Small subunit ribosomal DNA phylogeny of microsporidia that infect *Daphnia* (Crustacea: Cladocera)

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SUMMARY

Glugoides intestinalis, *Microsporidium* sp., *Ordospora colligata*, *Gurleya vavrai*, *Larssonia obtusa* and *Flabelliforma magnivora* are microsporidian parasites of planctonic freshwater crustaceans *Daphnia* spp. We performed a phylogenetic analysis of the small subunit ribosomal DNA which revealed their positions as polyphyletic. *G. intestinalis*, *Microsporidium* sp. and *O. colligata*, which are horizontally transmitted gut parasites with small spores and low virulence, group with different lineages. *G. intestinalis* is related to 2 microsporidia infecting lepidopterans and to *Vittaforma corneae*, which has been described as a human pathogen. It is thought that *V. corneae* may have an invertebrate as its natural host. *Microsporidium* sp. is a relative of the genera *Enterocytozoon* and *Nucleospora*, pathogens of man and fish respectively. *O. colligata* is the first species found to be closely related to the genus *Encephalitozoon*, which is comprised of 3 species that are parasites of homeothermic vertebrates. *G. vavrai* and *L. obtusa* are sister taxa that branch close to the Amblyosporidae, the only microsporidia with known intermediate hosts. This finding supports the presumption of secondary hosts for *G. vavrai* and *L. obtusa*, as it has not been possible to maintain these species in *Daphnia* in the laboratory. *F. magnivora* roots deep at the base of the phylum microsporidia with no close relative found so far.

Key words: *Gurleya daphniae*, maximum likelihood, parsimony, logDet/paralinear distance.

INTRODUCTION

Microsporidia are a phylum of amitochondriate, unicellular endoparasites of other eukaryotes that are transmitted by means of infective spores (Mathis, 2000). During the last decade there was a twofold renaissance in microsporidia research, beginning with their recognition as opportunistic pathogens in HIV-infected patients which stimulated studies on their epidemiology and on possible animal reservoir hosts (Deplazes, Mathis & Weber, 2000) and followed by an interest in the origin of the phylum itself. There is growing evidence that microsporidia are not ancient eukaryotes but are closely related to fungi and may have arisen from within them (Edlind *et al*. 1996; Keeling, Luker & Palmer, 2000).

Recently, several classifications of microsporidia, which are based on morphology, have been published (Larsson, 1986; Sprague, Becnel & Hazard, 1992). Whereas they are consistent in the classification of lower level taxa, the major divisions of the phylum differ according to the characters used. The minute size and the restricted number of useful characters make microsporidia prime candidates for the development of a molecular phylogeny. Since the sequencing of the small subunit ribosomal DNA (SSUrDNA) of *Vairimorpha necatrix* by Vossbrinck *et al*. (1987), this gene has been sequenced from many species and phylogenies based on the SSUrDNA have helped to yield insight into the evolution of this phylum (Baker *et al*. 1995, 1998; Adler, Becnel & Moser, 2000; Muller *et al*. 2000). Comparison with earlier taxonomies based on ultrastructural observations has revealed several examples where morphological characters were misleading (Baker *et al*. 1995, 1998; Cheney, Lafranchi-Tristem & Canning, 2000).

The planktonic crustaceans of the genus *Daphnia* are common inhabitants of lakes and ponds and are frequently infected with microsporidia (Green, 1974; Bengtsson & Ebert, 1998). Microsporidia of *Daphnia* are diverse regarding their virulence, mode of transmission and site of infection (Ebert, Lipsitch & Mangin, 2000).

In this study we have analysed the SSUrDNA of 6 microsporidian species that parasitize *Daphnia*. Three of them share several features: *Glugoides intestinalis*, *Microsporidium* sp. and *Ordospora colligata* have very small spores ($< 3\ \mu$m) and infect the gut epithelium of their host, using a waterborne transmission route (Larsson *et al*. 1996; Larsson, Ebert & Vávra, 1997). Their virulence is low, i.e. their hosts' longevity and reproduction are barely affected (Ebert *et al*. 2000). The other 3 species have

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a high virulence. Hosts infected by *Gurleya vavrai* and *Larssonia obtusa* have strongly reduced reproduction and survival (Bengtsson & Ebert, 1998; Ebert, unpublished observation). It has not been possible to maintain these two parasites in laboratory populations of *Daphnia*, either due to unfavourable laboratory conditions or, more likely, to the requirement of a second host to complete their life-cycle (Ebert, unpublished observation). *Flabelliforma magnivora* is vertically transmitted and reduces both host fecundity and longevity. Horizontal transmission is assumed but not proven for *F. magnivora* (Mangin, Lipsitch & Ebert, 1995, in which this species is called *Tuzetia* sp.). This species is possibly misclassified as its SSUrDNA differs substantially from that of the type species *F. montana* (Canning *et al*. 2001).

