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# α- and β-Tubulin Phylogenies Support a Close Relationship Between the Microsporidia *Brachiola algerae* and *Antonospora locustae*

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ABSTRACT. Microsporidia are a large and diverse group of intracellular parasites related to fungi. Much of our understanding of the relationships between microsporidia comes from phylogenies based on a single gene, the small subunit (SSU) rRNA, because only this gene has been sampled from diverse microsporidia. However, SSUrRNA trees are limited in their ability to resolve basal branches and some microsporidian affiliations are inconsistent between different analyses. Protein phylogenies have provided insight into relationships within specific groups of microsporidia, but have rarely been applied to the group as a whole. We have sequenced α- and β-tubulins from microsporidia from three different subgroups, including representatives from what have previously been inferred to be the basal branches, allowing the broadest sampled protein-based phylogenetic analysis to date. Although some relationships remain unresolved, many nodes uniting subgroups are strongly supported and consistent in both individual trees as well as a concatenate of both tubulins. One such relationship that was previously unclear is between *Brachiola algerae* and *Antonospora locustae*, and their close association with *Encephalitozoon* and *Nosema*. Also, an uncultivated microsporidian that infects cyclopoid copepods is shown to be related to *Edhazardia aedis*.

**Key Words.** *Edhazardia aedis*, *Encephalitozoon cuniculi*, fungi, molecular systematics, parasite.

MICROSPORIDIA are highly derived unicellular parasites thought to be closely related to fungi (Keeling and McFadden 1998). Morphologically, microsporidia are recognized by their distinct spore structure, which contains a suite of specialized structures that mediate infection. However, microsporidia lack canonical eukaryotic characteristics, such as 80S ribosomes, typical mitochondria, and peroxisomes (for reviews see Burri and Keeling 2007; Keeling and Fast 2002). This perceived lack of complexity, coupled with earlier phylogenetic analyses originally suggested that microsporidia are an early diverging lineage of eukaryotes (Cavalier-Smith 1989; Vossbrinck et al. 1987). Subsequent phylogenetic studies have shown that microsporidia are instead highly derived relatives of fungi, although whether they are sisters to fungi or members of fungi is still a matter of debate (Gill and Fast 2006; Hibbett et al. 2007; James et al. 2006; Keeling 2003; Liu, Hodson, and Hall 2006). In light of this, their unusual characteristics have been reinterpreted as resulting from parasitic adaptation to their hosts, rather than being primitive. The nature of the genome provides an extreme illustration of this: some microsporidia have reduced their genomes via gene loss and compaction, giving them the smallest primary eukaryotic genomes – some falling within the range of bacterial genome sizes (Keeling and Slamovits 2005; Keeling et al. 2005).

Our knowledge of the relationships between microsporidia is primarily based on analyses of small subunit rRNA (SSUrRNA) sequences. It remains the most widely sampled microsporidian gene, and for most species, is the only molecular sequence available. Early SSUrRNA phylogenies produced four main groups of microsporidia: Group I represented by *Ichthyosporidium*; Group II represented by *Endoreticulatus*; Group III represented by *Encephalitozoon*; and Group IV represented by *Nosema/Vairmorpha* (Baker et al. 1994, 1995; Keeling and McFadden 1998). In general, Groups III and IV form a clade, and Group II is a sister to this Group III–IV clade, with Group I being most basal. Subsequent analysis including the genus *Amblyospora*, parasites of aquatic larval and copepod hosts with a relatively complex life cycle, suggested this genus is the basal subgroup of microsporidia. This position is suspect because these trees were rooted using very distantly related sequences from *Giardia lamblia* and *Tritrichomonas foetus* (Baker et al. 1997), and indeed later analyses have suggested that the bee-parasite *Antonospora scoticae* is more basal (Fries et al. 1999).

