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Molecular phylogeny of the Microsporidia: ecological, ultrastructural and taxonomic considerations

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Abstract. The Microsporidia are a group of obligate intracellular parasites, now thought to be derived fungi. Presented here is a comparative small subunit rDNA (ssrDNA) analysis of 125 species of Microsporidia (sequences obtained from GenBank). This analysis shows that groups or clades are formed based largely on habitat and host. This result is supported by comparative molecular analyses of the past decade, and indicates that structural and ultrastructural characters are unreliable for distinguishing among higher-level microsporidian taxa. Our findings indicate the presence of five major clades of Microsporidia which group according to habitat. We present three new classes of Microsporidia based on natural phylogenetic groupings as illustrated by the ssrDNA analysis: Aquasporidia, Marinosporidia and Terresporidia. The names of the proposed classes reflect the habitat of each group. The class Aquasporidia, found primarily in freshwater habitats, is a paraphyletic group consisting of three clades. The Marinosporidia are found in hosts of marine origin and the Terresporidia are primarily from terrestrial environments.

A unique feature of the Microsporidia is the long, coiled polar filament present in the spore, which is used to inject the sporoplasm into the host cell upon spore germination. The polar filament is thought to evert, penetrating the host cell and pulling the sporoplasm into the host cell. This ability of the Microsporidia has apparently allowed these parasites to diverge into numerous animal hosts from many of the animal phyla. This apomorphic feature, and the unique diplokaryon arrangement of the nuclei (in many species) clearly define the Microsporidia structurally.

Microsporidia, like all organisms, are classified using common characters which appear as different character states. In the Microsporidia an example of a character used for classification is the nuclear condition, with uninucleate and diplokaryotic character states. Other examples of characters used in the classification of the Microsporidia include the number of coils in the polar filament around the periphery of the spore and the thickness of the polar filament (with isofilar and anisofilar character states). Development of taxonomies is an iterative process in which it is decided which characters change state rapidly and can be used to distinguish genera, species and populations and which characters change state slowly and can be used to distinguish higher levels such as class, order and family. Unfortunately, the characters which are used to determine the higher levels of classification in the Microsporidia (number of nuclei/cell, presence of a membrane surrounding the parasite (sporophorous vesicle), and type of nuclear division) appear to be characters which change states quickly at the genus, species and population levels. Comparative analyses of small subunit ribosomal (ssrDNA) sequence data show that the use of ultrastructural features for taxonomic divisions of the Microsporidia is untenable, and indicate that the Microsporidia can instead be divided into groups which reflect habitat and host.

To determine a plausible phylogeny for any group of organisms, a data set has to be made which includes each organism and all of the common characters and character states which can be identified for the group. After this data set is complete, a phylogenetic analysis can be conducted.

Four fundamentally different types of phylogenetic analyses are available: distance methods, parsimony analysis, maximum likelihood analysis (Swofford 2002) and Bayesian analysis (Huelsenbeck et al. 2001).

Distance methods are the least computationally intensive and involve calculating a matrix of differences of character states between each pair of taxa. Even with a large number of taxa a distance tree can be rapidly constructed. Each pair of taxa are then connected based on similarity (unpaired group mean analysis, UPGMA) and in the case of neighbour-joining analysis, calculations are made to adjust, as far as possible, for differences in evolutionary rates among taxa.

Maximum parsimony analysis involves the construction of every possible tree (the branch and bound

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routine will shorten the process while still guaranteeing finding the shortest tree) and places the character states for each character on each tree. The number of evolutionary steps required for each tree is determined and the tree which requires the fewest evolutionary steps is considered to be the best. Maximum parsimony analysis is therefore a minimum homoplasy (the tree described by the fewest convergent character states) analysis. While distance methods give a single tree, maximum parsimony methods can give more than one tree with the same number of evolutionary steps (more than one shortest tree). In addition, it is useful to see if trees which are one or two steps longer than the shortest tree, based on molecular data, might fit better with taxonomies based on non-molecular characters. This capability was implemented into the latest version of PAUP (http://paup.csit.fsu.edu/downl.html).

Maximum likelihood analysis is by far the most computationally intensive algorithm. The problem reduces to computing the probability of a particular set of sequences on a given tree and maximizing this probability over all evolutionary trees (Felsenstein 1981). It involves finding the evolutionary tree which yields the highest probability of containing the observed data. Each tree examined involves considerable computation; this method is good for data sets with a small number of taxa or for testing subsets of data where the resolution of a relationship among a few taxa is difficult.

Bayesian analysis (not yet included in the PAUP package), like maximum likelihood analysis, is a probabilistic method which (in the case of Bayesian inference) calculates *a posteriori*, the probability of finding a tree based on a set of assumptions. It has recently become a popular method (Mr. Bayes program; http://morphbank.ebc.uu.se/mrbayes/) for evaluating evolutionary trees as it seems to do a good job with tested data sets (Murphy et al. 2001) but as a purely mathematical approach claims no mechanistic basis.

Almost all editors now require a bootstrap analysis with any phylogenetically based tree. This computer algorithm randomly samples the sequence data and generates trees based on these sampled subsets of data. Typically one hundred trees will be generated (using any of the above procedures) and a consensus tree is produced showing the percentage of trees which gave a particular topology on the tree. Branch points on the tree with high consensus values (in the range of 95 to 100%) are thought, with fair certainty, to contain those taxa. This gives the reader a feel for the data in terms of which parts of the tree are better resolved and which areas need more data or more analysis.

As discussed above, all of these methods require a data set containing characters with character states for the Microsporidia being analysed. Larsson (1986, 1988, 1999) has made several bold attempts to make such an analysis. Larsson (1986) makes a gallant effort at gathering and listing characters and character states for the purpose of constructing a phylogeny based on "traditional" (morphological and developmental) features. In his review papers, Larsson clearly illustrates, with light and electron micrographs, the structural and ultrastructural features which could be used in differentiating various taxa of Microsporidia. Larsson (1986) lists 12 characters for 64 taxa covering a much broader group of Microsporidia (mostly different genera) than those considered here. In addition, Larsson (1986) presents some of the first attempts at a phylogenetic analysis of Microsporidia using such characters and character states. From a phylogenetic point of view, Larsson should be highly commended for his tremendous effort to develop characters and character states for the Microsporidia. In his 1999 review article Larsson lists 14 characters for the "identification of Microsporidia" but develops no phylogenetic tree. He attributes the failure to develop an accurate tree to having incomplete descriptions for many of the species and therefore an incomplete data set. The more likely explanation is that none of these characters clearly defines any higher level taxa.

