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ScienceDirect
Molecular Phylogenetics and Evolution 47 (2008) 339–352
MOLECULAR PHYLOGENETICS AND EVOLUTION
www.elsevier.com/locate/ympev

Phylogenetic placement of diverse amoebae inferred from multigene analyses and assessment of clade stability within 'Amoebozoa' upon removal of varying rate classes of SSU-rDNA

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Received 14 August 2007; revised 2 November 2007; accepted 19 November 2007
Available online 3 December 2007

Abstract

Placing amoeboid lineages on the eukaryotic tree of life is difficult due to the paucity of comparable morphological characters and the limited molecular data available for many groups. This situation has led to the lumping of distantly related lineages into large inclusive groups, such as Sarcodina, that do not reflect evolutionary relationships. Previous analyses of molecular markers with limited taxon sampling reveal members of Sarcodina are scattered in five of the six proposed supergroups. We have used multigene analyses to place seven diverse amoeboid lineages—two *Nolandella* spp., *Rhizamoeba* sp., *Pessonella* sp., *Arcella hemisphaerica*, *Arachnula* sp. and *Trichosphaerium* sp.—on the eukaryotic tree of life. Bayesian analysis of the concatenated data of the four genes sequenced (SSU-rDNA, actin, alpha-tubulin and beta-tubulin), including diverse representatives of eukaryotes, indicates that all seven taxa group within the 'Amoebozoa' supergroup. We further performed separate analyses of the well-sampled SSU-rDNA and actin genes using Bayesian and Maximum Likelihood analyses to assess the positions of our newly characterized taxa. In the case of SSU-rDNA, we performed extensive analyses with removal of the fastest rates classes to evaluate the stability and resolution of various taxonomic hypotheses within 'Amoebozoa'. Five of our seven amoeboid lineages fall within well-supported clades that are corroborated by morphology. In contrast, the positions of *Arachnula* sp. and *Trichosphaerium* sp. in the SSU-rDNA gene trees are unstable and vary by analyses. Placement of these taxa will require additional data from slowly evolving genes combined with taxon-rich phylogenetic analyses. Finally, the analyses without the fastest rate classes demonstrate that SSU-rDNA has a limited signal for deep relationships within the 'Amoebozoa'.

Keywords: Amoeba; Sarcodina; Multigene analysis; SSU-rDNA; Unicellular eukaryote; Supergroups

1. Introduction

The term amoeba is used to describe the many unicellular eukaryotes that move by temporary cytoplasmic projections known as pseudopods. The taxonomy of amoeboid lineages has had a turbulent history creating substantial confusion in the literature (Schaeffer, 1926; Singh, 1955; Loeblich and Tappan, 1961; Jahn and Bovee, 1965; Jahn et al., 1974; Bovee, 1985; Page and Blanton, 1985; Page, 1986, 1987; Rogerson and Patterson, 2002; Cavalier-Smith, 2000, 2003; Cavalier-Smith et al., 2004; Smirnov et al., 2005; Adl et al., 2005). Amoeboid lineages traditionally placed in broad taxonomic groups such as the Sarcodina (Schmarda, 1871) are now found scattered in five of the six proposed eukaryotic supergroups (Baldauf et al.,

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doi:10.1016/j.ympev.2007.11.015

2000; Simpson and Roger, 2002; Nikolaev et al., 2004; Adl et al., 2005), though many lineages remain homeless (Patterson, 1999; Smirnov et al., 2005; Nikolaev et al., 2006; Cavalier-Smith et al., 2004). Comparative systematics has failed for over a century to provide a robust comprehensive higher-level taxonomic framework for amoeboid protists, despite considerable effort (Bovee, 1953; Flickinger, 1974; Page, 1978, 1980, 1985, 1986, 1987, 1988, 1991; Page and Blakey, 1979; Page and Blanton, 1985; Rogerson and Patterson, 2002). This is due to few comparable morphological characters, though ultrastructural identities have been reported for lower-level taxonomic groups (Page, 1983, 1988; Patterson, 1999; Rogerson and Patterson, 2002). Molecular systematics is introducing new comparable characters that will ultimately yield insights into a more natural higher-level classification of amoeboid protists.

The supergroup 'Amoebozoa', whose members are the focus of this study, emerged from analyses of molecular data (e.g. Cavalier-Smith, 1998; Fahrni et al., 2003; Kudryavtsev et al., 2005; Nikolaev et al., 2006). The 'Amoebozoa' lack a clear morphological synapomorphy though they share a few broadly defined morphological characters such as dynamic pseudopodia and branched tubular mitochondrial cristae (Cavalier-Smith, 1998). This putative supergroup includes many of the naked amoebae (e.g. *Amoeba proteus*), testate lobose amoebae (e.g. *Arcella*) and pelobionts (e.g. *Pelomyxa*, *Entamoeba histolytica*) as well as cellular (dictyostelid), acellular (e.g. myxogastrid) and protostelid slime molds.

We performed multigene analyses to investigate the phylogenetic placement of seven newly characterized taxa within the eukaryotic tree of life. Multigene analyses are proving useful as a means of placing eukaryote lineages within phylogenies where morphological evidence and single gene analyses have not been successful (e.g. Nikolaev et al., 2004; Tekle et al., 2007). We obtained sequences of SSU-rDNA as well as three protein-coding genes (actin, alpha-tubulin and beta-tubulin) of several amoebae. Specifically, we characterized genes from two amoebae of unknown phylogenetic position: *Arachnula* sp. and *Trichosphaerium* sp. and other taxa including two *Nolandella* spp., *Pessonella* sp., *Rhizamoeba* sp. and *Arcella hemisphaerica* for which no molecular data were previously available.

We further performed single gene analyses of the relatively well-sampled genes (actin and SSU-rDNA) to determine the position of our newly characterized sequences within the supergroup 'Amoebozoa'. Particularly, the SSU-rDNA has been used as the basis for identifying major clades within the 'Amoebozoa' including Tubulinea, Flabellinea and Conosea (see Smirnov et al., 2005), though the relationship among these lineages is unknown and many of these clades are poorly supported. It is not known whether the lack of resolution in 'Amoebozoa' SSU-rDNA gene trees is due to the considerable rate heterogeneity and length variation among members of this supergroup. To better understand the evolution of 'Amoebozoa' and to test the validity of the different hypotheses, we performed extensive analyses of the SSU-rDNA data set with systematic removal of the fastest evolving rate classes (Philip et al., 2005). Ultimately, the validity of the clades based on SSU-rDNA analyses that conflict with morphology will require corroboration from other sources, including additional genes.

2. Materials and methods

2.1. Taxa studied and morphology

Arachnula sp. ATCC® 50593, *Nolandella* sp. ATCC® 50913, *Nolandella* sp. ATCC® PRA-27, *Pessonella* sp.