MATERIALS AND METHODS

Parasite species

G. intestinalis, synonym *Pleistophora intestinalis* Chatton, 1907, parasitizes *D. magna* and *D. pulex* (Larsson *et al*. 1996). Type locality is Southern Oxfordshire, UK. Our sample was collected in Munich, Germany and identified as *G. intestinalis* using electron microscopy. *O. colligata* parasitizes *D. magna* (Larsson *et al*. 1997). Type locality is North Oxfordshire, UK. Our sample was collected in Tvärminne, Finland and identified as *O. colligata* using electron microscopy.

An undescribed microsporidium (referred to as *Microsporidium* sp.) was collected from a population of *D. pulex* in Plön, Northern Germany. Electron microscopical examination revealed no similarities to any described species (Larsson, personal communication). The exospore is thin (as in *Glugoides* and *Ordospora*). The polaroplast has 2 regions: an anterior region with closely packed lamellae and a posterior region with distinct wide lamellae. The polar filament is isofilar with at least 6 coils. Presporal stages (and hence modes of reproduction) and nuclear configurations are unknown. Spores are grouped and in close contact with the host cytoplasm. There is no sporophorous vesicle and no parasitophorous vacuole. Spores of *G. intestinalis* (in sporophorous vesicles) and *O. colligata* (in parasitophorous vacuoles of host cell origin) are by these envelopes separated from the host cytoplasm and appear to lie in empty spaces.

L. obtusa was described by Vidtman & Sokolova (1994). Type locality is a pond in the zoological garden of Kaunas, Lithuania. As they published their description in Russian, we summarize the main points: *L. obtusa* infects fat cells and haemocytes of *D. pulex*. Muscles, hypodermis and ovaries remain uninfected. All developmental stages are uninucleate with the exception of a short intermediate stage between merogony and sporogony when a diplokaryon is visible. Spore clusters contain 4–32 spores, single spores measure 4·3–4·6 × 2·6–3 μm. Bengtsson & Ebert (1998) found *L. obtusa* in rockpools at the east coast of Sweden near Uppsala where it infected *D. pulex* and *D. longispina*. Our sample was collected in Tvärminne, Finland and identified using electron microscopy.

G. vavrai infects the epidermis of *D. longispina* (Green, 1974). Type localities are Hertfordshire and Norfolk, UK. Our sample was collected in Tvärminne, Finland, where the parasite infected *D. pulex*. The description mentions only spore size and shape which renders *G. vavrai* undistinguishable from *G. daphniae* that infects *D. pulex* (Friedrich *et al*. 1996). To elucidate the relationship between those 2 species; we obtained a sample of *G. daphniae* from the type locality in Graz, Austria.

F. magnivora infects fat body, hypodermis and ovaries of *D. magna* (Larsson *et al*. 1998). A detailed description of its biology was performed by Mangin *et al*. (1995) under the preliminary name *Tuzetia* sp. and a phylogenetic analysis has been done by Canning *et al*. (2001).

Molecular biology

To prepare samples for DNA extraction, 5 *Daphnia* infected with a single species were homogenized in a microfuge tube with a minipestle. DNA was extracted with the DNeasy Tissue Kit (Qiagen, Basel, Switzerland) according to the manufacturers instructions (Proteinase K digestion for 2 h and final elution in 200 μl of Buffer AE). Samples were stored at −20 °C. Amplification of microsporidian DNA was carried out in a 50 μl reaction mix that contained PCR buffer (Sigma, Buchs, Switzerland), 200 mM each dNTP, 1 mM each primer and 1 μl template. Amplification was carried out in a PTC-200 DNA Engine (MJ Research, Waltham MA, USA). After an incubation step of 5 min at 94 °C, 1·25 units of *Taq* DNA Polymerase (Sigma, Buchs, Switzerland) were added and 40 cycles were performed (denaturation for 30 s at 94 °C, annealing for 30 s at 54–60 °C (depending on primers used), elongation for 1–3 min (depending on expected fragment length) at 72 °C). Primers VI (caccaggttgattctgccctgac) (Zhu *et al*. 1993) and HG4r (ggtccgtgtttcaagacggg) (Gatehouse & Malone, 1998) were used to amplify SSU, ITS and partial LSU of *G. daphniae*, *G. vavrai*, *L. obtusa* and *O. colligata*. Primers 18f (caccaggttgattctgcc) and 1537r (ttatgatcctgctaatggttc) (Vossbrinck *et al*. 1993) were used to amplify the SSU of *Microsporidium* sp.