Issi (1986) also presents an important review of microsporidian features with a database consisting of 68 genera and 11 characters. The text of this publication has been translated into English by Professor Jerzy J. Lipa and is available through the Division of Microsporidia from the Society of Invertebrate Pathology. Issi (1986) presents detailed illustrations of microsporidian characters and proposes a taxonomic scheme based on Weiser (1977). Issi (1986) also mentions studies showing similarities between the Microsporidia and yeast cells with respect to the type of nuclear division (acentriolar cryptic intranuclear pleuromitosis) and points out that the lamellar plates in the spindles of Microsporidia resemble those of yeast nuclei, but does not claim a connection between the Microsporidia and the Fungi.

Early classifications of the Microsporidia were developed based on characters which were visible with light microscopy (Balbiani 1882, Stempell 1909, Léger and Hesse 1922) and were attempts to separate the Microsporidia into logical groups. Over time more emphasis was placed on developing taxonomies which did not differ unreasonably from perceived evolutionary relationships (Hennig 1966). With the advent of the electron microscope, new ultrastructural characters, such as type of nuclear division, were described for the Microsporidia (Tuzet et al. 1971, Weiser 1977, Sprague 1977, Sprague et al. 1992). These ultrastructural characters were then incorporated into classifications of the Microsporidia (Sprague et al. 1992).

Higher-level taxonomic classifications of the Microsporidia differ in the placement of the Chytridiopsidae and Hessidae. Weiser (1977) includes the Metchnikovellidae, Chytridiopsidae and Hessidae in the Metchnikovellidea ("primitive" Microsporidia) while Sprague (1977, 1982) places the Chytridiopsidae and Hessidae with the "higher" Microsporidia. The "higher" Micro-

sporidia are then divided into two groups based on the presence (Pansporoblastina) or absence (Apansporoblastina) of an external cover around the sporoblast (the pansporoblast, Tuzet et al. 1971). Sprague et al. (1992) introduce a different classification system, based on whether the species is diplokaryotic at some point in the life cycle (Dihaplophasea) or uninucleate throughout its life cycle (Haplophasea). The Dihaplophasea are further separated into those in which the diplokaryon is formed through meiosis (Meiodihaplophasida) and those in which the diplokaryon is formed through nuclear dissociation (Dissociodihaplophasida).

MATERIALS AND METHODS

All sequences presented in Table 1 are available from the United States National Center for Biotechnology Information DNA sequence repository and shared with the European Molecular Biological Laboratory. Sequences were aligned using the alignment program CLUSTAL_X.

Table 1. Microsporidian and three outgroup sequences used for ssrDNA phylogenetic analysis.

Organism	GenBank Acc. No.
Amblyospora bracteata	AY090068
Amblyospora californica	U68473
Amblyospora canadensis	AY090056
Amblyospora cinerei	AY090057
Amblyospora connecticus	AF025685
Amblyospora crenifera	AY090061
Amblyospora excrucii	AY090043
Amblyospora ferocious	AY090062
Amblyospora indicola	AY090051
Amblyospora khaliulini	AY090045
Amblyospora opacita	AY090052
Amblyospora salinaria	U68474
Amblyospora stictici	AY090049
Amblyospora stimuli	AF027685
Amblyospora weiseri	AY090048
Amblyospora sp. 1	AY090053
Amblyospora sp. 2	AY090055
Amblyospora sp. 3	AJ252949
Ameson michaelis	L15741
Antonospora scoticae	AF024655
Basidiobolus ranarum	AY635841
Bacillidium vesiculoformis	AJ581995
Bacillidium sp.	AF104087
Berwaldia schaefernai	AY090042
Brachiola algerae	AF069063
Bryonosema plumatellae	AF484690
Caudospora palustris	AF132544
Caudospora simulii	AY973642
Conidiobolus coronatus	AF296753
Culicospora magna	AY090054
Culicosporella lunata	AF027683
Cystosporogenes legeri	AY233131
Cystosporogenes operophterae	AJ302320
Dictyocoela cavimanum	AJ438960
Dictyocoela deshayesum	AJ438961
Dictyocoela muelleri	AJ438956
Dictyocoela duebenum	AF397404
Dictyocoela berillonum	AJ438957
Dictyocoela gammarellum	AJ438958
Edhazardia aedis	AF027684
Encephalitozoon cuniculi	L39107
Encephalitozoon hellem	L39108
Encephalitozoon intestinalis	L39113
Encephalitozoon lacertae	AF067144
Enterocytozoon bieneusi	L07123
Endoreticulatus schubergi	L39109
Endoreticulatus bombycis	AY009115
Flabelliforma magnivora	AJ302318
Flabelliforma montana	AJ2052962
Glugea americanus	AF056014
Glugea anomala	AF044391
Glugea atherinae	U15987
Glugea stephani	AF056016
Glugoides intestinalis	AF394525
Gurleya daphniae	AF439320
Gurleya vavrai	AF394526
Hazardia milleri	AF090067
Hazardia sp.	AF090066
Heterococcus pleurococcoides	AJ579335
Heterosporis anguillarum	AF387331
Heterosporis sp.	AF356225
Hyalinocysta chapmani	AF483837
Ichthyosporidium giganteum	L39110
Intrapredatorus barri	AY013359
Janacekia debaisieuxi	AY090070
Kabatana takedai	AF356222
Larssonia obtusa	AF394527
Loma acerinae	AJ252951
Loma salmonae	U78736
Loma embiotocia	U78815
Loma sp.	AF104081
Marssoniella elegans	AY090041
Microgemma caulleryi	AY033054
Microgemma sp.	AJ252952
Microsporidium prosopium	AF151529
Nadelspora canceri	AY958070
Nosema apis	X73894
Nosema bombi	AY008373
Nosema bombycis	L39111
Nosema carpocapsae	AF426104
Nosema ceranae	U26533
Nosema furnacalis	U26532
Nosema granulosis	AJ011833
Nosema oulemae	U27359
Nosema portugal	AF033316
Nosema pyrausta	AY958071
Nosema spodopterae	AY211392
Nosema trichoplusiae	U09282
Nosema tyriae	AJ012606
Nosema vespula	U11047
Nucleospora salmonis	U78186
Nucleospora sp.	AF186007
Oligosporidium occidentalis	AF495379
Ordospora colligata	AF394529
Orthosomella operophterae	AJ302317