Phylogenetic reconstruction of microsporidian relationships based on protein-coding genes has been limited due to the lack of available sequences. The first analysis of relationships within the group based on protein-coding genes was based on sequences of RPB1, the largest subunit of RNA Polymerase II (Cheney et al. 2001). That study focused on resolving the relationships of polysporous microsporidia, largely limiting the taxa to those species infecting fish (Group I). Some taxa from Group IV were also represented and both groups were shown to be monophyletic. The only other study was based on α- and β-tubulins (Keeling 2003), where a greater overall diversity was sampled, but only included a few members of each of Groups I–IV and none from the putatively basal *Amblyospora* group. These relationships based on tubulin phylogenies were in agreement with previous classifications based on SSUrRNA (Baker et al. 1995, 1997).

Many more microsporidian SSUrRNA-based analyses have been performed since then, with variable degrees of sampling, and focusing on specific subgroups (Elizabeth-McClymont et al. 2005; Lom and Nilsen 2003; Refardt et al. 2002; Vossbrinck et al. 2004). Vossbrinck and Debrunner-Vossbrinck (2005) also conducted a comprehensive SSU rRNA study comprised of over 125 microsporidian species that included members of *Amblyospora* and Groups I–IV. These analyses suggest five clades, and also three natural classes of microsporidia that coincide with the habitats of their respective hosts. These analyses supported several conclusions: (1) the most basal clade includes species infecting freshwater-dwelling hosts, suggesting an aquatic origin for microsporidia; (2) the ''freshwater'' class of microsporidia is polyphyletic (Vossbrinck et al. 2004); (3) a sister relationship between microsporidia infecting largely marine- and those infecting terrestrial-dwelling hosts (Cheney et al. 2001); and (4) the basal position of the clade consisting of *Amblyospora* species is not supported (Lom and Nilsen 2003; Refardt et al. 2002; Vossbrinck and Debrunner-Vossbrinck 2005).

Indeed, the position of the root of microsporidia is problematic, as to which microsporidian lineages are basal remains unclear and in some cases evidence is contradictory. The *Amblyospora* clade,

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which includes *Edhazardia aedis*, has long been considered to be the most basal (Baker et al. 1997). However, the *Brachiola algerae* lineage has most recently been suggested to be part of the most basal clade (Vossbrinck and Debrunner-Vossbrinck 2005). Placement of *Antonospora locustae* has also been problematic due to its association with *A. scoticae*, which has been shown to be early diverging (Fries et al. 1999; Lom and Nilsen 2003). We should note that we use *B. algerae* and *A. locustae* as these names are in common usage, however these species are also sometimes referred to as *Anncaliia algerae* and *Paranosema locustae*, respectively (Franzen et al. 2006; Sokolova et al. 2003). Here we assess microsporidian relationships using the α- and β-tubulins, independently and combined. We sampled microsporidia that were not included in previous protein analyses, including *E. aedis*, *B. algerae*, and an uncultivated copepod-infecting microsporidian, in an attempt to determine phylogenetic affinities between representatives of major subgroups.

## MATERIALS AND METHODS

**Strains.** *Edhazardia aedis* spores were cultured from *Aedes aegypti* larvae and were a generous gift from Dr. James Becnel (United States Department of Agriculture, Gainesville). *Brachiola algerae* spores were a generous gift from Dr. Louis Weiss (Albert Einstein College of Medicine, New York City). Spores from an unclassified microsporidium were isolated from freshwater cyclopoid copepods from a roadside ditch in Vancouver, British Columbia, Canada. We refer to this undescribed species by the initials AMVB (Brown 2005).