PCR products were cloned with the TOPO TA Cloning Kit (Invitrogen, Groningen, Netherlands). Colonies were selected by blue/white-screening and plasmids were isolated with an alkaline lysis miniprep (Sambrook, Fritsch & Maniatis, 1989). Plasmids were digested with *Eco*RI to check whether

Table 1. Species list of SSUrDNA sequences included in the phylogenetic analysis, host from which they were obtained and GenBank accession number

(*Gurleya daphniae* has been sequenced but not included in the phylogenetic analysis as its sequence is identical to *G. vavrai*.)

Organism	Host*	Accession number
(A) Microsporidian sequences from GenBank		
Amblyospora californica	*Culex tarsalis* (I)	U68473
Amblyospora sp.	*Simulium* sp. (I)	AJ252949
Ameson michaelis	*Callinectes sapidus* (C)	L15741
Culicosporella lunata	*Culex pilosus* (I)	AF027683
Edhazardia aedis	*Aedes aegypti* (I)	AF027684
Encephalitozoon cuniculi	*Homo sapiens*	L17072
Encephalitozoon hellem	*Homo sapiens*	AF118142
Encephalitozoon intestinalis	*Homo sapiens*	L39113
Enterocytozoon bieneusi	*Homo sapiens*	AF024657
Endoreticulatus schubergi	*Lymantria dispar* (I)	L39109
Flabelliforma magnivora	*Daphnia magna* (C)	AJ302319
Glugea atherinae	*Atherina presbyter* (fish)	U15987
Nosema apis	*Apis mellifera* (I)	U97150
Nosema bombycis	*Bombyx mori* (I)	L39111
Nosema granulosis	*Gammarus duebeni* (C)	AJ011833
Nucleospora salmonis	salmonid fish	U10883
Nucleospora sp.	*Pleuronectes vetulus* (fish)	AF186007
Parathelohania anophelis	*Anopheles quadrimaculatus* (I)	AF027682
Perezia nelsoni	*Litopenaeus setiferus* (C)	AJ252959
Spraguea lophii	*Lophius americanus* (fish)	AF033197
Trachipleistophora hominis	*Homo sapiens*	AJ002605
Tuzetia sp.	*Farfantepenaeus aztecus* (C)	AJ252958
Vairimorpha necatrix	*Malacosoma americanum* (I)	Y00266
Vairimorpha sp.	*Solenopsis richteri* (I)	AF031539
Vavraia oncoperae	*Weiseana* sp. (I)	X74112
Vittaforma corneae	*Homo sapiens*	U11046
(B) New sequences of microsporidia		
Glugoides intestinalis	*D. magna*, *D. pulex* (C)	AF394525
Gurleya daphniae	*D. pulex* (C)	AF439320
Gurleya vavrai	*D. longispina* (C)	AF394526
Larssonia obtusa	*D. pulex* (C)	AF394527
Microsporidium sp.	*D. pulex* (C)	AF394528
Ordospora colligata	*D. magna* (C)	AF394529
(C) Other species from GenBank		
Basidiobolus ranarum (Fungi: Zygomycetes)		D29946
Giardia intestinalis (Diplomonadida)		M54878
Tritrichomonas foetus (Parabasalidea)		M81842

* C = crustacean; I = insect.

they contained the desired insert and purified with the NucleoSpin Extract Kit (Macherey-Nagel, Oensingen, Switzerland). Sequencing was done by automated means by a private company (Microsynth, Balgach, Switzerland).