Ovipleistophora mirandellae	AF356223
Paranosema grylli	AY305325
Paranosema locustae	AY305324
Paranosema whitei	AY305323
Parathelohania anophelis	AF027682
Parathelohania obesa	AF090065
Pleistophora anguillarum	U47052
Pleistophora mirandellae	AF104085
Pleistophora ovariae	AJ278955
Pleistophora typicalis	AF044387
Polydispyrenia simulii	AY090069
Pseudoloma neurophilia	AF322654
Pseudonosema cristatellae	AF484694
Schroedera plumatellae	AY135024
Spraguea lophii	AF033197
Tetramicra brevifilum	AF364303
Thelohania contejeani	AF492593
Thelohania parastaci	AF294779
Thelohania solenopsae	AF031538
Trachipleistophora hominis	AJ002605
Trichonosema algonquinensis	AY582742
Trichonosema pectinatellae	AF484695
Trichotuzetia guttata	AY326268
Vairimorpha sp.	AF031539
Vairimorpha cheracis	AF327408
Vairimorpha imperfecta	AJ131645
Vairimorpha lymantriae	AF033315
Vairimorpha necatrix	Y00266
Vavraia culicis	AJ252961
Vavraia oncoperae	X74112
Visvesvaria acridophagus	AF024658
Vittaforma corneae	L39112
Weiseria palustris	AF132544

RESULTS AND DISCUSSION

A Molecular Classification of the Microsporidia based on ssrDNA analysis

Fig. 1 is a phylogenetic tree of the 125 species of Microsporidia for which relatively complete small subunit rDNA (ssrDNA) sequences are available. Fig. 1 shows a consensus tree resulting from 20,000 bootstrap replicates using neighbour-joining analysis. Fig. 2 is a maximum parsimony tree showing the same basic relationships among the 5 major groups except that the rooting of the parsimony tree separates clade V into two separate groups. The parsimony tree given was one of 30 shortest trees generated. Since the 30 shortest trees differed only in relationships within the *Nosema*/*Vairimorpha* and *Dictyocoela* groups, the parsimony consensus tree is not shown as it does not show relative distances among taxa. Considering the large number of taxa analysed, the trees resulting from these two very different methods are remarkably similar. The relative positions of the three proposed classes are the same for Figs. 1 and 2. The parsimony tree of Fig. 2 gives a distinct rooting but the trichotomy of Fig. 1 indicates that the rooting of this tree is still in question.

The ssrDNA gene has not been sequenced for any of the "primitive" Microsporidia from the family Metchnikovellidae, so this analysis includes only the "higher Microsporidia". The Metchnikovellidae are considered primitive (Sprague 1977) because they lack polaroplasts and have a very short, thick polar filament. The metchnikovellids are parasites of gregarines (which in turn are parasites of polychaete annelids) and this modification of the polar filament could represent a derived state for parasitizing a specialised host. At this time it is not known whether the short polar filament of the Metchnikovellidae is a plesiomorphic (primitive) character, as Sprague's (1977) classification would suggest, or whether this character state has been secondarily derived (apomorphic) in response to the small host, as the classification of Tuzet et al. (1971) would imply. If the short polar filament is a plesiomorphic character then a *Metchnikovella* species would be an extremely important outgroup for the analysis of the phylum Microsporidia.

Due to recent reports that Microsporidia are derived fungi (Edlind et al. 1996, Keeling and Doolittle 1996) specifically related to the Zygomycota (Keeling et al. 2000), we selected two fungi, *Basidiobolus ranarum* and *Conidiobolus coronatus* from the family Entomophthoraceae in the Zygomycota, as outgroups. The yellow-green eukaryotic alga, *Heterococcus pleurococcoides* is also included as an outgroup. The analysis in Fig. 1 reveals clade V, members of the Class Aquasporidia, to be the sister group to the remaining "higher" Microsporidia. This placement agrees with the results of Canning et al. (2002), who used Bayesian inference and maximum likelihood analysis of ssrDNA from 44 species, with the zygomycote *Basidiobolus ranarum* as an outgroup. The Canning et al. (2002) analysis shows a group of Microsporidia (clades G and H) primarily from freshwater habitats which appears to be the sister group to the remaining Microsporidia. Clades G and H correspond to clade V in Fig. 1. It has been shown previously (Vossbrinck et al. 2004a) for 82 species of Microsporidia that there is a correlation between phylogenetic group and habitat (freshwater, marine and terrestrial) for the three major groups of Microsporidia.

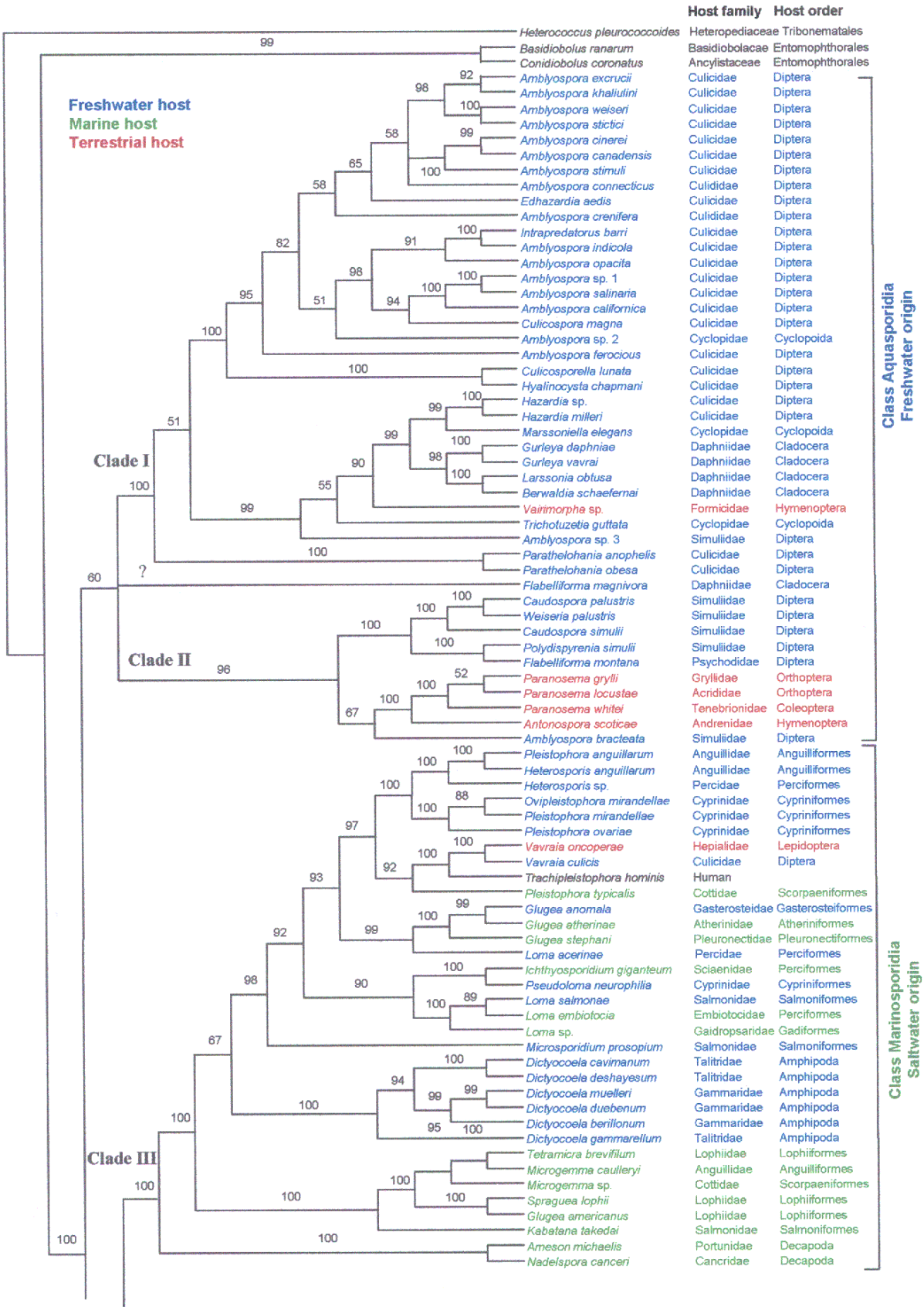
The phylogeny in Fig. 1 illustrates a number of points that have emerged over the past decade as a result of molecular analysis of the Microsporidia. Analysis of partial large subunit rRNA sequences (Baker et al. 1994) showed that the genus *Nosema* (defined as being diplokaryotic throughout the life cycle) was composed of a group of "true *Nosema*" as defined by the type species *Nosema bombycis*, and several additional groups of unrelated *Nosema* species including *Nosema locustae* (now *Paranosema locustae*), *N. algerae* (now *Brachiola algerae*) and *N. kingi*. This finding suggested that the character state of being diplokaryotic throughout the life cycle was not necessarily an indication of relatedness.

