

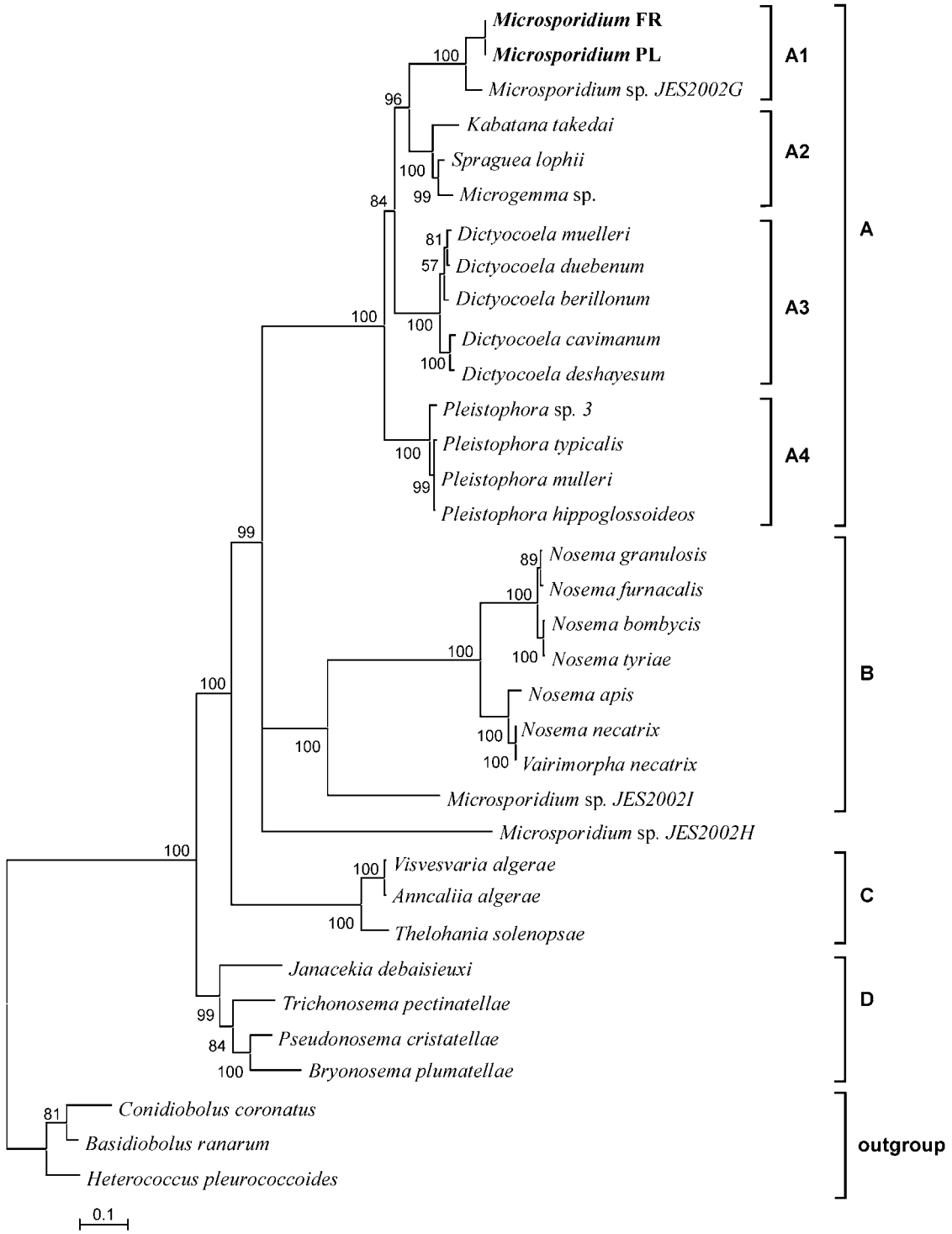

Fig. 5. Microsporidian phylogenetic tree based on a 1046 bp region of SSU rDNA. The 2 haplotypes: Polish and French infecting *Dikerogammarus villosus* are highlighted in bold.

phylogenetically distant from the type species *Nosema bombycis* and hence should be ascribed to other genera (Silveira and Canning, 1995; Müller *et al*. 2000; Canning *et al*. 2002; Slamovits *et al*. 2004; Franzen *et al*. 2005, 2006; Sokolova *et al*. 2005).