Part of the SSUrDNA of *G. intestinalis* was amplified with primers MicU1 (tgattctgcctgacgtrgrygc) and MicU9r (rrcathktttactgcwrgaacta) that were designed based on an alignment of microsporidian SSUrDNA. Based on the sequence obtained, additional primers were designed and an inverse PCR was performed to amplify DNA adjacent to known sequence: genomic DNA was digested with 5 μl of *Pst*I (overnight at 37 °C and 20 min at 65 °C) and religated (2 μl of T4 Ligase, 2 h at RT). DNA was purified and digested with 1 μl of *Ear*I (1 h at 37 °C). Ten μl of the reaction were used to perform a PCR with the Expand Long Template PCR System (Roche Molecular Biochemicals, Rotkreuz, Switzerland). The amplicon was checked on a 1·5 % agarose gel and sequenced directly. With the obtained sequence data new primers were designed to check whether the results could be repeated.

Phylogenetic analyses

Sequences of the SSUrDNA of 26 microsporidia and of *Basidiobolus ranarum*, *Giardia intestinalis* and *Tritrichomonas foetus* were obtained from Genbank (Table 1). Sequences AJ252959 and AJ252958 were thought to be from species of *Pleistophora* when the DNA from isolated spores was processed and sequenced. Subsequent electron microscopy re-

Table 2. Length and G/C content of the SSUrDNA of microsporidian species analysed

Microsporidian species	Length of SSUrDNA (bp)	Percentage G/C
Flabelliforma magnivora	1304	49·54
Glugoides intestinalis	1260*	46·16
Gurleya daphniae	1320*	49·32
Gurleya vavrai	1320	49·32
Larssonia obtusa	1318	53·57
Microsporidium sp.	1297	36·93
Ordospora colligata	1352	50·44

* The SSU of *G. intestinalis* and *G. daphniae* has been partially sequenced (1211 and 1292 bp resp.). As the missing 5′ ends lie in a conserved region it was possible to estimate total length confidently.

vealed them respectively to be *Perezia nelsoni* (Sprague, 1950) and a new species assigned to the genus *Tuzetia* (Canning, Curry & Overstreet, 2002). Microsporidian sequences were chosen to cover all prominent groups of the phylum, all parasites of crustaceans and important human pathogens. Sequences were automatically aligned with Clustal W (Thompson, Higgins & Gibson, 1994) and edited visually using BioEdit (Hall, 1999). Primer sites at the 3′ and 5′ ends were truncated and characters that could not be aligned unambiguously as well as characters containing gaps were removed leaving an alignment of 777 bp length. Analyses were carried out using distance, maximum likelihood and parsimony methods in PAUP* version 4.0b4a (Swofford, 2000). All searches were done heuristically with random stepwise addition (10 replicates) and TBR branch swapping. *Giardia intestinalis* (G), *Tritrichomonas foetus* (T) and *Basidiobolus ranarum* (B) were used as outgroup species and combined differently to control for their influence on tree topology (no outgroup; B; G; T; BG; BT; GT; BGT).

Maximum likelihood. A first tree was generated with the HKY85 model (transition/transversion ratio = 2). On this tree we estimated the substitution rate matrix, among-site rate variation (discrete gamma approximation with 12 categories) and proportion of invariable sites using the tree score option. Then a new search was run using the General Time Reversible model including among-site rate variation and proportion of invariable sites with the parameters estimated. On the resulting tree, parameters were again optimized and the search was repeated with the new parameters. This procedure was iterated until parameters of consecutive trees were identical. Where topologies varied in respect of the placement of our new sequences because of method and outgroup used, we compared them using SHTests v1.0 that implements Shimodaira–Hasegawa tests of phylogenetic hypotheses (Shimodaira & Hasegawa, 1999). Competing topologies were redrawn omitting branch lengths with TreeView (Page, 1996) and the test was run with a Markov General Reversible model, resampling estimated log-likelihood, 1000 bootstrap replicates and 12 categories for the discrete gamma rate heterogeneity. Values for the substitution rate matrix and among-site rate variation were taken from the corresponding maximum likelihood search.

Distance. A distance matrix was calculated using the logDet/paralinear model with the proportion of invariable sites included as estimated in the maximum likelihood search. Pairwise distances were calculated using both minimum evolution and least squares objective function. The search was bootstrapped 100 times and summarized in a 50 % majority rule consensus tree.

Parsimony. A 90 % majority rule consensus tree was generated by bootstrapping 100 times the original dataset. The consensus tree was imported into MacClade version 3.01 (Maddison & Maddison, 1997) together with the original dataset. Data were weighted using the rescaled consistency index (RC) and polytomies were treated as soft. The weighted dataset was then imported back into PAUP*. A 50 % majority rule consensus tree was generated by bootstrapping 100 times the weighted dataset, using the 90 % majority rule consensus tree as a constraint.