The analysis also indicated that the *Vairimorpha* (based on the type species *Vairimorpha necatrix*) were the sister group to the "true" *Nosema* species. Baker et al. (1994) observed that the formation of octospores during the life cycle occurred in some species which clustered with the genus *Nosema* (as defined by *N. bombycis*) and did not occur in some species which clustered with the genus *Vairimorpha* (as defined by *V. necatrix*). Fig. 1 shows a mixture of *Nosema* (diplokaryotic throughout the life cycle) and *Vairimorpha* (octospores present at some point in the life cycle) species in two clades of the terrestrial Microsporidia. At the same time, primers for amplification of microsporidial ribosomal DNA were also being developed (Vossbrinck et al. 1993). Two primers (ss530F and ls580R) were constructed to amplify a segment of DNA containing a large portion of the small subunit rDNA, the internal transcribed spacer (ITS) region and a portion of the large subunit rDNA. Vossbrinck et al. (1993) showed that restriction analysis of the resulting PCR products gave distinctive patterns, allowing differentiation among three isolates (*Encephalitozoon hellem*, *Encephalitozoon cuniculi* and *Nosema corneum*) from AIDS patients, showing clearly for the first time that *E. hellem* and *E. cuniculi* were two separate species. The ITS region was shown to be highly variable. Didier et al. (1996) found differences in this region among isolates of *E. cuniculi* corresponding to the host from which the strain was isolated. Attempts were made to standardise the primers for microsporidial rDNA amplification (Vossbrinck et al. 1993, Kent et al. 1996), but some species of Microsporidia did not amplify well in this region (ss530F to ls580R). As a result the small subunit rDNA (18F to 1492R), which was amplified successfully for the majority of the Microsporidia tested, became the standard sequence used for molecular analysis of microsporidial rDNA (Weiss and Vossbrinck 1999), excluding the variable internal transcribed spacer (ITS) region from the analysis.

Analysis of ssrDNA (Baker et al. 1995) again demonstrated the *Vairimorpha/Nosema* relationship and showed the *Encephalitozoon* species to be the sister group to the *Vairimorpha/Nosema* clade. Placement of the ultrastructural characters on the molecular tree revealed that characters such as nuclear condition, membrane surrounding the sporoblast, sporogony and chromosome cycle did not seem to be indicators of relatedness (Baker et al. 1995). The molecular analysis also indicated that *Septata intestinalis* was a member of the genus *Encephalitozoon* as originally described by Didier et al. (1991). Baker et al. (1997) proposed that the complex life cycles of *Amblyospora* (Andreadis 1985, Sweeney et al. 1985) may represent the plesiomorphic state for Microsporidia. Phylogenetic analysis indicated that the *Amblyospora* were a sister group to the remainder of the higher Microsporidia. The most parsimonious explanation for the ssrDNA phylogeny is that the ancestral microsporidian was aquatic and had a complex life cycle, and that groups with simpler life cycles represent losses of various life-cycle components. Sequencing of four additional *Amblyospora* and *Amblyospora*-like Microsporidia (Baker et al. 1998) showed a possible evolutionary correlation between *Aedes* and *Culex* hosts and *Amblyospora* parasites and between *Anopheles* hosts and *Parathelohania* parasites. Further work (Vossbrinck et al. 2004b) reinforced these results with additional examples of *Amblyospora* species which separated along the lines of *Culex* and *Aedes/Ochlerotatus* host clades. Small subunit rDNA sequence information was also used for the identification of intermediate hosts (Voss-brinck et al. 1998) by sequencing spores from possible copepod intermediate hosts and comparing these sequences to those obtained from spores from mosquito hosts.