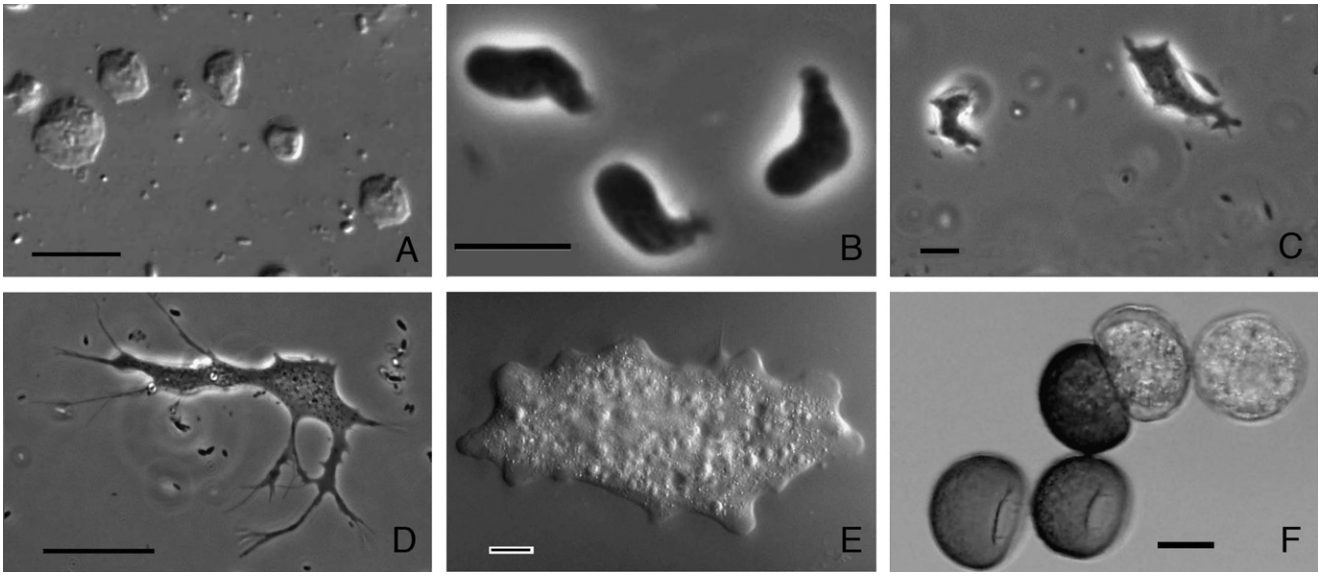

Fig. 1. Light micrographs: (A) *Pessonella* sp. ATCC® PRA-29 (scale bar = 10 μm). (B) *Nolandella* sp. ATCC® 50913 (scale bar = 5 μm). (C) *Rhizamoeba* sp. ATCC® 50933 (scale bar = 5 μm). (D) *Arachnula* sp. ATCC® 50593 (scale bar = 10 μm). (E) *Trichosphaerium* sp. ATCC® 40318 (scale bar = 20 μm). (F) *Arcella hemisphaerica* (scale bar = 25 μm).

ATCC® PRA-29, 50933, *Trichosphaerium* sp. ATCC® 40318 and *Rhizamoeba* sp. ATCC® 50933 were obtained from the American Type Culture Collection (Manassas, VA). *Arcella hemisphaerica* was isolated from a culture of *Arcella vulgaris* (Carolina Biological Supply Company Catalogue No. 13-1310).

All species, except *Arcella hemisphaerica*, were identified only to the genus level based on preliminary gross light microscopy (Fig. 1), as well as, TEM analyses for three taxa (Fig. 2). There is compelling evidence that the *Rhizamoeba* sp., *Arachnula* sp., *Pesssonella* sp., and both isolates of *Nolandella* are new species. A formal description

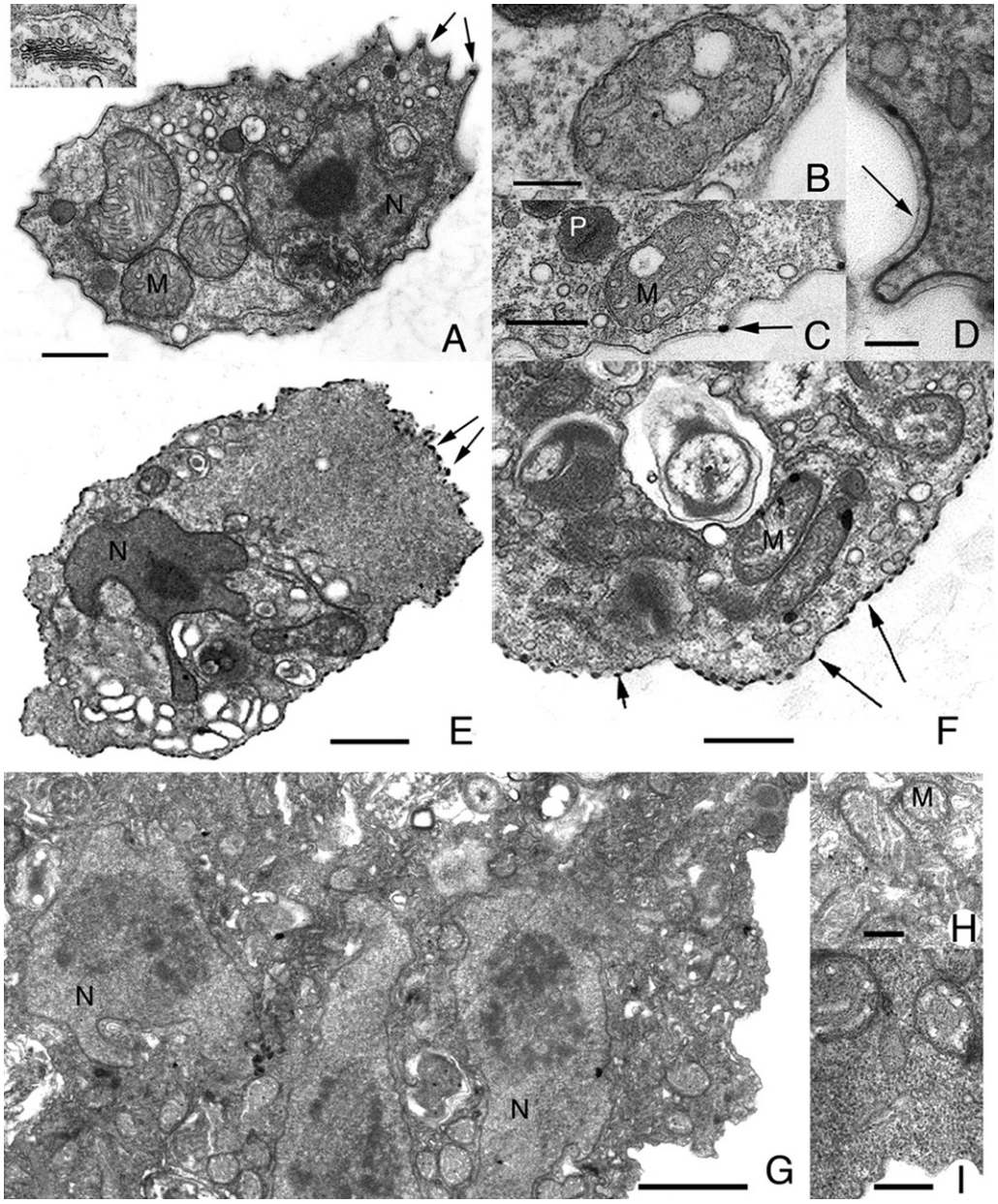

Fig. 2. Electron micrographs: (A–D) *Nolandella* sp. ATCC® 50913, (E–F) *Rhizamoeba* sp. ATCC® 50933, (G–I) *Arachnula* sp. ATCC® 50593 (A) section of *Nolandella* cell showing the nucleus (N), tubulocristate mitochondria (M) and electron-dense 50 nm collosomes (arrows). Inset: detail of Golgi body, typically nestled within a concave portion of the nuclear envelope (scale bar = 0.5 μm). (B and C) Details of mitochondria (M) showing tubular cristae. Peroxisomes (P) contain internal lamellae. Occasional dense collosomes (arrow) are located on protrusions of the plasma membrane (scale bars = 0.1 and 0.5 μm, respectively). (D) High magnification image of the cell surface showing the characteristic 30 nm thick cuticle (arrow) of *Nolandella* (Scale bar = 0.1 μm). (E) Section of *Rhizamoeba* sp. cell showing the irregular perimeter of the nucleus (N) with an elongated protrusion. Collosomes (arrows) are scattered along the plasmalemma (scale bar = 1 μm). (F) Detail of the cytoplasm and cell surface showing tubulocristate mitochondria (M) and dense collosomes (arrows) on the cell surface (scale bar = 0.5 μm). (G) Overview of nuclear region of *Arachnula* sp. Showing dense cytoplasm and multiple, irregularly shaped nuclei (N), each with a large centrally located nucleolus (scale bar = 1 μm). (H) Branching tubulo-cristate mitochondria (M) (scale bar = 0.5 μm). (I) Enlarged view of mitochondrial cristae and cell periphery, exhibiting the naked plasma membrane (scale bar = 0.5 μm).

of these isolates is underway and will be published elsewhere. Here we provide brief preliminary morphological descriptions to complement the genetic data and provide additional verification of their taxonomic identification using light microscopic and transmission electron microscopy (TEM) preparations (Anderson et al., 1997).