RESULTS

Sequences obtained for the 6 microsporidia from *Daphnia* spp. have been deposited in GenBank under the accession numbers given in Table 1. The lengths of the SSUrDNA sequences from our microsporidia were within the range for microsporidian SSUrDNA (Table 2). We did not sequence the SSUrDNA of *G. intestinalis* and *G. daphniae* at the 5′ end completely. Because the 5′ end of the SSU is conserved, we can estimate a total length of 1260 bp and 1320 bp respectively. The G/C contents were within the range of the lineages with which our species grouped. An exception was *Microsporidium* sp. which showed the lowest content of its clade (Table 2).

The primers V1/HG4r did not amplify the rDNA of *G. intestinalis*. An inverse PCR enabled us to read 1211 bp of the 3′ end of the SSUrDNA and 753 bp of the 5′ end of the ITS. The sequences of *G. vavrai* and *G. daphniae* had an identity of 99·6 %. Only the sequence of *G. vavrai* was used in the phylogenetic analysis.

Phylogenetic analysis

All methods that were applied (maximum likelihood, parsimony and logDet/paralinear distance) found trees with a similar topology. These topologies can

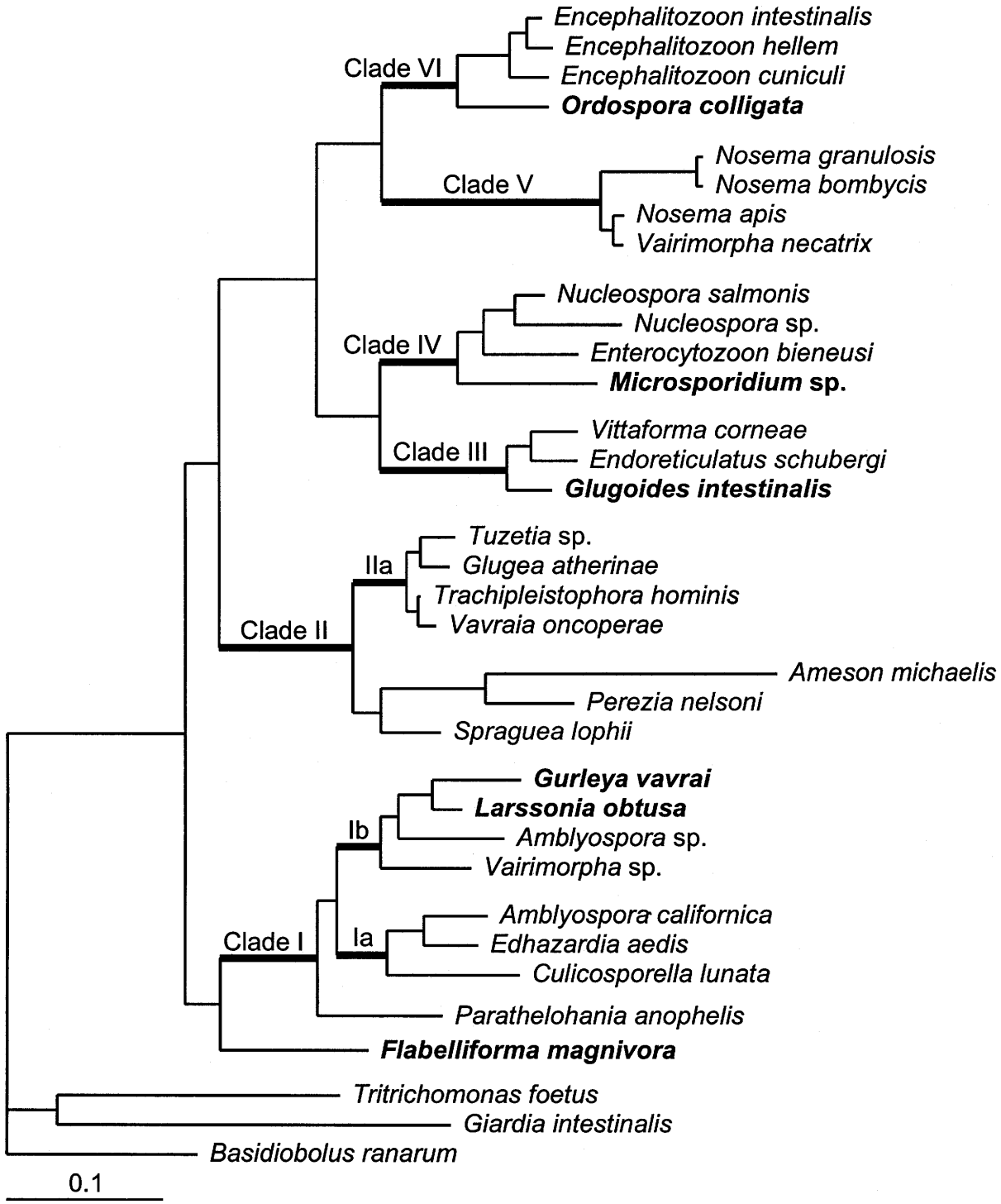

Fig. 1. Maximum likelihood phylogenetic tree of microsporidian small subunit ribosomal DNA. Parasites of *Daphnia* spp. are set in bold letters.

be subdivided into 6 clades that were consistent irrespective of method and outgroup used (Fig. 1). Parsimony and distance methods produced $\geqslant 97\%$ bootstrap support for these clades. Exceptions occurred with some outgroups where clade I had lower support when parsimony (BT: 94%; GT: 88%) or distance with minimum evolution objective function (T: 82%) were used.

The taxa *G. intestinalis*, *Microsporidium* sp. and *O. colligata* were basal in clades III, IV and VI respectively. Parsimony and distance methods gave bootstrap values $\geqslant 99\%$. All methods displayed *G. vavrai* and *L. obtusa* as sister taxa with *Amblyospora* sp. and *Vairimorpha* sp. being basal to them. Bootstrap support for the monophyly of *G. vavrai* and *L. obtusa* was $\geqslant 88\%$ (distance minimum evolution), $\geqslant 94\%$ (distance least squares) and $\geqslant 77\%$ (parsimony). The branching order of *Ameson michaelis*, *Perezia nelsoni* and *Spraguea lophii* within clade II differed with the applied methods. Maxi-