The great discrepancies between the evolutionary relationships among the Microsporidia based on small subunit rDNA analysis and the published taxonomic designations based on morphological characters must be resolved. It is proposed here that taxonomic divisions of the Microsporidia consider phylogenetic relatedness and that major taxonomic divisions be based on habitat and host. Taxa are already being renamed based on their evolutionary relationships as determined by ssrDNA analysis. Three *Nosema* species have been renamed as a result of reports of phylogenetic relatedness. *Nosema algerae* is now *Brachiola algerae* (Cali et al. 1998) and *Nosema corneum* is now *Vittaforma corneae* (Silveira and Canning 1995). Interestingly, *Nosema locustae* has been renamed by two different groups. Sokolova et al. (2003) renamed *Nosema locustae* as *Paranosema locustae* based on ultrastructure and small subunit rDNA analysis, while Slamovits et al. (2004) renamed *Nosema locustae* as *Antonospora locustae* because of its phylogenetic relationship to *Antonospora scoticae*. Taxonomic divisions which result in polyphyletic clades, as with the true *Nosema* species and *Vairimorpha* species, are not acceptable. Generic names within the *Nosema* and *Vairimorpha* groups should be determined by phylogenetic placement, with those in the *Nosema bombycis* clade designated as *Nosema* species, and those in the *Vairimorpha necatrix* clade designated as *Vairimorpha* species. Fig. 1 and other studies (Vossbrinck et al. 2004b) clearly show that the genera *Edhazardia*, *Intrapredatorus* and *Culicospora* fall within the *Amblyospora* clade and may have to be designated as *Amblyospora* in the future. There is an increasing reliance on ssrDNA analysis to determine generic designation (Maddox et al. 1999) and researchers are including ssrDNA sequence analyses as part of their species descriptions (Andreadis and Vossbrinck 2002, Canning et al. 2002, Sokolova et al. 2003).

Small subunit rDNA sequence data for the Microsporidia has been accumulating for nearly two decades, and as the database has grown to include sequences from a large number of taxa, ssrDNA has become the


Freshwater host
Marine host
Terrestrial host
Host family
Host order
Heterococcus pleurococcoides Heteropediaceae Tribonematales
Basidiobolus ranarum Basidiobolacae Entomophthorales
Conidiobolus coronatus Ancylistaceae Entomophthorales
Amblyospora excrucii Culicidae Diptera
Amblyospora khaliulini Culicidae Diptera
Amblyospora weiseri Culicidae Diptera
Amblyospora stictici Culicidae Diptera
Amblyospora cinerei Culicidae Diptera
Amblyospora canadensis Culicidae Diptera
Amblyospora stimuli Culicidae Diptera
Amblyospora connecticus Culididae Diptera
Edhazardia aedis Culicidae Diptera
Amblyospora crenifera Culididae Diptera
Intrapredatorus barri Culicidae Diptera
Amblyospora indicola Culicidae Diptera
Amblyospora opacita Culicidae Diptera
Amblyospora sp. 1 Culicidae Diptera
Amblyospora salinaria Culicidae Diptera
Amblyospora californica Culicidae Diptera
Culicospora magna Culicidae Diptera
Amblyospora sp. 2 Cyclopidae Cyclopoida
Amblyospora ferocious Culicidae Diptera
Culicosporella lunata Culicidae Diptera
Hyalinocysta chapmani Culicidae Diptera
Hazardia sp. Culicidae Diptera
Hazardia milleri Culicidae Diptera
Marssoniella elegans Cyclopidae Cyclopoida
Gurleya daphniae Daphniidae Cladocera
Gurleya vavrai Daphniidae Cladocera
Larssonia obtusa Daphniidae Cladocera
Berwaldia schaefernai Daphniidae Cladocera
Vairimorpha sp. Formicidae Hymenoptera
Trichotuzetia guttata Cyclopidae Cyclopoida
Amblyospora sp. 3 Simuliidae Diptera
Parathelohania anophelis Culicidae Diptera
Parathelohania obesa Culicidae Diptera
Flabelliforma magnivora Daphniidae Cladocera
Caudospora palustris Simuliidae Diptera
Weiseria palustris Simuliidae Diptera
Caudospora simulii Simuliidae Diptera
Polydispyrenia simulii Simuliidae Diptera
Flabelliforma montana Psychodidae Diptera
Paranosema grylli Gryllidae Orthoptera
Paranosema locustae Acrididae Orthoptera
Paranosema whitei Tenebrionidae Coleoptera
Antonospora scoticae Andrenidae Hymenoptera
Amblyospora bracteata Simuliidae Diptera
Pleistophora anguillarum Anguillidae Anguilliformes
Heterosporis anguillarum Anguillidae Anguilliformes
Heterosporis sp. Percidae Perciformes
Ovipleistophora mirandellae Cyprinidae Cypriniformes
Pleistophora mirandellae Cyprinidae Cypriniformes
Pleistophora ovariae Cyprinidae Cypriniformes
Vavraia oncoperae Hepialidae Lepidoptera
Vavraia culicis Culicidae Diptera
Trachipleistophora hominis Human
Pleistophora typicalis Cottidae Scorpaeniformes
Glugea anomala Gasterosteidae Gasterosteiformes
Glugea atherinae Atherinidae Atheriniformes
Glugea stephani Pleuronectidae Pleuronectiformes
Loma acerinae Percidae Perciformes
Ichthyosporidium giganteum Sciaenidae Perciformes
Pseudoloma neurophilia Cyprinidae Cypriniformes
Loma salmonae Salmonidae Salmoniformes
Loma embiotocia Embiotocidae Perciformes
Loma sp. Gaidropsaridae Gadiformes
Microsporidium prosopium Salmonidae Salmoniformes
Dictyocoela cavimanum Talitridae Amphipoda
Dictyocoela deshayesum Talitridae Amphipoda
Dictyocoela muelleri Gammaridae Amphipoda
Dictyocoela duebenum Gammaridae Amphipoda
Dictyocoela berillonum Gammaridae Amphipoda
Dictyocoela gammarellum Talitridae Amphipoda
Tetramicra brevifilum Lophiidae Lophiiformes
Microgemma caulleryi Anguillidae Anguilliformes
Microgemma sp. Cottidae Scorpaeniformes
Spraguea lophii Lophiidae Lophiiformes
Glugea americanus Lophiidae Lophiiformes
Kabatana takedai Salmonidae Salmoniformes
Ameson michaelis Portunidae Decapoda
Nadelspora canceri Cancridae Decapoda
Clade I
Clade II
Clade III
Class Aquasporidia
Freshwater origin
Class Marinosporidia
Saltwater origin