Seven named and 2 unnamed *Nosema* species have been reported to date in amphipods of Boreal-Atlantic, Ponto-Caspian and Mediterranean origins (Spraque, 1977; Larsson, 1983; Ovcharenko and Kurandina, 1987; Terry *et al*. 1999). However, only *Nosema granulosis* was completely described, including phylogenetic analysis of the small submit ribosomal RNA gene sequences (Terry *et al*. 1999). Morphological and molecular study of the *Nosema* sp. infecting the North American amphipod *Crangonyx pseudogracilis* gave ground to description of a novel genus *Fibrillanosema* (Slothouber-Galbreath *et al*. 2004).

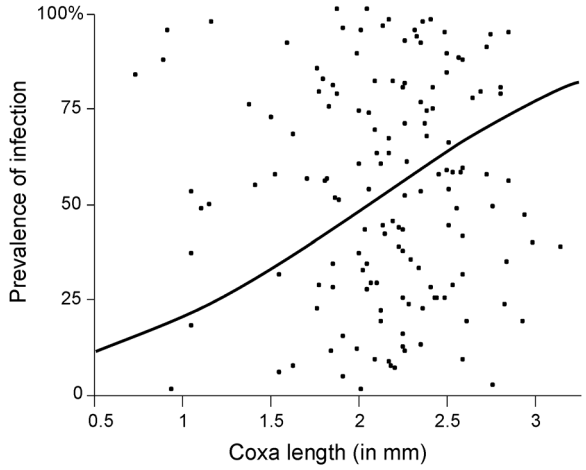

Fig. 6. Size-dependent probability of infection in *Dikerogammarus villosus* by *Cucumispora dikerogammari*.

In the present study we show that the previously described *Nosema dikerogammari* and *Microsporidium* sp. *D* are definitely a single parasite species. Morphological analysis (light and electron microscopy) of *D. villosus* parasites from Poland allowed microsporidia to be identified as the previously described *Nosema dikerogammari* (Ovcharenko and Vita, 1996). However, molecular phylogenetic analysis of parasites from the same individual hosts revealed that this microsporidian species could not be included in the genus *Nosema sensu stricto*. Second, SSU rDNA sequences obtained for parasites infecting *D. villosus* from France (*Microsporidium* sp. *D*) and from Poland have 99·7% identity, sufficiently high to indicate that they are unlikely to represent 2 different species. These parasites were grouped within a clade closely related to *Pleistophora* parasites (parasites infecting fish and amphipod) and *Dictyocoela* parasites (infecting amphipods only). Ultrastructural analysis of spores indicated several details that can be used as diagnostic features to differentiate the microsporidium infecting *D. villosus* from other *Nosema*-like parasites. These are elongate (cucumiform) spores, sometimes slightly curved; short polaroplast covered with a well-developed anchoring disk; relatively short polar filament turned into several coils with various tilt of anterior turns; thin spore wall and relatively small posterior vacuole, containing posterosome.

We therefore propose to create a new genus *Cucumispora* for this parasite. Since it was discovered in *Dikerogammarus villosus*, we propose to call this microsporidium *Cucumispora dikerogammari*. Given that the undescribed parasite *Microsporidium* sp. *G* infecting *Gammarus chevreuxi* which has a closely-related SSU rDNA sequence should also be placed in *Cucumispora*. However, in the absence of ultrastructural data, an adequate taxonomic description of this parasite is not yet possible.

Wattier *et al*. (2007) who studied French individuals of *D. villosus* infected with *Cucumispora dikerogammari* showed that male and female hosts had an equal probability of being infected. Our data obtained for the Polish population confirmed these results. In females, infection was equally likely in somatic tissues and gonads, and only 1 female was infected in the ovaries only. In addition, symptoms were obvious in muscles, where a rough estimate of up to a million spores could be seen in most infected individuals. These features contrast with those of asymptomatic, vertically-transmitted sex ratio distorting microsporidia found in other amphipod species, where infection is limited almost entirely to the ovaries of females (Bulnheim and Vávra, 1968; Haine *et al*. 2004, 2007; Mautner *et al*. 2007). However, the presence of parasites in embryos showed that vertical transmission occurs, but since our molecular investigation was made on entire broods, we cannot assess the precise rate of this vertical transmission. The whole brood can be infected, but a single embryo per brood can be infected as well.