mum likelihood placed them as a sister clade to clade IIa. Distance (minimum evolution) and parsimony separated *S. lophii* and placed it basal to clade IIa (bootstrap support $\geqslant 97\%$). Distance (least squares) generated a subsequent branching of *A. michealis*, *P. nelsoni* and *S. lophii* basal to clade IIa (bootstrap support $\geqslant 82\%$). These inconsistencies were not further examined as no *Daphnia* parasites branched within clade II.

Maximum likelihood. All combinations of outgroups in the searches yielded the same overall topology. Two taxa could not be placed unambiguously: *Parathelohania anophelis* was either basal to clade I (no outgroup, G, T, BG, GT, BGT) or basal to clade Ib (B, BT). *F. magnivora* was either basal to all clades (G, T, GT) or basal to clade I (B, BT, BG, BGT). A comparison of these competing topologies (and a hypothetic placement of *F. magnivora* basal to clades II–VI) with a Shimodaira–Hasegawa test found no significant differences.

Distance. Overall topologies were identical with those of the maximum likelihood search. As in the maximum likelihood search, positions of *P. anophelis* and *F. magnivora* could not be resolved exactly. Using least squares objective function, all outgroups produced a polytomy for the branching of *P. anophelis*, clade Ia and clade Ib. *F. magnivora* was placed either basal to clade I (G, BG), basal to clades II–VI (B, T, BT, BGT) or was not resolved (GT). All positions had low bootstrap support. When minimum evolution was used as objective function, *P. anophelis* was consistently placed basal to clade Ib with moderate bootstrap support. *F. magnivora* was placed either basal to clade I (G, BG, BT, GT, BGT), basal to clades II–VI (T) or was not resolved (B). All positions had moderate bootstrap support.

Parsimony. Overall topologies were identical with those of the maximum likelihood search. Again, positions of *P. anophelis* and *F. magnivora* were not fully resolved. *P. anophelis* was either basal to clade I (no outgroup), basal to clade Ib (B, T) or branched polytomous with clades Ia and Ib (G, BG, BT, GT, BGT). All positions had low bootstrap support. *F. magnivora* was either basal to clade I (BGT), basal to clades II–VI (G) or unresolved (B, T, BG, BT, GT).