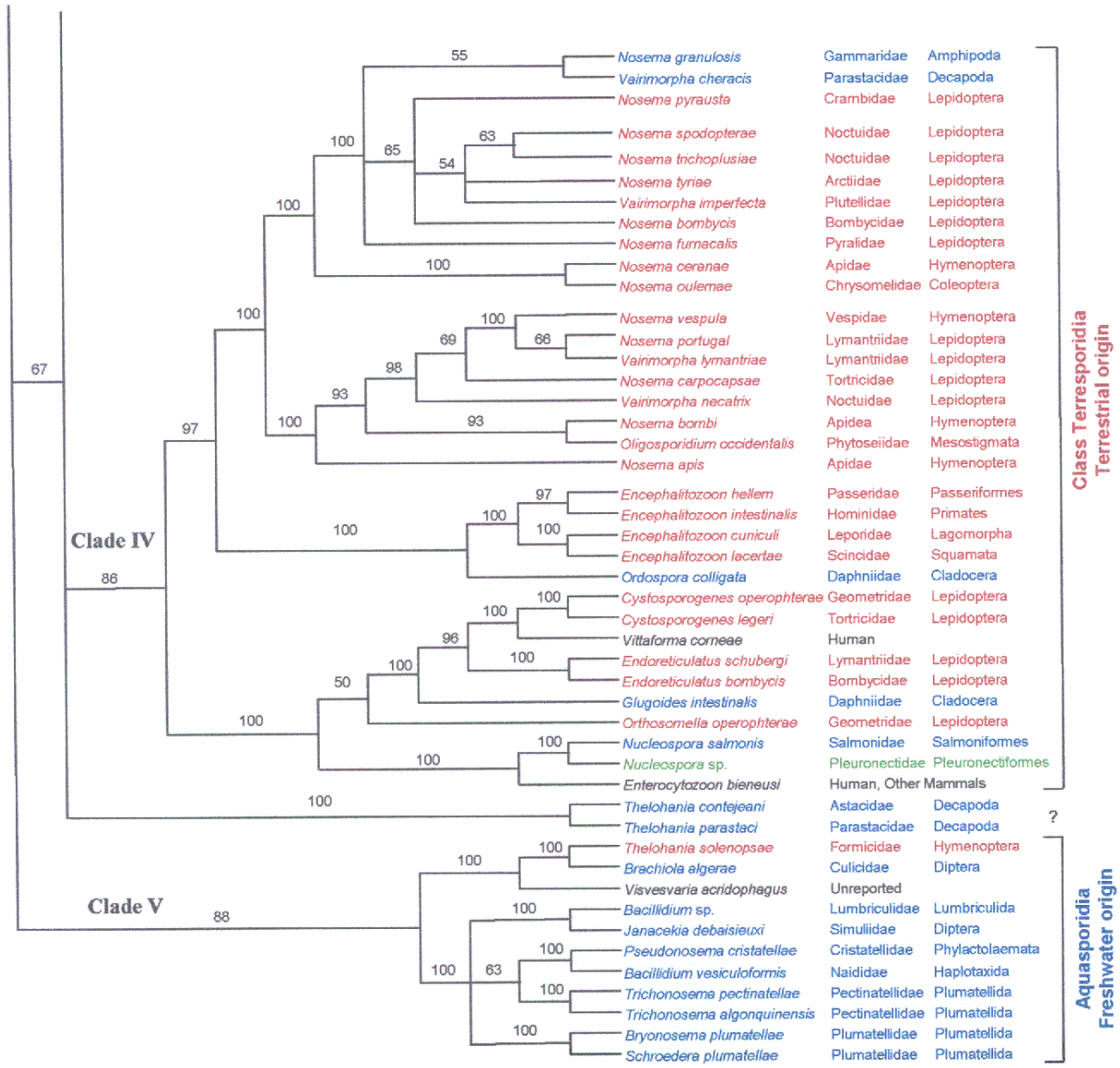
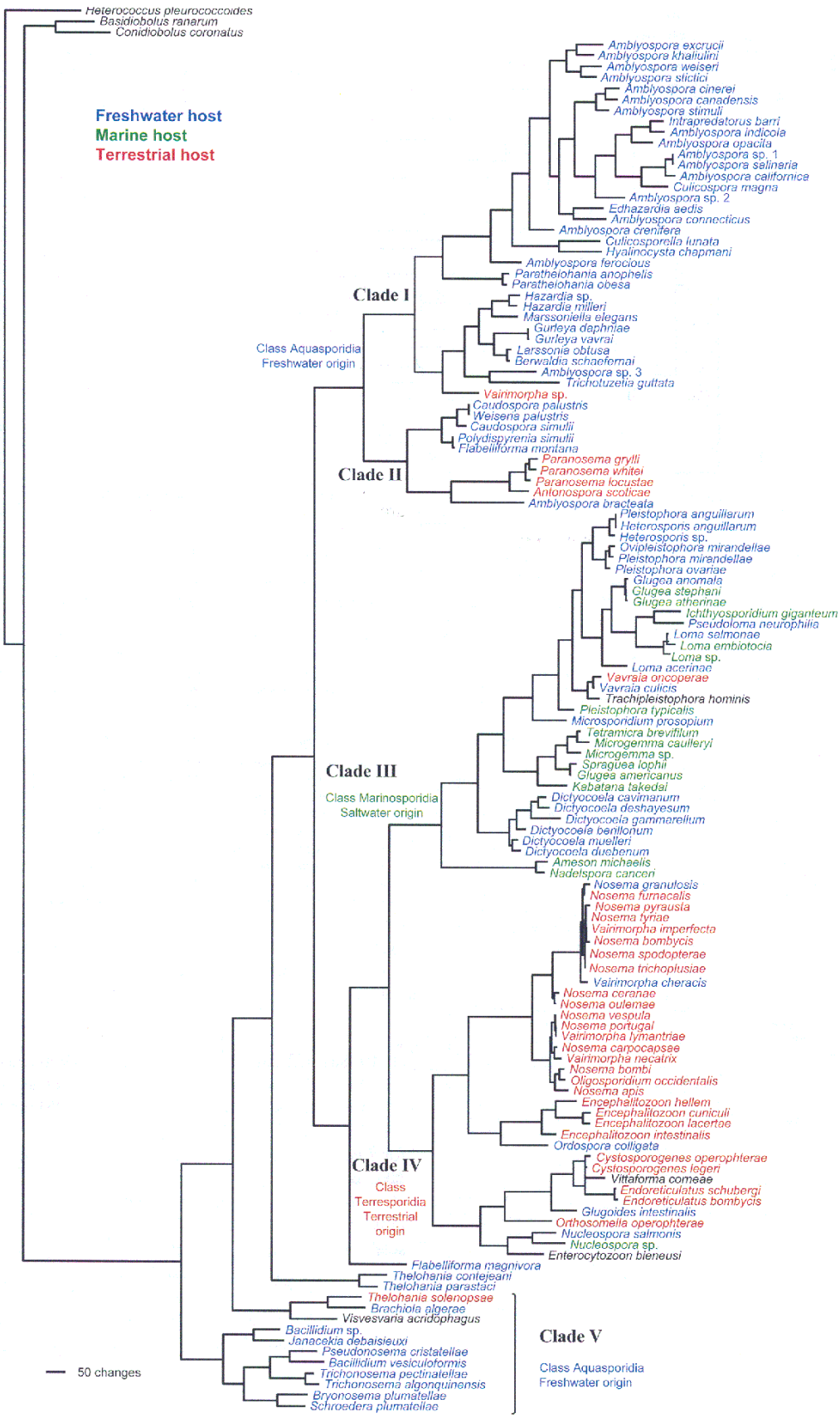