**DNA isolation, PCR, and sequencing.** *Edhazardia aedis* and *B. algerae* spores were ruptured by glass bead beating, and genomic DNA was purified by the standard phenol–chloroform method. For the microsporidian AMVB, spores were heated in lysis buffer (10 mM Tris, 1 mM EDTA, 10 mM NaCl, 1% SDS) at 100 °C and digested with 0.5 mg/ml proteinase K before phenol–chloroform extraction. α-tubulin genes were amplified from *E. aedis*, *B.algerae*, and AMVB using the primers 5′-TCCGAATTCARGTNGG NAAYGCNGGYTGGGA-3′ and 5′-CGCGCCATNCCYTCNCC NACRTACCA-3′. β-tubulin genes were amplified from *E.aedis*, *B. algerae*, and AMVD using the primers 5′-GCCTGCAGGNCART GYGGNAAYCA-3′ and 5′-GGCCTCAGTRAAYTCCATYTCRT CCAT-3′. PCR products that were well separated on agarose gels were excised and purified using Ultraclean15 MOBIO DNA purification kit (BIO/CAN Scientific, Mississauga, On, Canada) according to the manufacturer's instructions. Amplicons were cloned into the pCR 2.1 vector using the TOPO TA Cloning kit (Invitrogen, Burlington, ON, Canada). Several independent clones were sequenced on both strands using ABI's Big Dye 3.1 chemistry. New α-tubulin sequences have been deposited into GenBank: *E. aedis* (EU486986), *B. algerae* (EU625354), and AMVB (EU625356). New β-tubulin sequences have also been deposited: *E. aedis* (EU486987) and *B. algerae* (EU625355). AMVB has two different β-tubulins which have been deposited into GenBank as EU625357 and EU625358 – the ORF translations between the two versions differ at only four positions, and only EU625357 was used for phylogenetic analyses.

**Phylogenetic analyses.** New microsporidian sequences obtained in this study and all available microsporidian sequences in GenBank were added to existing α- and β-tubulin protein alignments in MacClade and edited by eye (Maddison and Maddison 1989). The α-tubulin alignment was composed of 13 taxa and consisted of 373 characters. For the β-tubulin alignment there were 15 taxa and 325 characters. Phylogenies were inferred from these alignments using maximum likelihood (ML), maximum likelihood-distance (ML-D), and Bayesian methods. In addition, phylogenies were also inferred by combining α- and β-tubulin, as in a previous and similar phylogenetic study (Keeling 2003). For the combined analyses, there were 13 taxa and 698 characters. Additional analyses were carried out to test fungal outgroups and these trees possessed similar in-group topologies to those found in the unrooted trees. The exception, however, was that the rooted trees recovered inconsistent and erroneous basal microsporidian lineages. Therefore, trees were left unrooted.

For ML, all analyses were performed using the JTT substitution matrix with eight γ rate categories plus one invariable (Jones, Taylor, and Thornton 1992). The parameter α and the proportion of invariable sites were calculated using Tree-Puzzle version 5.2 (Schmidt et al. 2002). The α-parameters were determined to be 1.37, 0.79, and 0.98 for α-tubulin, β-tubulin, and combined. The fractions of invariable sites were estimated as 0.35, 0.30, and 0.32, respectively. ML analyses were performed using PROML version 3.6b (Felsenstein et al. 2000) with slow analyses, global rearrangements, randomized input orders, and with jumbling 10 times as the settings. For ML-D analyses, distances were calculated with Puzzleboot (M. Holder and A. Roger, http://www.tree-puzzle.de) and the tree constructed using Fitch version 3.6b (Felsenstein et al. 2000) with global rearrangements, randomized input orders, and jumbling 10 times. Bootstrapping was performed with Phyml (Guindon and Gascuel 2003) for ML analyses, and for ML-D analyses as described above using Puzzleboot (http://www.tree-puzzle.de), with 100 bootstrap replicates performed using each method for all three datasets.

For Bayesian analyses, MrBayes version 3.0b4 (Huelsenbeck and Ronquist 2001) was used with options set for JTT substitution, eight γ rate categories and proportion of invariable sites, and four Markov Chain Monte Carolo (MCMC) chains. For each analysis, MrBayes was used to perform 2,000,000 generations, with trees sampled every 1,000 generations and with a prior burn-in of 400,000 generations. After the 400 sampled trees were discarded, a majority rule consensus tree was constructed from the 1,601 post-burn-in trees.

Alternative topologies differing in the relative positions of *E. aedis*, AMVB, *Spraguea lophii*, *B. algerae*, and *A. locustae* were generated with MacClade for the combined tubulin dataset. Approximately unbiased (AU) tests (Shimodaira 2002) were performed with Consel (Shimodaira and Hasegawa 2001) using the site likelihoods determined previously by Tree-Puzzle and evaluated at a 5% significance level.