Pessonella sp. has hyaloplasmic cone-like bosses typical of the genus (Fig. 1A), though the bosses in our isolate are less numerous compared to the type species. The morphology of our isolate *Arcella hemisphaerica* (Fig. 1F) fits the description of Deflandre (1928).

Three of the amoebae (*Nolandella* sp., *Rhizamoeba* sp. and *Arachnula* sp.) have tubulocristate mitochondria (Fig. 2A–C, F, H and I) and the nucleolus is centrally located in the nucleus (Fig. 2A, E and G). *Nolandella* sp. ATCC® 50913 is considerably smaller relative to the type species, though the cuticle is characteristic of the genus, consisting of closely spaced subunits that produce a hyaline layer with thin electron dense boundary (Fig. 2D). A Golgi body is nestled within a concave depression of the nuclear envelope (Fig. 2A) and beneath the cell surface of this isolate are regularly spaced numerous small electron dense collosomes (Fig. 2A).

The *Rhizamoeba* ATCC® 50933 isolate had been tentatively assigned to the genus *Biomyxa* when deposited, but based on the TEM and molecular genetic evidence, we transferred it to the genus *Rhizamoeba*. This *Rhizamoeba* sp. (c. 6–10 μm) is smaller than other described species and is characterized by a comet-shaped nucleus, electron dense collosomes and an uncoated surface (Fig. 2E and F).

Arachnula ATCC® 50593 is a multinucleate amoeba with branching pseudopodia (Fig. 1D) and has a dense, finely granular cytoplasm fenestrated by hyaline lacunae that may represent deep penetrations of the surface plasmalemma (Fig. 2G). The *Arachnula* sp. ATCC® 50593 (Fig. 1D) isolate studied here matches the description for the genus in that it forms large multinucleate sheets (not illustrated).

Our strain of *Trichosphaerium* ATCC® 40318 has a non-spiculate, membranous test as in all described gamonts. The dactylopodia (Fig. 1E) are a common feature of all *Trichosphaerium* species at all stages. Further our SSU-rDNA sequences are very similar (~95% identical, with polymorphisms largely confined to variable regions among our three sequences (see Section 3.4.1 below)) to the sequence obtained from the smooth stage of another isolate *Trichosphaerium sieboldi* CCAP 1585/2 (Pawlowski and Fahrni, 2007; Jan Pawlowski, pers. comm., 2007).

2.2. DNA extraction, PCR amplification, alignment and phylogenetic analysis

DNA samples of *Nolandella* sp. ATCC® 50913, *Nolandella* sp. ATCC® PRA-27, *Rhizamoeba* sp., *Pessonella* sp., *Arcella hemisphaerica*, *Arachnula* sp. and *Trichosphaerium* sp. were extracted using DNA Stat60™ (Tel-Test, Inc., Friendswood, Texas, Cat. No. TL-4220) per manufacture's instructions and with the addition of a phenol–chloroform-isoamyl step using Phase Lock Gel Heavy tubes (Eppendorf AG, Hamburg, Germany, Cat. No. 955154070). Primers for SSU-rDNA genes are from Medlin et al. (1988) with three additional primers used to generate overlapping sequences from each clone as described in Snoeyenbos-West et al. (2002). Primers for actin, alpha-tubulin and beta-tubulin are from Tekle et al. (2007). Phusion DNA Polymerase, a strict proofreading enzyme, was used to amplify the genes of interest and Lucigen PCRSmart, Novagen Perfectly Blunt and Invitrogen Zero Blunt Topo cloning kits were used for cloning. Sequencing of cloned plasmid DNA, was accomplished using vector- or gene-specific primers and the BigDye terminator kit (Perkin-Elmer). Sequences were run on an ABI 3100 automated sequencer. We have fully sequenced 2–4 clones of each gene and surveyed up to 8 clones per taxon in order to detect paralogs.

To align SSU-rDNA sequences, we used the Hmmer package (Eddy, 2001), version 2.1.4 with default settings. Hmmer used a set of previously aligned sequences to model the secondary structure of a sequence. The training alignment for building the model, consisting of all available SSU-rDNA eukaryote sequences (as of December 2006) aligned according to their secondary structure (for the concatenated analyses) and all 'Amoebozoa' and some representative of the outgroup taxa (Opithokonta) (for the separate analysis), were downloaded from the European Ribosomal Database (Wuyts et al., 2002). The alignment was further edited manually in MacClade v4.05 (Maddison and Maddison, 2002).

Insertions and variable regions in the SSU-rDNA alignment that could not be aligned unambiguously were removed and two sets of data matrices were prepared that differed only in the number of characters included: conservative mask (1238 bp retained) and liberal mask (1483 bp retained) (Table 1). The liberal mask data set was prepared to assess phylogenetic inferences from regions conserved in the majority of the ingroup taxa but where there was some ambiguity in positional homology for most divergent taxa. We hoped inclusion of more characters of an alignment based on secondary structure of the molecule, might contribute useful signal. Protein-coding genes (including paralogs) were aligned as amino acids using Clustal W (Thompson et al., 1994) as implemented in DNAstar's Lasergene software and adjusted by eye in Se-Al, Sequence Alignment Editor (Rambaut, 1996). All protein data were analyzed as amino acids. The data matrices analyzed are deposited in TreeBASE. The selection of paralogs used in our multigene analyses was based on preliminary phylogenetic analyses. Paralogs that clustered together representing most of the ingroups were used. All paralogs examined in our ingroup species were sister to one another, indicating that we are not dealing with ancient gene families.

To assess the effect of rate heterogeneity on the SSU-rDNA topologies, we partitioned the two data matrices

Table 1
Support values (BPP/BS) for some of the proposed taxonomic hypotheses within 'Amoebozoa' based on SSU-rDNA analyses with systematic removal of the fastest rate classes

Taxonomic hypothesis	Liberal mask			Conservative mask		
	Full (1482 bp)	Minus rate class 8 (6.7%)	Minus rate classes 7 and 8 (21.2%)	Full (1238 bp)	Minus rate class 8 (11.2%)	Minus rate classes 7 and 8 (21.6%)
Lobosea	–/–	–/–	–/–	–/–	–/–	–/–
Tubulinea	0.83/<50	0.79/<50	0.85/<50	0.95/<50	0.86/<50	0.87/<50
Flabellinea	0.82/<50	0.53/<50	0.50<50	0.92/<50	0.66/<50	–/–
Conosea	–/–	–/–	–/–	–/–	–/–	–/–
Mycetozoa (Myxogastria + Dictyosteliida)	–/–	–/–	–/–	–/–	–/–	–/–
Leptomixda	1.00/99	1.00/100	1.00/99	1.00/95	1.00/79	0.96/60
Myxogastria	1.00/100	1.00/100	1.00/100	1.00/100	1.00/100	1.00/99
Tubulinea + Echinamoebae	0.82/<50	0.75/<50	0.85/<50	0.72/<50	0.66/<50	0.77/<50
Archeamoeba/Pelobionts	1.00/<50	1.00/52	1.00/-	0.94/<50	0.98/-	1.00/–
Entamoeaba + *Pelomyxa palustris*	1.00/74	1.00/67	1.00/72	1.00/60	0.98/82	1.00/63
Acanthamoeba + Thecamoebidae	0.98/<50	0.78/<50	–/–	0.63/<50	<0.50/–	–/–
Myxogastrea + *Trichosphaerium*	0.99/<50	0.61/<50	<0.50/<50	0.98/<50	<0.50/<50	–/<50
Acanthamoeba + *Balamuthia*	1.00/93	1.00/77	1.00/70	1.00/84	1.00/65	1.00/57
Vanellidae + *Platyamoeba*	1.00/100	1.00/100	1.00/99	1.00/97	1.00/96	1.00/100
Vanellidae + (*Pessonella* + *V. minutissima*)	0.69/<50	–/<50	–/–	–/<50	–/–	–/–
Dactylopodida without *V. minutissima*	1.00/88	1.00/81	1.00/79	1.00/90	1.00/78	0.87/59
Thecamoebidae + *Platyamoeba stenopodia*	1.00/<50	0.79/<50	–/–	0.72/<50	–/–	<0.50/<50