DISCUSSION

We sequenced the SSUrDNA of 6 microsporidia that parasitize *Daphnia* spp. and generated a phylogeny, together with other microsporidian sequence data. Our study shows that several lineages of microsporidia have independently established themselves as parasites in *Daphnia* spp. i.e. they are of polyphyletic origin. This pattern was consistently supported by all methods used irrespective of the outgroups. In our phylogeny, parasites of *Daphnia* spp. group with lineages infecting insects (*G. vavrai*, *L. obtusa*) and vertebrates (*O. colligata*, *Microsporidium* sp.). The clade containing, *G. intestinalis* includes parasites of Lepidoptera and 1 species from a vertebrate.

Three of the microsporidia analysed (*G. intestinalis*, *O. colligata* and *Microsporidium* sp.) have small spores and infect the gut wall of their hosts but can be distinguished by ultrastructural and genetic methods. The distant placement of *Glugoides* and *Ordospora* suggested by their ultrastructure (Larsson *et al*. 1996, 1997) has been confirmed in the molecular phylogeny.

O. colligata and *Microsporidium* sp. are basal to clades of parasites that are only known to infect vertebrates (Canning, 1998). *O. colligata* is the first microsporidium to be identified as a relative of the genus *Encephalitozoon*, of which 3 species are described. They infect homeothermic vertebrates and are known as human pathogens (Weber *et al*. 1994). *O. colligata* and *Encephalitozoon* share the following features: isolated nuclei in all stages of the life-cycle, development in a parasitophorous vacuole of host cell origin, di- and tetrasporoblastic sporogony and uninucleate spores. The sporogony differs in that, in *O. colligata*, the tetrasporoblastic sporogony is preceded by binary division into 2 sporoblast mother cells (Larsson *et al*. 1997). The 2 genera are remarkably similar.

Microsporidium sp. is basal to the family Enterocytozoonidae that contains the 2 species *Enterocytozoon bieneusi* and *Nucleospora salmonis*. *E. bieneusi* has been reported from several mammalian hosts, with some strains apparently naturally-occuring in primates (Canning, 2001). *N. salmonis* infects salmonid fish and an undescribed *Nucleospora* species has been detected in English sole but has not been described morphologically (Hedrick, Groff & Baxa, 1991; Khattra *et al*. 2000). Unfortunately our ultrastructural data on the development of *Microsporidium* sp. are scarce and do not allow a profound discussion of the relation of its morphology to *E. bieneusi* and *N. salmonis*. *Microsporidium* sp. as well as *E. bieneusi* and *N. salmonis* develop in close contact with the host cell cytoplasm.

The only groups from which parasites of crustaceans have been genetically analysed are clades II and V (Terry *et al*. 1999; Cheney *et al*. 2000). None of our parasites fell in these clades. Clade IIa contains pairs of parasites which have been reported previously as closely related: *Trachipleistophora hominis* and *Vavraia oncoperae*, parasites of man and fish respectively (see Nilsen, 2000) and *Glugea atherinae* and *Tuzetia* sp. parasites of fish and crustaceans respectively (see Cheney *et al*. 2000). *Tuzetia* sp. was referred to as *Pleistophora* sp. (PA) by Cheney *et al*. (2000) but has since been described as a

new species of *Tuzetia* (Canning *et al*. 2002). When described (Larsson *et al*. 1996), *G. intestinalis* was placed in the family Glugeidae, represented in our analysis by *G. atherinae*. This classification has to be reconsidered as our molecular data indicate that *G. intestinalis* is related to *Vittaforma corneae* and *Endoreticulatus schubergi*. Another species that has not been included in our analysis is *Cystosporogenes operophterae*, which on SSUrDNA has been shown to be close to *Endoreticulatus* (Canning *et al*. 2001).