## RESULTS

**α-tubulin and β-tubulin trees.** Based on a 373-character alignment of 13 α-tubulin sequences, phylogenetic trees were inferred. The inferred ML-tree generated the same topology as the Bayesian analysis (not shown). Many of the expected relationships were recovered: *Encephalitozoon, Nosema*, and *Endoreticulatus* form a clade, although the genus *Encephalitozoon* is unexpectedly polyphyletic, with no support (Fig. 1). The expected pairing of *Glugea plecoglossi* and *Trachipleistophora hominis* was also recovered with high support. Novel relationships were also recovered, including the strongly supported sisterhood of *E. aedis* and AMVB, and also of *A. locustae* and *B. algerae*. The latter clade also branches with moderate support as a sister to the *Nosema/Encephalitozoon/Endoreticulatus* grouping (Groups II–IV).

Two expected relationships were not recovered: *S. lophii*'s affiliation with other Group I microsporidia, such as *Trachipleistophora* and *Glugea*, was not supported, and an absence of a clear sister relationship between Group I microsporidia and Groups II–IV. These regions of the α-tubulin tree were generally unsupported and no strongly supported alternative relationships were recovered.

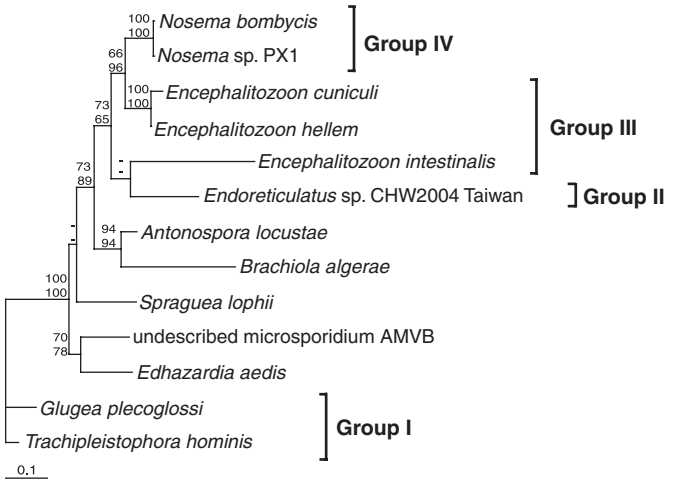


Fig. **1.** γ-corrected protein maximum likelihood (ML) phylogeny of microsporidia based on α-tubulin protein sequences. Bootstrap support values are presented as percentages at each node, and are shown if >60%. Support values are shown for ML (upper value) and maximum likelihood-distance (lower value). Schematic groupings indicated by the brackets represent common microsporidial divisions suggested by others (Baker et al. 1994, 1995; Keeling and McFadden 1998).

Based on a 325-character alignment of 15 taxa, the β-tubulin phylogenies inferred from ML (Fig. 2) and Bayesian analyses (not shown) were congruent. The β-tubulin phylogeny shares many branches in common with that of α-tubulin: *Glugea* and *Trachipleistophora* branch together with strong support, as do *E. aedis* and AMVB. Group II microsporidia such as *Vittaforma corneae* and *Enterocytozoon bieneusi*, group together and this clade is sister to the *Nosema/Encephalitozoon* (Groups III–IV) lineage, forming a strongly supported monophyletic group. Unlike α-tubulin, *Encephalitozoon* is monophyletic in β-tubulin phylogeny, as expected. Similarly, the sisterhood of *A. locustae* and *B. algerae* is not recovered, although the position of *S. lophii* between them is not strongly supported (Fig. 2).