In addition, we showed that an efficient horizontal transmission mode of *C. dikerogammari* can occur through consumption of infected tissue, a mode of transmission similar to that of *Pleistophora mulleri* infecting *Gammarus duebeni celticus* (MacNeil *et al*. 2003). The importance of this mode of transmission is emphasized by our observation that hosts of large size have a higher probability of being infected than hosts of small size. It is known that individuals of *D. villosus* are cannibalistic predators (Kinzler and Maier, 2003; Kinzler *et al*. 2009) and their main source of infection may therefore be preying upon infected conspecifics. All gammaridean species are known to be omnivorous, often necrophagous and predacious (Kelly *et al*. 2002). This makes the microsporidium *C. dikerogammari* a potential danger for other gammaridean species which co-occur with *D. villosus*, including the native species in invaded areas. On the other hand, the parasite may function as a factor controlling the population dynamics of its invasive host (Henry and Oma, 1981; Sweeney and Becnel, 1991). Further ecological studies on this parasite are therefore necessary to understand whether *C. dikerogammari* should be considered as an emerging disease in Central and Western European rivers.

Description of the genus

Name: *Cucumispora* n. gen.
Diagnosis: In all developmental stages the nuclei are diplokaryotic and develop in direct contact with the host cell cytoplasm. Merogonic and sporogonic stages divide by binary fission. Each sporont produces 2 elongate sporoblasts which develop into 2 elongate spores with thin spore walls, uniform

exospores and isofilar polar filaments arranged in 6–8 coils. The angle of the anterior 3 coils differs from that of subsequent coils. A thin, umbrella-shaped, anchoring disc covers the anterior region of the polaroplast, which has 2 distinct lamellar regions, occupying approximately one fourth of the spore volume. The parasite infects gammaridean hosts and infects primarily muscle tissue but can also occur in other tissues.

Description of the species

Name: *Cucumispora dikerogammari* (Ovcharenko and Kurandina, 1987).
Synonym: *Nosema dikerogammari* Ovcharenko and Kurandina, 1987
GenBank Accession numbers: GQ246188, GQ258752
Type host: *Dikerogammarus villosus* (Sowinsky, 1894)
Type locality: Estuarine region of Dnieper River (Ukraine)
Other localities: Dnieper's Reservoirs (Ukraine), Danube River, the Vistula, the Bug, the Oder Rivers; Rhine, Rhône, Seine, Loire, Saone Rivers.
Location in the host: Sarcoplasm of muscle cells (primary site); somatic and adipose tissues, haemolymph and gonads.
Type specimens: Not available. The re-description based on material collected in the Oder River (N51°14′54·79″; E16°50′47·61″) and in the Zegrzynski Reservoir on the Bug River, Poland (N52°27′30·79″; E21°01′04·52″). The paratype slides containing mature free spores were deposited in Protozoan Type Slide Collection at Smithsonian Institution Washington, DC 20560, USA. Other slides were deposited at the Laboratory of the corresponding author at the Institute of Parasitology of the Polish Academy of Sciences, Warszawa 00-818, Poland.
Etymology: *Cucumispora* after the spore shape, *dikerogammari* after the host genus.
Diagnosis: Like the genus. The spore sizes averaged $3{\cdot}82\ (2{\cdot}50-5{\cdot}03)\ \pm\ 0{\cdot}48\times2{\cdot}21\ (1{\cdot}32-3{\cdot}07)\ \pm\ 0{\cdot}33\ \mu$m. Giemsa stained spores measured $3{\cdot}74\ (2{\cdot}50-4{\cdot}78)\ \pm0{\cdot}39\times1{\cdot}91\ (1{\cdot}21-2{\cdot}47)\pm0{\cdot}24\ \mu$m.
Transmission: Horizontal, per os. Transovarial transmission cannot be dismissed because some clutches were infected, but more studies are necessary to confirm the significance of this route of transmission.

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