(conservative mask and liberal mask) into eight rate classes using the GTR model with invariable sites and rate variation among sites following a discrete gamma distribution, as implemented in HyPhy version .99b package (Kosakovsky Pond et al., 2005). Class 1 and 8 represents the slowest and the fastest rate classes, respectively. We then ran analyses eliminating the fastest rate class (8) and the fastest two rate classes (7 and 8).

Gene trees were constructed in MrBayes (Huelsenbeck et al., 2001) and RaxML (Stamatakis et al., 2005a, 2005b). Data analyzed include: concatenated four gene (2291 characters), actin (258 aa), and a total of six SSU-rDNA data sets: (1) full conservative mask (1238 bp) and the conservative mask minus rate class/es 8 (1103 bp) and 8 + 7 (971 bp), (2) full liberal mask (1483 bp) and liberal mask minus rate class/es 8 (1382 bp) and 8 + 7 (1167 bp). For analyses of single genes (actin and SSU-rDNA), a restricted number of outgroups were chosen to enable assessment of relationships among 'Amoebozoa'—such an approach is necessary given the inability of single gene trees to reconstruct deep relationships. Clearly, future studies need to be both taxon and gene rich to provide sufficient power to confirm relationships that emerge from single gene trees.

MrModeltest (Nylander, 2004) and ProtTest (Abascal et al., 2005) were used to select the appropriate model of sequence evolution for the nucleotide and amino acid data, respectively. In both methods, models were evaluated under default settings that use a fixed topology generated either by neighbor-joining (NJ) with Jukes-and-Cantor distances (for MrModeltest) or BIONJ (for ProtTest). Bayesian analyses were performed with the parallel version of MrBayes 3.1.2 using the GTR+I+Γ (for nucleotide) and rtREV (for amino acid) models of sequence evolution (Ronquist and Huelsenbeck, 2003). In the concatenated analyses the same model of amino acid and parameters were used for the three proteins. Four simultaneous MCMCMC chains were run for 4 million generations sampling every 100 generations. Stationarity in likelihood scores was determined by plotting the $-\ln L$ against the generation. All trees below the observed stationarity level were discarded, resulting in a "burnin" of 25% of the number of generations used. The 50% majority-rule consensus tree was determined to calculate the posterior probabilities for each node. RaxML was run for 100 iterations using the GTRGAMMA model for nucleotide data and PROTGAMMA with matrix rtREV for amino acid data. A total of 100 independent bootstrap analyses were run in RaxML and a 50% majority rule consensus were calculated to determine the support values for each node.

3. Results

3.1. Concatenated four-gene analysis

Bayesian analysis of the concatenated data (SSU-rDNA and amino acid sequences of actin, alpha-tubulin and beta-tubulin) including a total of 100 representatives from diverse eukaryotic groups (Fig. 3) is generally concordant with previously published trees (e.g. Baldauf et al., 2000).

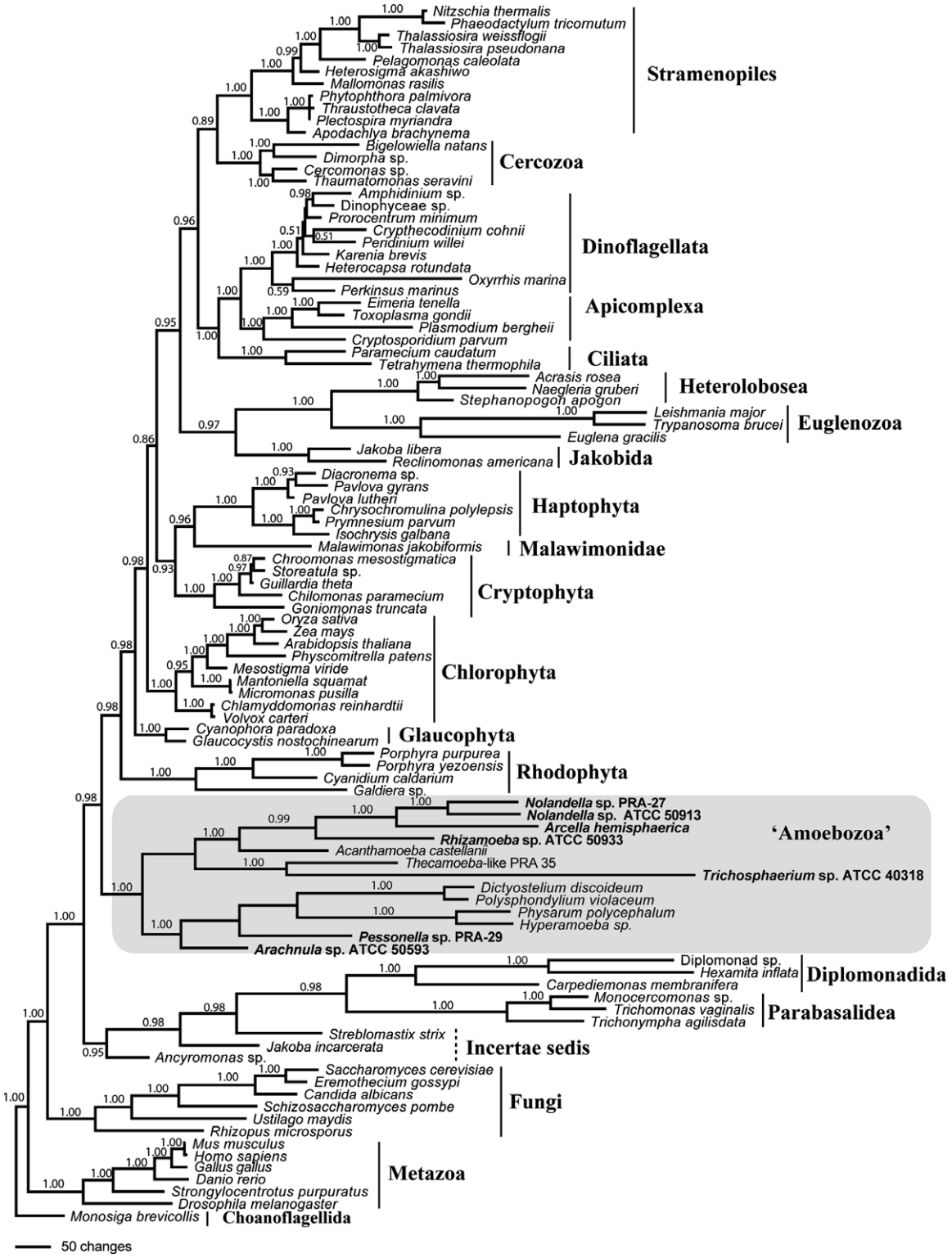

Fig. 3. Concatenated Bayesian phylogeny ($-\ln L = 71019.269$) inferred from four genes (SSU-rDNA and amino acid sequences of actin, alpha and beta tubulins) showing the placement of the seven diverse amoebae (in bold and shaded) within 100 eukaryote taxa covering the major groups. BPP supports above 50% are shown at the nodes. All branches are drawn to scale.