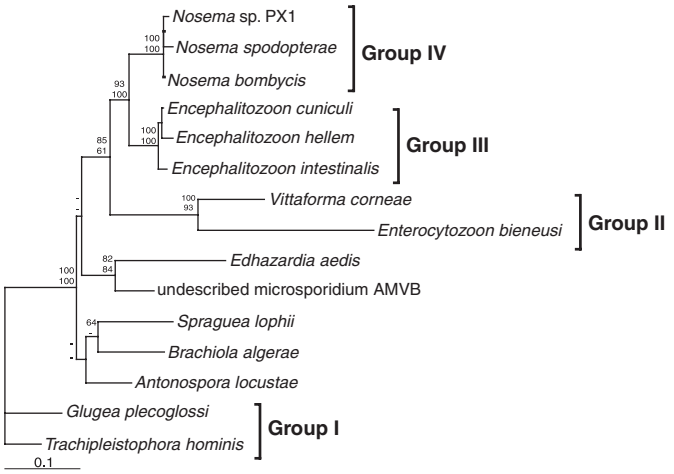


Fig. **2.** γ-corrected protein maximum likelihood (ML) phylogeny of microsporidia based on β-tubulin protein sequences from microsporidia. Bootstrap support values are presented as percentages at each node, and are shown if >60%. Support values are shown for ML (upper value) and maximum likelihood-distance (lower value). Schematic groupings indicated by the brackets represent common microsporidial divisions suggested by others (Baker et al. 1994, 1995; Keeling and McFadden 1998).

**Combined trees.** The two tubulin trees share many nodes in common, but many nodes also lack support, so we combined the two genes to infer a single tree. The combined analysis largely supports and strengthens the relationships recovered in the individual phylogenies (Fig. 3). Briefly, Groups III and IV group with strong support, and three other nodes of interest are recovered with high support: *T. hominis* with *G. plecoglossi* (Group I), *E. aedis* with AMVB, and a monophyletic group containing *A. locustae* and *B. algerae*, which are together a sister to the *Nosema/Encephalitozoon* clade (Groups III–IV).

To further examine the significance of several of these groups, AU tests were performed on the combined data set. *Spraguea lophii*, *E. aedis*, AMVB, *A. locustae*, and *B. algerae* were all individually moved to all possible positions in the tree in order to assess the likelihood of alternative topologies. For *A. locustae, E. aedis* and AMVB, all alternatives (other than their position as shown in Fig. 3) were rejected at the 5% level. For *S. lophii* and *B. algerae* most alternative positions were also rejected, but with two exceptions: (1) *B. algerae* basal to an *A. locustae* plus *Nosema/Encephalitozoon* group was not rejected ($P = 0.072$) and (2) *S. lophii* as a sister to the *Antonospora/Brachiola* grouping was not rejected ($P = 0.17$).

## DISCUSSION

In general, tubulin phylogenies are in agreement with microsporidian relationships that have been proposed based on analyses of SSU rRNA sequences. In addition, the tubulin phylogenies suggest two unexpected relationships that were previously unclear from SSU rRNA. The first is a relationship between *A. locustae* and *B. algerae*. This result is strongly supported in both the α-tubulin tree and in the combined analysis, although in β-tubulin trees the unsupported position of *S. lophii* disrupts this relationship. The same relationship was weakly recovered in a previous SSU rRNA analysis (Slamovits, Williams, and Keeling 2004), so these results are not contrary to SSU rRNA analyses, which are not clearly resolved on the position of either taxon. Second, tubulin trees suggest *A. locustae* and *B. algerae* are together sisters to the large clade consisting of Groups II–IV. This relationship was strongly supported in the combined tree, and received moderate support in the α-tubulin tree. This association is also supported by

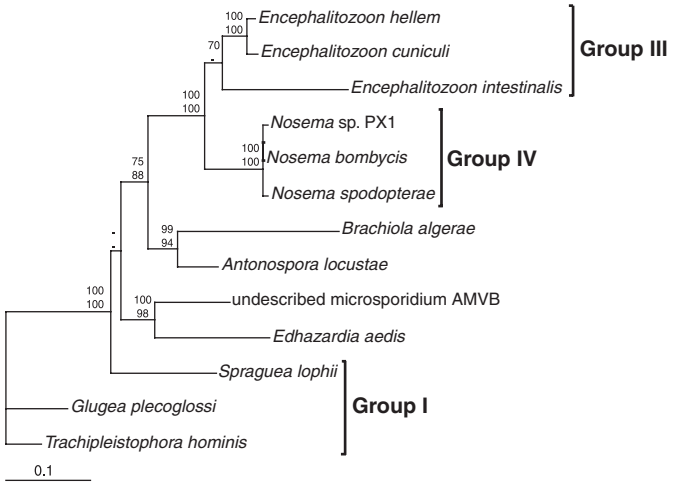


Fig. **3.** γ-corrected protein maximum likelihood phylogeny of microsporidia based on combined α- and β-tubulin protein sequences. Bootstrap support values are presented as percentages at each node, and are shown if >60%. Support values are shown for maximum likelihood (upper value) and maximum likelihood-distance (lower value). Schematic groupings indicated by the brackets represent common microsporidial divisions suggested by others (Baker et al. 1994, 1995; Keeling and McFadden 1998).