All species in clade III are polysporoblastic. *E. schubergi*, C. *operophterae* and *G. intestinalis* are similar in having isolated nuclei throughout development and all stages lying within a parasitophorous vacuole, the membrane of which originates from host endoplasmatic reticulum (Canning *et al*. 1985; Brooks, Becnel & Kennedy, 1988; Larsson *et al*. 1996). *V. corneae* appears to be unrelated on morphological criteria, as all stages are diplokaryotic. However, the envelopment of each life-cycle stage by a cisterna of endoplasmatic reticulum is a unifying factor and this must have some evolutionary significance. Although in some classification systems, including that of Sprague *et al*. (1992), emphasis is placed on nuclear data, the occurence of both uninucleate and diplokaryotic sequences in the life-cycles of several microsporidia e.g. *Spraguea* and *Vairimorpha*, suggests that the diplokaryotic state may be ancestral, with loss of one or other state in the evolution of many of the extant genera. *V. corneae* is also exceptional in clade III in parasitizing a vertebrate (man) while the others infect arthropods. However, it has been hypothesized that *V. corneae* is an opportunistic parasite of man and may have a natural host among insects (Weiss & Vossbrinck, 1998). It has been recorded only once as a corneal infection, which is an immunoprivileged site (Shadduck *et al*. 1990) and a renal infection in an AIDS patient (Deplazes *et al*. 1998).

The sequences of *G. daphniae* and *G. vavrai* are nearly identical and the differences appear to be amplification/sequencing artifacts. Measurements of the spore size of fresh material of both species gave no differences. The only other difference mentioned in the species description of *G. daphniae* (Friedrich *et al*. 1996) lies with the host species, which are *D. longispina* for *G. vavrai* and *D. pulex* for *G. daphniae*. There is no information on the host specificity of the 2 parasites. An ultrastructural analysis of the type material of *G. vavrai* is needed to establish whether these are 2 different species.

G. vavrai and *L. obtusa* are sister taxa. They branch within or close to the *Amblyospora* group (Baker *et al*. 1998) with *Vairimorpha* sp. and *Amblyospora* sp. being their closest relatives. This *Vairimorpha* sp. is very probably misclassified and belongs to another genus (Moser *et al*. 1998). It infects the black fire ant *Solenopsis richteri*. The failure of attempts to maintain *Vairimorpha* sp. in laboratory colonies of its host suggests that it may have an alternate host (Moser, personal communication). *Amblyospora* sp. was obtained from the black fly *Simulium* sp. (either *S. equinum* or *S. ornatum*) (Cheney *et al*. 2000). There is no further information available on this parasite.

The phylogenetic position of *G. vavrai* and *L. obtusa* supports the presumption of an obligate secondary host for the completion of their life-cycles. They are closely related to the *Amblyospora* group that contains the only microsporidia known to have 2 alternating hosts (Baker *et al*. 1998). *A. californica* (and other species of its genus) and *P. anophelis* infect mosquitoes and copepods (Sweeney, Graham & Hazard, 1988; Avery & Undeen, 1990; Becnel, 1992). There is evidence that the other 2 genera of this group, *Culicosporella* and *Edhazardia*, underwent regressive evolution and reduced their life-cycle from 2 hosts to 1 (Becnel, 1994). All intermediate hosts detected so far have been copepods. Our current knowledge of development of *G. vavrai* and *L. obtusa* in *Daphnia* allows no statement whether this host corresponds to the mosquito or to the copepod.

Microsporidia of the *Amblyospora* group have intricate life-cycles involving multiple spore types with different nuclear phases. In all microsporidia with intermediate hosts of which the life-cycle has been described, the generation in the copepod is entirely haplophasic (Becnel, 1994). All developmental stages of *L. obtusa* in *D. pulex* are uninucleate with the exception of a short stage probably between merogony and sporogony, where 2 associated nuclei are visible (Vidtman & Sokolova, 1994). This needs to be investigated further. The life-cycle of *G. vavrai* has not been described. *G. daphniae* has only uninucleate stages (Friedrich *et al*. 1996). Larsson (1986) used the absence of diplokaryotic stages as a key for the identification of the genus *Gurleya*. A second explanation for failed attempts to maintain *G. vavrai* and *L. obtusa* in laboratory populations of *Daphnia* may be unsuitable environmental conditions. Friedrich *et al*. (1996) reported a deterioration in spore development of *G. daphniae* when kept in the laboratories but reported that horizontal transmission was successful. We were not able to repeat these findings. *F. magnivora* falls into none of the known clades of microsporidia. It branches deep in the phylum without any relation to any other taxa that have been sequenced and does not group with *F. montana*, the type species of its genus (Canning *et al*. 2001). We were not able to resolve its position unambiguously. A comparison of maximum likelihood topologies where *F. magnivora* was either basal to clade I, clades II–VI or both with a Shimodaira–Hasegawa test found no significant differences. Bootstrap support in distance and parsimony was low for any of the positions.

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