Given our taxon selection, which includes only a limited sample of non-amoeboid lineages, most of the traditionally well-defined lower taxonomic groups (i.e. dinoflagellates, apicomplexa, ciliates, stramenopiles, 'cercozoa', euglenozoa, heterolobosea, jakobids, haptophytes) are recovered with full posterior probability (BPP) supports. All of our

newly characterized taxa, *Arachnula* sp., *Arcella hemisphaerica*, *Pessonella* sp., *Rhizamoeba* sp., *Trichosphaerium* sp. and the two *Nolandella* spp., fall within the 'Amoebozoa' with full BPP support.

3.2. SSU-rDNA analyses

Because of the very limited taxon sampling of amoebae for multigene analyses, we turned to taxon-rich SSU-rDNA gene trees to assess the position of our amoeboid lineages within the 'Amoebozoa' territory. We present the ML gene tree (Fig. 4) from a conservative mask as a representative of all the analyses performed since this tree is well resolved and most of the clades found in the other data sets are well represented. We did not find any well-supported incongruencies among our various analyses. The SSU-rDNA gene tree including the diverse amoebae is generally concordant with previous publications based on this gene (e.g. Smirnov et al., 2005; Nikolaev et al., 2006).

3.2.1. Placement of the seven taxa based on analyses with and without fast rate classes

Four of our newly characterized taxa: *Rhizamoeba* sp., *Arcella hemisphaerica* and the two *Nolandella* isolates fall within the Tubulinea clade (BPP = 0.72) (Fig. 4). This grouping is consistent in all of our analyses including the small partitions without the fast rate classes, but it is only supported in Bayesian analyses (Table 1). *Rhizamoeba* sp. forms a sister group relationship with *Paraflabellula reniformis* (BPP = 0.88, maximum likelihood bootstrap (BS) = 58) within the Leptomyxida (BPP = 1.00, BS = 95). This relationship is supported in all of our analyses (not shown). The two *Nolandella* isolates group together (BPP = 1.00, BS = 100) and fall at the base of the clade that includes Amoebidae and Hartmannellidae with weak support (below 50%) (Fig. 4). Similarly, the position of the *Nolandella* isolates within the Tubulinea varied by analysis (not shown). *Arcella hemisphaerica* falls within Arcellinida (Fig. 4) but the support is very weak and its position within the 'Amoebozoa' varied greatly by analyses.

The position of *Arachnula* sp. and *Trichosphaerium* sp. were neither supported nor stable in any of our analyses. *Arachnula* sp. was shown to fall at the base of myxogastrids (not shown) or within myxogastrids + dictyostelids + pelobionts (not shown) or basal to dictyostelids (Fig. 4). The *Trichosphaerium* sp. consistently grouped at the base of myxogastrid clade (Fig. 4), except when the fast rate classes were systematically removed (Table 1).

3.2.2. Comparison of full conservative and full liberal mask analyses

The gene trees from the full conservative (Fig. 4) and liberal mask (not shown) data sets show similar topologies with no support for deep level relationships. Support (BS and BBP) for the lower nested clades such as *Leptomyxa*, Myxogastria, *Acanthamoeba* + *Balamuthia*, *Vannella* + *Platyamoeba* and others was higher in the full liberal mask than the full conservative mask and generally dropped with successive removal of rate classes (Table 1). On the contrary the support (only BBP) for the more inclusive clades such as Tubulinea and Flabellinea was higher in the conservative mask and its two-subset data sets than the respective liberal mask data sets.

3.2.3. Analyses without the fastest rate classes

Results of the analyses upon removal of the fastest rate classes demonstrate some clades are unstable (Table 1). Proposed high-level taxonomic groups such as Lobosea, Conosea and Mycetozoa were not recovered in any of our analyses (Fig. 4 and Table 1). Spurious taxonomic relationships such as Myxogastria + *Trichosphaerium* sp. and Thecamoebidae + *Platyamoeba stenopodia* that were supported in the full data sets (conservative and liberal) were shown to collapse with methodical removal of fast rate classes (Table 1).

3.3. Actin analyses

As there is also reasonable taxon sampling for actin, 23 amoebozoan lineages characterized, we also analyzed single-gene trees for this locus. The actin gene trees from ML (Fig. 5) and Bayesian (not shown) analyses are generally concordant, though resolution and support are limited. The Tubulinea clade, which includes four of our taxa: *Rhizamoeba* sp., *Arcella hemisphaerica* and the two isolates of *Nolandella*, is supported in both analyses (BS = 68, BPP = 1.00). Paralogs of *Arachnula* sp. (BS = 89, BPP = 1.00), *Trichosphaerium* sp. (BS = 68, BPP = 0.83), *Arcella hemisphaerica* (BS = 99, BPP = 0.99) and the isolates of *Nolandella* (BPP = 0.66) each form a monophyletic group (Fig. 5).

3.4. Molecular evolution

3.4.1. Putative paralogs

Putative paralogs of protein-coding genes were detected in some of the taxa studied here. To distinguish allelic variation from paralogy, we use a working definition of paralogs as sequences that differ by >1% within a species. Such a definition will obscure recent gene duplications but will maintain gene family members that may confound phylogenetic inferences. In *Arachnula* sp. two putative paralogs were found for each of the actin and beta-tubulin genes that diverge by 2.4% and 3.7%, respectively. We found two putative paralogs for alpha-tubulin in *Rhizamoeba* sp. (nucleotide divergence = 3.3%). The two *Nolandella* isolates each had two putative paralogs for actin. Three actin putative paralogs of *Arcella hemisphaerica* were obtained from another study. We also found three putative paralogs for the actin gene in *Trichosphaerium* sp. diverging from 3.3% to 7.0%. Intriguingly, we found three haplotypes among SSU-rDNA clones from a population of cultured *Trichosphaerium* sp. (nucleotide divergence = 1.4–1.8%)),