Fig. 1. Bootstrap analysis (25,000 neighbour-joining replicates) of 125 microsporidian isolates and 3 outgroups. Clades I–V are indicated. Microsporidia from freshwater hosts are indicated in blue, from marine hosts in green and from terrestrial hosts in red. Outgroups and microsporidians from human or unreported hosts are in black.

gene of choice for phylogenetic analysis of the Microsporidia. As additional genes are sequenced for a large number of microsporidial species, these new genes may become equal or superior candidates for use in the phylogenetic analysis of the Microsporidia. Various microsporidial genes have been sequenced to explore the phylogenetic placement of the Microsporidia among the eukaryotes (see Weiss and Vossbrinck 1999 for a complete list). These genes include alpha and beta tubulin (Edlind et al. 1996, Keeling and Doolittle 1996), elongation factors 1a/Tu and 2/G (Hashimoto and Hasegawa 1996), RNA polymerase II (Hirt et al. 1999), polar tube protein (Keohane et al. 1998), 5s rRNA (Peyretaillade et al. 1998a), U2 snRNA (DiMaria et al. 1996, Fast et al. 1998), hsp 70 homologue (Peyretaillade et al. 1998b), tRNA synthases (Brown and Doolittle 1995), and chitin synthase (Hinkle et al. 1997). In addition, the genome for *Encephalitozoon cuniculi* has been sequenced (Katinka et al. 2001) and sequencing of the genome for *Paranosema locustae* is partially completed (http://jbpc.mbl.edu/Nosema/). This information may help with the selection of likely gene candidates for phylogenetic analysis, by aiding in the search for genes with common microsporidial primers. Cheney et al. (2001) create a phylogeny based on the largest subunit of RNA polymerase II regions A–G for 14 species, including those


Heterococcus pleurococcoides
Basidiobolus ranarum
Conidiobolus coronatus
Freshwater host
Marine host
Terrestrial host
Amblyospora excrucii
Amblyospora khalulini
Amblyospora weiseri
Amblyospora stictici
Amblyospora cinerei
Amblyospora canadensis
Amblyospora stimuli
Intrapredatorus barri
Amblyospora indicola
Amblyospora opacita
Amblyospora sp. 1
Amblyospora salinaria
Ambiyospora californica
Culicospora magna
Amblyospora sp. 2
Edhazardia aedis
Amblyospora connecticus
Ambiyospora crenifera
Culicosporella lunata
Hyalinocysta chapmani
Amblyospora ferocious
Parathelohania anophelis
Parathelohania obesa
Clade I
Hazardia sp.
Hazardia milleri
Marssoniella elegans
Gurleya daphniae
Gurleya vavrai
Larssonia obtusa
Berwaldia schaefernai
Amblyospora sp. 3
Tricholuzetia guttata
Class Aquasporidia
Freshwater origin
Vairimorpha sp.
Caudospora palustris
Weisenia palustris
Caudospora simulii
Polydispyrenia simulii
Flabelliforma montana
Clade II
Paranosema grylli
Paranosema whitei
Paranosema locustae
Antonospora scoticae
Ambiyospora bracteata
Pleistophora anguillarum
Heterospora anguillarum
Heterosporis sp.
Ovipleistophora mirandellae
Pleistophora mirandellae
Pleistophora ovariae
Glugea anomala
Glugea stephani
Glugea atherinae
Ichthyosporidium giganteum
Pseudoloma neurophilia
Loma salmonae
Loma embiotocia
Loma sp.
Loma acerinae
Vavraia oncoperae
Vavraia culicis
Trachipleistophora hominis
Pleistophora typicalis
Microspondium prosopium
Tetramicra brevifilum
Microgemma caulleryi
Microgemma sp.
Spraguea lophii
Glugea americanus
Kabatana takedai
Clade III
Class Marinosporidia
Saltwater origin
Dictyocoela cavimanum
Dictyocoela deshayesum
Dictyocoela gammarellum
Dictyocoela berillonum
Dictyocoela muelleri
Dictyocoela duebenum
Ameson michaelis
Nadelspora canceri
Nosema granulosis
Nosema furnacalis
Nosema pyrausta
Nosema tyrae
Vairimorpha imperfecta
Nosema bombycis
Nosema spodopterae
Nosema trichoplusiae
Vairimorpha cheracis
Nosema ceranae
Nosema oulemae
Nosema vespula
Nosema portugal
Vairimorpha lymantriae
Nosema carpocapsae
Vairimorpha necatrix
Nosema bombi
Oligosporidium occidentalis
Nosema apis
Encephalitozoon hellem
Encephalitozoon cuniculi
Encephalitozoon lacertae
Encephalitozoon intestinalis
Ordospora colligata
Clade IV
Class
Terresporidia
Terrestrial
origin
Cystosporogenes operophterae
Cystosporogenes legeri
Vittaforma comeae
Endoreticulatus schubergi
Endoreticulatus bombycis
Glugoides intestinalis
Orthosomella operophterae
Nucleospora salmonis
Nucleospora sp.
Enterocytozoon bieneusi
Flabelliforma magnivora
Thelohania contejeani
Thelohania parastaci
Thelohania solenopsae
Brachiola algerae
Visvesvaria acridophagus
Bacillidium sp.
Janacekia debaisieuxi
Pseudonosema cristatellae
Bacillidium vesiculoformis
Trichonosema pectinatellae
Trichonosema algonquinensis
Bryonosema plumatellae
Schroedera plumatellae
Clade V
Class Aquasporidia
Freshwater origin
50 changes

from the marine and terrestrial groups. While much of their phylogeny is in agreement with that shown in Fig. 1, including the major division between marine and terrestrial Microsporidia (a member of the aquatic group was not available), there are some differences. *Spraguea lophii*, for example, a parasite of the angler fish *Lophius americanus*, becomes a member of the terrestrial Microsporidia rather than the marine. Cheney et al. (2001) point out that the largest subunit RNA polymerase II sequence of the species they were working with changes much more rapidly than the ssrDNA. We conclude that the RNA polymerase II gene may be much more useful than ssrDNA for examining relationships at the generic and species levels, but for understanding the basic relationships among the Microsporidia as presented here, ssrDNA may be a better choice. While these studies illustrate that there may not be total phylogenetic agreement among genes, they also highlight the fact that molecular analysis has shown us a tremendous amount about the evolution of the ecological and morphological characters.