the fact that these microsporidia possess many shared characteristics, including diplokaryotic merogony, disporoblastic merogony, and development in direct contact with the host cytoplasm (Slamovits et al. 2004; Visvesvara et al. 2005). In fact, these morphological features were used to previously place both *A. locustae* and *B. algerae* within the *Nosema* clade (both *A. locustae* and *B. algerae* were previously classified as species of *Nosema*) (Baker et al. 1994). Although both molecular and morphological evidence now make it clear that *A. locustae* is not a member of the *Nosema* group (Slamovits et al. 2004; Sokolova et al. 2003), the relationship we propose based on tubulin phylogenies suggests that some of the characteristics they share may be derived from a common ancestor after all.

Both *B. algerae* and *A. locustae* have also previously been proposed to be among the more basal microsporidian lineages (Fries et al. 1999; Lom and Nilsen 2003; Slamovits et al. 2004; Vossbrinck et al. 2004). However, the sisterhood of *A. locustae*/*B. algerae* plus *Nosema*/*Encephalitozoon*, coupled with the derived characters found within the *Nosema*/*Encephalitozoon* group (Katinka et al. 2001; Vossbrinck and Debrunner-Vossbrinck 2005), would seem to argue against this notion as it would suggest that all other microsporidia were derived from this group and had lost the characters they share. Analysis of tubulin trees cannot suggest an alternative because they were not rooted.

Another novel and strongly supported grouping recovered is the pairing of *E. aedis* with the uncultivated, copepod parasite AMVB. *Edhazardia aedis* infects the mosquito *A. aegypti*, a known carrier of the yellow fever and dengue hemorrhagic fever viruses. The *Ambylospora* clade, of which *E. aedis* is part, contains microsporidia with the most complex life cycles. For example, life cycles of members of this clade often require horizontal and vertical routes of transmission, produce multiple distinct spore types, require an intermediate copepod host, and require multiple host generations (Becnel, White, and Shapiro 2005). Although the life cycle of *E. aedis* is moderately complex, involving at least four spore types and often two generations of its mosquito host, it does not require an intermediate copepod host (Becnel et al. 2005). The uncultivated and little-studied microsporidian AMVB was isolated from a copepod, but other aspects of its life cycle are unknown (Brown 2005). However, linking AMVB with *Amblyospora* as proposed here is reasonable given previous SSU rRNA phylogenies that show a strong relationship between this unclassified microsporidian and *Marsoniella elegans* (Brown 2005): *M. elegans* is known to branch as a sister to the *Amblyospora* clade (Vossbrinck et al. 2004) and like *M. elegans*, AMVB has spores arranged within a distinct mucocalyx structure and infects a cyclopoid copepod (Brown 2005).

Currently, microsporidian sequence data are limited, but as more genome sequencing projects and EST surveys are carried out on diverse microsporidian species, there will be increased opportunities for undertaking protein phylogenies based on combining multiple markers. Such studies will likely allow for a much greater degree of phylogenetic resolution. Our understanding of microsporidian relationships would also benefit from including sequences from an appropriate outgroup. Whether this will be a fungus or perhaps the metchnikovellids, proposed to be the most ancestral microsporidia (Sprague 1977; Vossbrinck and Debrunner-Vossbrinck 2005) is not clear. It also remains possible that a period of generally accelerated rates of molecular sequence substitution around the time microsporidia originated will make it very difficult to find a molecule that links the microsporidia to other eukaryotes sufficiently closely to allow outgroup sequences to be used.

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