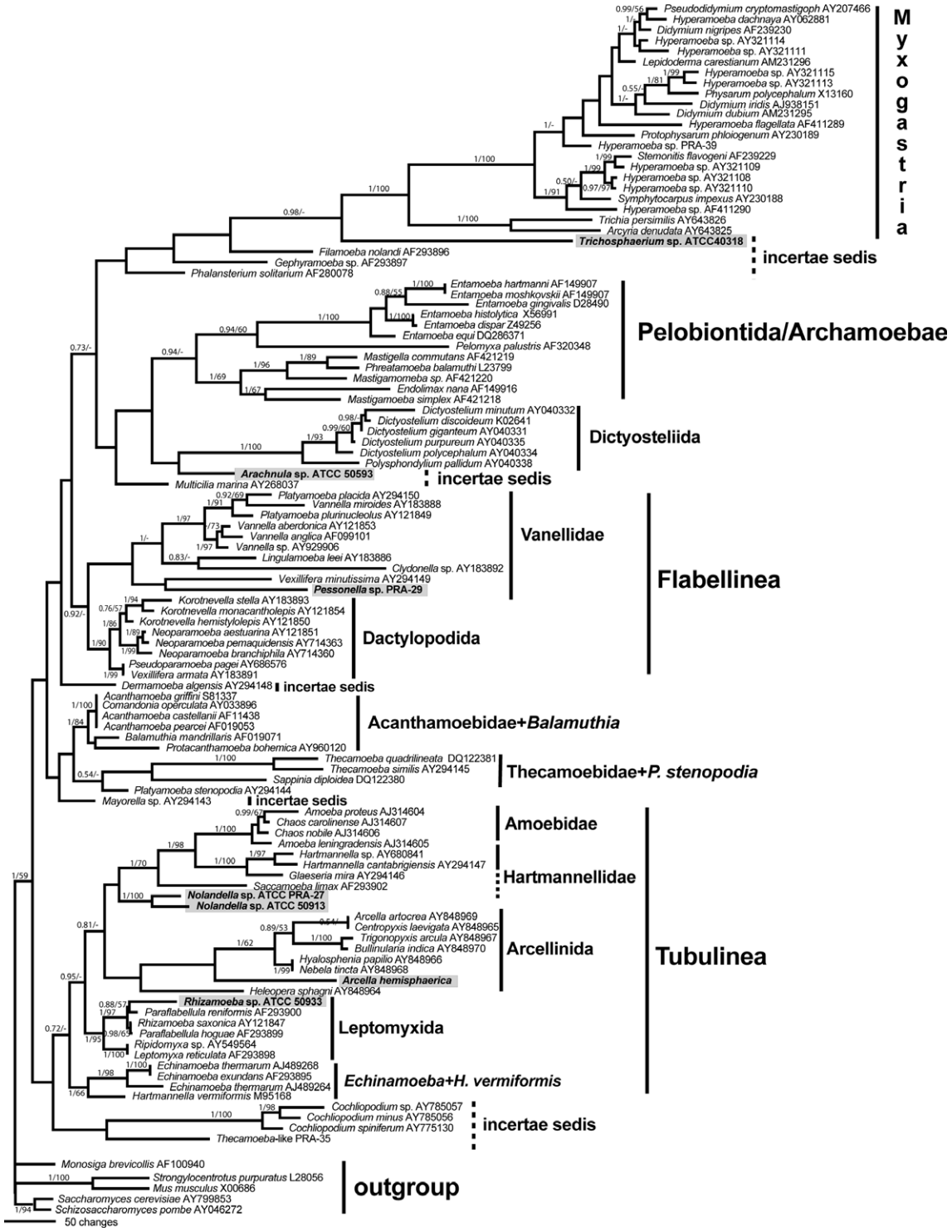

Fig. 4. SSU-rDNA gene tree, inferred from maximum likelihood ($-\ln L = 33501.736$), showing the positions of the seven diverse amoebae (in bold and shaded) within the 'Amoebozoa'. BPP/BS supports above 50% are shown at the nodes. Hyphen (-) represents support values below 50% (BPP/BS). All branches are drawn to scale.

suggesting the presence of either divergent paralogs or alleles. Genbank accession numbers of the four genes sequenced in this study are: *Arachnula* sp. (EU273436-40), *Arcella hemisphaerica* (EU273441-5), *Nolandella* sp. ATCC 50913 (EU273446-51), *Nolandella* PRA-27 (EU273452-6), *Pessonella* sp. (EU273457-8), *Rhizamoeba* sp. (EU273459-63), and *Trichosphaerium* sp. (EU273464-71).

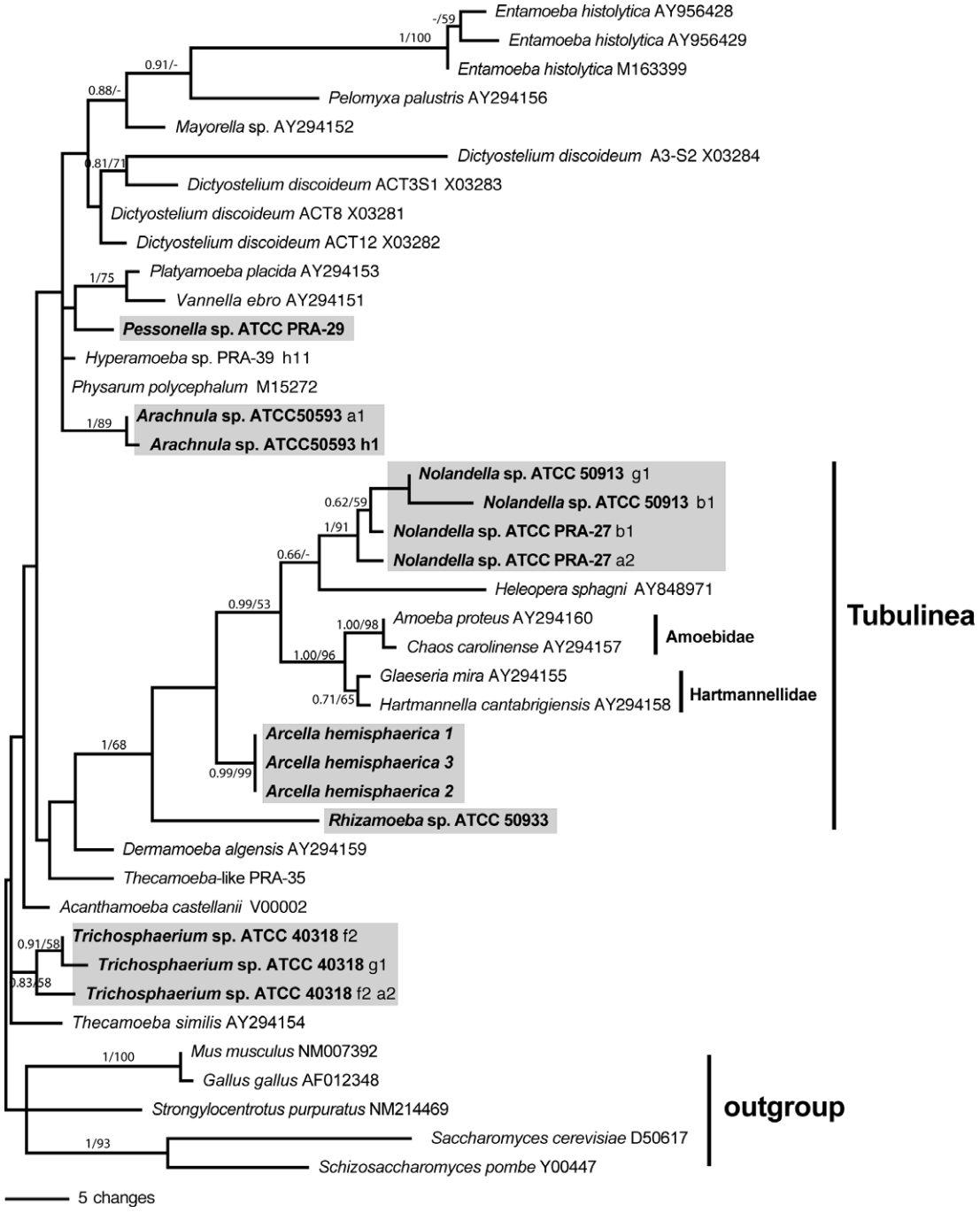

Fig. 5. Actin gene tree, inferred from Bayesian inference ($-\ln L = 2539.250$), showing the positions of the seven diverse amoebae within the 'Amoebozoa'. In these analyses, we have included available divergent paralogs that differed by 1%, which are indicated by types or clone numbers, as described in GenBank. BPP/BS supports above 50% are shown at the nodes. Hyphen (-) represents support values below 50% (BS/BPP). All branches are drawn to scale.

3.4.2. Introns

Introns with canonical acceptor and donor sites were detected for many of the taxa studied here, suggesting these amoebae may have intron-rich genomes. Although these introns are putative in that they have not been confirmed by RNA analyses, exclusion of the predicted introns yielded open reading frames. In *Rhizamoeba* sp., we detected four introns in alpha-tubulin and two in beta-tubulin. *Nolandella* PRA-27 had two introns in each of the three protein-coding genes. In *Pessonella* sp. two introns were found in the actin gene while the alpha-tubulin of *Arcella hemisphaerica* had three introns. *Trichosphaerium* sp. appears to be particularly intron rich as the alpha-tubulin gene contained 15 introns and the beta-tubulin gene contained five introns.