Aquatic Microsporidia

The three most divergent taxa of the Microspordia presented in Figs. 1 and 2, designated here as the Class Aquasporidia, are primarily parasites of freshwater organisms. The *Amblyospora*, which have complex life cycles, are members of this group. It will be interesting to see if other groups of the Microsporidia have complex life cycles as suggested by Lom and Nilsen (2003) for Microsporidia of marine organisms. The *Paranosema* species seem to fall within the Aquasporidia. It is possible that the position of *Paranosema* has not been properly resolved, as indicated by low bootstrap values. It would also appear from Figs. 1 and 2 that one of the clades within the Aquasporidia gave rise to those organisms isolated from hosts of the Marinosporidia and Terresporidia.

Marine Microsporidia

The majority of the marine Microsporidia, which we designate here as the Class Marinosporidia, represent parasites of marine hosts. Exceptions include parasites of freshwater fish and the *Dictyocoela* parasites of freshwater amphipods. It could be argued that these Microsporidia were originally parasites of marine organisms whose hosts have adapted to freshwater habitats bringing their parasites with them. The anomalous placements of *Vavraia culicis* and *Vavraia oncoperae* cannot be explained so easily, but might represent broad host transfers from marine to freshwater and terrestrial habitats.

Terrestrial Microsporidia

Isolation of Microsporidia from terrestrial hosts, designated here as the Class Terresporidia, has focused on parasites of insects with economic importance. The *Nosema/Vairimorpha* group parasitize many Lepidoptera as well as Hymenoptera and Coleoptera. The *Encephalitozoon* species represent a taxonomically based clade isolated exclusively from vertebrates (Wright and Craighead 1922) including mammals, birds and reptiles (Koudela et al. 1998). The clade containing *Cystosporogenes* and *Endoreticulatus* represents organisms which infect insects. *Vittaforma corneae* falls within this clade, but was isolated from humans. It has been speculated that *Vittaforma corneae* is not a true human parasite, and that its isolation from humans represents an opportunistic infection. New research indicates that a number of *Nosema/Vairimorpha* species are parasites of aquatic hosts (Terry et al. 1999, Moodie et al. 2003), which may require adjustment of the taxonomy presented here. Based on Figs. 1 and 2, one might hypothesize that *Nosema granulosis* and *Vairimorpha cheracis* are two related *Nosema* species which have evolved from terrestrial Microsporidia. It is possible that other aquatic hosts may be found for members of this group of parasites isolated primarily from terrestrial hosts.

Flabelliforma magnivora and two *Thelohania* species (*T. parastaci* and *T. contejeani*) are also unresolved and will perhaps be resolved in the future after further analysis.

Conclusion

Five major clades with three taxonomic (class) designations in the phylum Microsporidia are identified in the phylograms in Figs. 1 and 2. Assuming a reasonable sequence alignment, the similarities between the neighbour-joining analysis of Fig. 1 and the parsimony analysis of Fig. 2 indicate a fair degree of certainty concerning the relationships among the major groups of Microsporidia presented here. We now see a much clearer picture of the correlation between host and parasite (see host taxa as indicated in Fig. 1). Traditional classification schemes (Sprague 1977) often group together species isolated from disparate hosts. Molecular phylogenetic analysis has revealed that genera such as *Nosema*, *Vairimorpha*, *Amblyospora*, *Thelohania* and *Pleistophora* are polyphyletic in origin, and efforts are being made to reclassify species unrelated to the type species. It has been proposed that the complex life cycle which involves an obligate intermediate host is an ancestral condition and therefore may exist in all groups of Microsporidia (Baker et al. 1997). As derived fungi,

Fig. 2. Maximum parsimony analysis of 125 microsporidian isolates and 3 outgroups using the heuristic search method (one of 30 trees at 13,468 steps). Clades I–V are indicated. The trees generated differed in the relationships among the *Nosema/Vairimorpha* group, and to a small extent, in the relationships among the *Dictyocoela* species. The main branching patterns remained the same for all 30 trees. Microsporidia from freshwater hosts are indicated in blue, from marine hosts in green and from terrestrial hosts in red. Outgroups and microsporidians from human or unreported hosts are in black.

perhaps microsporidia such as *Nosema* species, defined as being diplokaryotic throughout their life cycle, represent organisms which have lost their ability to produce gametes (uninucleate spores). *Pleistophora*, which are uninucleate throughout their life cycle, may represent organisms for which the definitive host has been lost from the life cycle.

We are at an exciting time in the study of the phylogeny and taxonomy of the Microsporidia. Studies (Edlind et al. 1996, Keeling and Doolittle 1996) showing the Microsporidia to be derived fungi have resulted in the inclusion of more appropriate outgroups for the analysis of phylogeny within the Microsporidia (Canning et al. 2002, Refardt et al. 2002). Over the past decade it has become increasingly apparent that diplokaryotic and uninucleate stages can be lost or gained very rapidly over evolutionary time in response to environmental circumstances such as the lack of an intermediate host, or even due to the effect of temperature on development in the case of *Vairimorpha* species (Malone and McIvor 1996). In short, the characters used to distinguish among the higher taxonomic levels of the Microsporidia change state very rapidly and taxonomies based on these characters result in unacceptable polyphyletic clades (clades which are defined by character states which have evolved separately many times and are not characteristic of a common ancestor).

Neither the taxonomic designations given here nor the phylogeny presented in Figs. 1 and 2 represent a final classification. We have included only 125 of the more than 1,200 known species of Microsporidia (Wittner 1999) in our analysis. Many described genera are not included in this phylogeny, and there may be many genera yet undescribed. The Metchnikovellidae, Chytridiopsidae and Hessidae, possible microsporidial outgroups, have not been sequenced. As discussed earlier, several researchers have found true *Nosema* species in aquatic crustaceans, which may necessitate changes in the taxonomy proposed here. In addition further analysis may be needed for rooting the microsporidial tree. Nonetheless, the phylogeny described here reveals a number of relationships among the Microsporidia which were unknown prior to the use of comparative rDNA sequence analysis. Grouping the Microsporidia into classes based on habitat (Aquasporidia, Marinosporidia, Terresporidia) appears more consistent with evolutionary relationships than do previously proposed schemes for higher level classification based on morphology and life-cycle characters.

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