4. Discussion

4.1. Placement of the newly characterized amoeboid lineages

A concatenated multigene analysis places the seven diverse amoeboid lineages including *Arachnula* sp. and *Trichosphaerium* sp. within the supergroup 'Amoebozoa' (Fig. 3). All of our taxa share the broad morphological features characteristic of most of this supergroup including the presence of dynamic pseudopodia and branched tubular mitochondrial cristae. However, these characteristics are also found elsewhere on the eukaryotic tree of life, including in some members of the supergroup 'Rhizaria' (see Patterson, 1999) and hence not defining characters of the 'Amoebozoa'.

In discussing the positions of our newly characterized taxa within the 'Amoebozoa', based on separate analyses of SSU-rDNA and actin, we follow the taxonomic scheme of 'Amoebozoa' after Smirnov et al. (2005).

4.1.1. Members of the Tubulinea clade

Both SSU-rDNA (Fig. 4) and actin (Fig. 5) analyses place *Rhizamoeba* sp., *Arcella hemisphaerica* and the two isolates of *Nolandella* sp. (ATCC® PRA-27 and ATCC® 50913) within the Tubulinea.

The placement of the *Rhizamoeba* sp. within Leptomyxida and *Rhizamoeba* + *Paraflabellula* clades is well corroborated by its morphology. The order Leptomyxida *sensu* Page, 1987 was shown to be non-monophyletic (Amaral Zettler et al., 2000), while a small subset of this order that includes *Leptomyxa*, *Paraflabellula* and *Rhizamoeba*, equivalent to suborder Rhizoflabellina *sensu* Page, 1987 (i.e. to the exclusion of *Stereomyxa* and *Gephyramoeba* under the suborder Leptoramosina), is consistently recovered with strong support (Table 1, see also Smirnov et al., 2005; Nikolaev et al., 2006). The Leptomyxida is described as being comprised of naked amoebae with an ability to alter their locomotive form from a flattened, expanded, and branched morphology to a subcylindrical, monopodial shape and uroidal structures with adhesive threads. *Rhizamoeba* sp. shares fine structural features consistent with those of the genus, which include shape of nucleus and dense collosomes (Fig. 2E and F) though its grouping with *Paraflabellula reniformis* conflicts the traditional classification (Page, 1987). Given the current taxonomic sampling, the genera *Paraflabellula* and *Rhizamoeba* are shown to be paraphyletic in our SSU-rDNA analyses (Fig. 4) (see also Smirnov et al., 2005). The two genera are morphologically distinct and this grouping needs further investigation.

The position of the two isolates of *Nolandella*, ATCC® 50913 and ATCC® PRA-27, within the Tubulinea varied with analysis. *Nolandella* is a hartmannellid amoeba with some eruptive pseudopodial activity (Page, 1980; Rogerson and Patterson, 2002). Based on our current taxonomic sampling and in previous studies (Fahrni et al., 2003; Nikolaev et al., 2006) the family Hartmannellidae is not monophyletic. With the exception of *Hartmannella vermiformis*, whose generic assignment has been questioned (Fahrni et al., 2003; Nikolaev et al., 2006), the SSU and actin sequences of *Hartmannella* together with *Glaeseria* form a strongly supported sister group relationship with members of the Amoebidae (Figs. 4 and 5), while *Saccamoeba* followed by two of our *Nolandella* isolates form a paraphyletic relationship with the above clade (Fig. 4). The sister group relationship of *Hartmannella* + *Glaeseria* and the Amoebidae was not affected by the removal of the fastest sites nor we recover a monophyletic Hartmannellidae (not shown).

Arcella hemisphaerica groups with the rest of Arcellinida within the Tubulinea in SSU-rDNA gene tree (Fig. 4), but the monophyly of Arcellinida was not recovered in the actin tree with limited taxon sampling (Fig. 5). In agreement with previous ribosomal studies (Nikolaev et al., 2005) the Arcellinida are a monophyletic group and sister group to Tubulinida (Amoebidae + Hartmannellidae); however, the support was weak in our analyses. Nikolaev et al. (2005) reported characters surrounding the composition of test, the main characters used in higher-level classification of Arcellinida, are homoplasious. Similarly, our analyses indicate taxa with proteinaceous and agglutinated shells are nested and do not form distinct groups, which conflicts with the traditional classification (e.g. Meisterfeld, 2002).

The position of *A. hemisphaerica* within Arcellinida was neither stable nor recovered every time. *A. hemisphaerica* was never shown to group with *A. artocrea* including in the analyses with the fastest rate classes removed, suggesting the non-monophyly of the genus. The non-monophyly of *Arcella* might be explained due to a heterogeneous rate of evolution in *A. hemisphaerica*. Alternatively, the high degree of similarity between the *A. artocrea* and *Centropyxis laevigata* sequences (>99% identity) raises the possibility that the *A. artocrea* is misidentified.

4.1.2. A member of the Flabellinea clade

The PRA-29 isolate, tentatively identified as *Pessonella* sp., shows an affinity with members of the family Vannellidae (Fig. 4), which are characterized by a prominent hyaline zone and lack of subpseudopodia. *Pessonella* is traditionally placed within this family, distinguished from other members of vannellids by small bosses on the surface of its hyaloplasm. The position of PRA-29 within the Vannellidae is not well supported (Table 1); it branches as sister to *Vexillifera minutissima* at the base of the vannellid clade with weak or no support (Fig. 4). The SSU sequence of *V. minutissima* is never shown to group with its closest relative *V. armata* (Fig. 4, Nikolaev et al., 2006; Kudryavtsev et al., 2005; Smirnov et al., 2005), even in our analyses with the fastest rate classes removed. *Vexillifera minutissima* is among long-branch lineages in the Flabellinea clade and this grouping might be due to long-branch attraction or to misidentification (see also Smirnov et al., 2005).

4.1.3. Incertae sedis amoebozoan taxa

The ATCC® 50593 isolate identified as *Arachnula* sp. and ATCC® 40318 *Trichosphaerium* sp. are among other amoeboid lineages whose taxonomic positions remain uncertain within the 'Amoebozoa' (Fig. 3). Besides these two isolates, 9 different species find no stable homes in SSU-rDNA gene trees (Fig. 4): *Dermamoeba algensis*, *Thecamoeba*-like PRA-35, *Gephyramoeba* sp., *Mayorella* sp., *Phalansterium solitarium*, *Filamoeba nolandi*, and the three species of *Cochliopodium* (see also Smirnov et al., 2005; Nikolaev et al., 2006). The genus *Arachnula* (family Vampyrellidae Zopf, 1885) includes a giant multinucleated branching amoeba that has a wide range of food sources (Old and Darbyshire, 1980). The taxonomic position of *Arachnula* has been disputed, as this taxon has been placed within the Granuloreticulosa (Margulis et al., 1990) or as a vampyrellid (Page and Siemensma, 1991) or as close relative of *Biomyxa* (Lee et al., 2000). Multigene analyses shows the ATCC® 50593 isolate as a member of the 'Amoebozoa' cluster (Fig. 3) but the position of this lineage within the supergroup is unstable.

The genus *Trichosphaerium* is an unusual multinucleate marine amoeba that is reported to have an alternation of generations with gamont (sexual) and schizont (asexual) stages (Schneider, 1878; Schaudinn, 1899; Angell, 1976). Due to the complex life cycle, the description and taxonomic position of *Trichosphaerium* has been a formidable task (see Sheehan and Banner, 1973), but detailed EM work has been done (Schuster, 1976; Angell, 1976). *Trichosphaerium* has been placed in different groups such as granuloreticulopodia (Loeblich and Tappan, 1964; Le Calvez, 1953) or Lobosia *sensu* Carpenter, 1861 (Parker and Haswell, 1940). More recent reviews place it among members of the 'Amoebozoa' supergroup as *incertae sedis* (Smirnov et al., 2005; Adl et al., 2005) or Arcellinida (Margulis et al., 1990). The *Trichosphaerium* sp. studied here has an unusually accelerated rate of mutation in SSU-rDNA, among the longest branch within the group (Fig. 4). It is shown to group consistently, in the SSU-rDNA tree, at the base of the highly divergent Myxogastrid clade (Fig. 4). There is no morphological evidence supporting this relationship. Moreover, as the fastest rate classes were removed this relationship was either weakly supported or completely collapsed (Table 1), indicating that this grouping may be spurious due to long-branch attraction. The evolutionary determination of *Trichosphaerium* requires evidence from slowly evolving genes.

4.2. The utility of SSU-rDNA in the evolution of 'Amoebozoa'

The SSU-rDNA analyses excluding the fastest rate classes coupled with increased taxonomic sampling have enabled us to test the stability of previously proposed hypotheses and to evaluate the resolving power of SSU-rDNA within this putative supergroup. Several taxonomic hypotheses, including Lobosea and Conosea as well as Mycetozoa were not recovered in any of our analyses with the taxonomic sampling used here (Table 1). Lobosea includes most of the naked and the lobose testate amoebae with broad lobed or finger-shaped pseudopodia and simple life cycle (Page, 1987). In the recent classification scheme of 'Amoebozoa' *sensu* Smirnov et al. (2005), Lobosea includes among others two of the consistently recovered ribo-clades: Flabellinea and Tubulinea, which are never shown to group together in our analyses. Our analyses refute the monophyly of Lobosea in agreement with previous studies (e.g. Smirnov et al., 2005; Nikolaev et al., 2006).

The Conosea (Conosa) is another higher-level amoebozoan group that includes the pelobionts and mycetozoans, and is defined by a cone of microtubules arising from the basal body (Cavalier-Smith, 1998). The monophlyly of this cluster receives no support in our analyses of rDNA gene trees (Fig. 4 and Table 1) nor in other published studies (Fahrni et al., 2003; Cavalier-Smith et al., 2004) and weak support in others (e.g. Kudryavtsev et al., 2005; Nikolaev et al., 2006). Support of Conosea comes from multigene analyses (Bapteste et al., 2002) of a limited taxonomic sample. The clustering and instability of Conosea in ribosomal studies may be expected as members of these taxa include the most divergent taxa characterized by long branches (Fig. 4). Even if we used algorithms that reduce artifactual grouping due to long-branch attraction and successively removed the fast rate class we were unable to recover the Conosea, indicating the lack of signal in the SSU-rDNA gene for this group (Table 1). The Conosea has been abandoned by Smirnov et al. (2005) due to its instability and we support this.

Similarly, the Mycetozoa, a subset of Conosea that unites myxogastrids and dictyostelids, did not form a monophyletic group in the SSU-rDNA analyses (Table 1), though the monophyly is supported in the concatenated analyses with very limited taxon sampling (Fig. 3) (see also Baldauf and Palmer, 1993; Drouin et al., 1995; Keeling and Doolittle, 1996; Baldauf and Doolittle, 1997). Ribosomal studies generally fail to recover the Mycetozoa as a monophyletic group (Hinkle and Sogin, 1993; Hendriks et al., 1991; De Rijk et al., 1995; Krishnan et al., 1990).

Despite lack of support at deep nodes within the SSU-rDNA gene trees of 'Amoebozoa', several clades with known morphological identities are consistently recovered, though with varying support. These include Tubulinea, Flabellinea, Pelobionta (mastigamoebae, entamoebae and *Pelomyxa*), and most of the lower groups nested in these clades are strongly supported (Table 1 and Fig. 4).

The Tubulinea is recovered in all of our analyses including analyses that excluded the fastest rate classes, with weak to moderate support from Bayesian analyses (Fig. 4 and Table 1). Additional evidence for this clade also comes from actin gene trees (Fig. 5, Fahrni et al., 2003). Morphological synapomorphies for the Tubulinea include monopodial locomotive forms that are tubular to subcylindrical in cross-section and clear monoaxial cyptoplasmic streaming

(Smirnov et al., 2005). This clade includes the Amoebidae, Hartmannellidae, Leptomyxida, Arcellinida and *Echinamoeba*. The latter taxon is designated *incertae sedis* by Rogerson and Patterson (2002). However, subsequent molecular studies based on SSU-rDNA confirm Echinamoebidae as a member of Tubulinea (Bolivar et al., 2001; Nikolaev et al., 2005, 2006; Smirnov et al., 2005). The placement of Echinamoebidae at the base of Tubulinea is not affected by the removal of the fastest class rates (Table 1), further corroborating its placement within this clade.

The Flabellinea clade (Vanellidae and Dactylopodida) receives lower support than the Tubulinea clade and collapsed when the fastest rates were removed. Members of Flabellinea clade are generally characterized as flattened cells lacking tubular pseudopodia and with polyaxial cytoplasmic flow (Smirnov et al., 2005). However, these characteristics are not unique to the group and no clear synapomorphy is yet known. More data are required to evaluate this hypothesis further.

The Pelobionta (Archamoebae *sensu* Cavalier-Smith, 1991) are amitochondriate and are composed of amoeboflagellates that include *Mastigamoeba*, *Mastigella*, *Pelomyxa* as well as the amoeboid Entamoebidae. This clade is generally recovered but its support decreases as the fast sites were removed, suggesting that this taxon may be the result of long-branch attraction (Table 1). The smaller Entamoebidae + *Pelomyxa* clade was recovered consistently in all of our analyses (Figs. 4 and 5) with strong support from both Bayesian and ML analyses and its support was less effected by the removal of the fastest rate sites.

Other taxa that group together in SSU-rDNA gene tree include *Acanthamoeba* + *Balamuthia*, (*Acanthamoeba* + *Balamuthia*) + *Thecamoeba* and Thecamoebidae + *Platyamoeba stenopodia*. The grouping of *Acanthamoeba* + *Balamuthia* has been reported in several studies (Amaral Zettler et al., 2000; Bolivar et al., 2001; Cavalier-Smith et al., 2004; Fahrni et al., 2003; Nikolaev et al., 2006). Our analyses shows that this relationship is robust to removal of fast sites (Table 1) and the placement of *Balamuthia* with *Acanthamoeba* by Smirnov et al. (2005) is reinforced. By contrast, the relationship between (*Acanthamoeba* + *Balamuthia*) + Thecamoebidae seems to be the result of long-branch attraction, as this clade collapses with removal of the fast sites (Table 1). The grouping of Thecamoebidae + *P. stenopodia* also collapses with the removal of the fast sites in our analyses. As has been reported elsewhere (e.g. Fahrni et al., 2003; Smirnov et al., 2005; Nikolaev et al., 2006), *P. stenopodia* never groups with other *Platyamoeba* suggesting that it may have been misidentified. Smirnov et al. (2007) synonymized and transferred *P. stenopodia* into Thecamoebidae as a new genus *Stenamoeba* based on gross morphology and SSU-rDNA analysis. Our finding indicates that this revision requires further evidence from other independent markers.

Acknowledgments

The authors thank Erika Barbero for providing actin sequences of *Arcella hemisphaerica*. This work is supported by the National Science Foundation Assembling the Tree of Life Grant (043115) to Debashish Bhattacharya, D.J.P., and L.A.K. This is Lamont-Doherty Earth Observatory Contribution Number 7117.

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