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The Testate Lobose Amoebae (Order Arcellinida Kent, 1880) Finally Find their Home within Amoebozoa

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Testate lobose amoebae (order Arcellinida Kent, 1880) are common in all aquatic and terrestrial habitats, yet they are one of the last higher taxa of unicellular eukaryotes that has not found its place in the tree of life. The morphological approach did not allow to ascertain the evolutionary origin of the group or to prove its monophyly. To solve these challenging problems, we analyzed partial small-subunit ribosomal RNA (SSU rRNA) genes of seven testate lobose amoebae from two out of the three suborders and seven out of the 13 families belonging to the Arcellinida. Our data support the monophyly of the order and clearly establish its position among Amoebozoa, as a sister-group to the clade comprising families Amoebidae and Hartmannellidae. Complete SSU rRNA gene sequences from two species and a partial actin sequence from one species confirm this position. Our phylogenetic analyses including representatives of all sequenced lineages of lobose amoebae suggest that a rigid test appeared only once during the evolution of the Amoebozoa, and allow reinterpretation of some morphological characters used in the systematics of Arcellinida.

Key words: testate amoebae; phylogeny; evolution; SSU rRNA gene; actin; Amoebozoa.

Introduction

The testate lobose amoebae (order Arcellinida Kent, 1880) are common in a wide range of moist and freshwater habitats. These small amoebae feed mostly on bacteria, algae, and fungi. The larger species also prey on other protozoans and small metazoans (Heal 1963; Mast and Root 1916). The Arcellinida are distinguished by their tests, comprising a single aperture and composed of

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either secreted proteinaceous material or agglutinated. Proteinaceous tests can be flexible, with rigid sheets of fibrous material or with regularly arranged hollow building units that form an areolate surface. Agglutinated tests can be either calcareous or siliceous (Ogden 1990; Ogden and Ellison 1988). Dictyosomes are involved in the secretion of the organic building units and the cement. Mitochondria have branched tubular cristae. Contractile vacuoles are present. Arcellinida have either endolobopodia that are granular or completely hyaline, or ectolobopodia that are generally fingerlike and in some species can anastomose (reticulolobopodia) (Bonnet 1961, 1963). Under unfavorable environmental conditions, most testate lobose amoebae produce cysts.

Classification of the Arcellinida is based mainly on characters of the test. Kudo (1954) presents the arcellinids as two unrelated families, Arcellinidae (with a membranous shell) and Difflugiidae (shell with foreign bodies, platelets, or scales). A more recent classification (Meisterfeld 2002) considers Arcellinida as a monophyletic order divided into three suborders: Arcellinina (membranous test, digitate pseudopodia), Difflugiina (test rigid with mineral particles, digitate pseudopodia), and Phryganellina (test with siliceous material, pseudopodia conical).

Testate lobose amoebae were for a long time considered as part of the Testacea (Rhizopoda), a taxon uniting all amoeboid protists that are enveloped by a single-chambered shell (Kudo 1954). In the first edition of the "Illustrated Guide to Protozoa" (Bovee 1985), the Testacea are not treated as a monophyletic taxon. The shape of pseudopodia (lobose or filose) is considered as an important taxonomic feature and testate lobose amoebae are placed within the class Lobosea Carpenter, 1861; while the testate filose amoebae are placed within the class Filosea Leidy, 1879. In the second edition of the "Illustrated Guide to the Protozoa" (Meisterfeld 2002), the Arcellinida are treated as a group of amoebae of uncertain affinities, because their evolutionary origins are unclear and molecular data are awaited to solve the problem.

Molecular data have already helped to resolve the phylogenetic status of testate filose amoebae which form at least three independent lineages within the recently defined super-group Rhizaria (Burki et al. 2002; Nikolaev et al. 2003, 2004; Wylezich et al. 2002). In contrast, the Arcellinida is one of the last widespread and well-known groups of eukaryotes that is not represented in molecular databases. To test the monophyly of Arcellinida, determine their position in the eukaryotic tree, and to resolve their internal relationships, we obtained partial small-subunit ribosomal RNA (SSU rRNA) gene sequences from representatives of two out of the three suborders and seven out of the 13 families of Arcellinida. We show that testate lobose amoebae are monophyletic and belong to the Amoebozoa, and confirm this result using complete SSU rRNA gene sequences from two species, and a partial actin sequence from one species.

Results

Seven species of testate lobose amoebae (*Arcella artocrea, Bullinularia indica, Centropyxis laevigata, Heleopera sphagni, Hyalosphenia papilio, Nebela tincta var. major, and Trigonopyxis arcula*) were examined (Fig. 1, Table 1). The partial SSU rRNA gene tree (Fig. 2) shows that the seven species form a monophyletic group within Amoebozoa. Monophyly of the Arcellinida is supported with both MrBayes and Maximum Likelihood analyses. The relationships within Arcellinida are relatively well resolved. *Heleopela sphagni* branches at the base of the testate lobose amoebae with strong support (PP of 1.00 and BV of 82%). The next branch is comprised of *H. papilio and N. tincta var. major*, which group together with high support (PP of 1.00 and BV of 100%). The four remaining species groups together with moderate support (PP of 0.82 and BV of 64%) and are divided in two highly supported (PP of 1.00 and BV of 100%) clusters: *T. arcula+B. indica* and *A. artocrea+C. laevigata*.

Analysis of complete SSU rRNA sequences (Fig. 3), including two arcellinid species (*H. sphagni and C. laevigata*), further supports the monophyly of Arcellinida, recovered here with strong support (PP of 0.97 and BV of 83%). In both SSU rRNA gene trees (Figs 2, 3), the testate lobose amoebae branch within a highly supported clade (PP of 1.00, BV of 94—100%) comprising the genus *Echinamoeba*, the order Leptomyxida, and the families Hartmannellidae and Amoebidae. This clade was named Tubulina in a recent classification of lobose amoebae (Smirnov et al. 2005), and we follow this classification here. Within Tubulina, a close relationship was recovered between testate lobose amoebae, and the families Amoebidae and Hartmannellidae. Support for this relation is moderate in the partial SSU rRNA analysis (PP of 0.63 and BV of 68%; Fig. 2), but it is high using complete SSU rRNA sequences

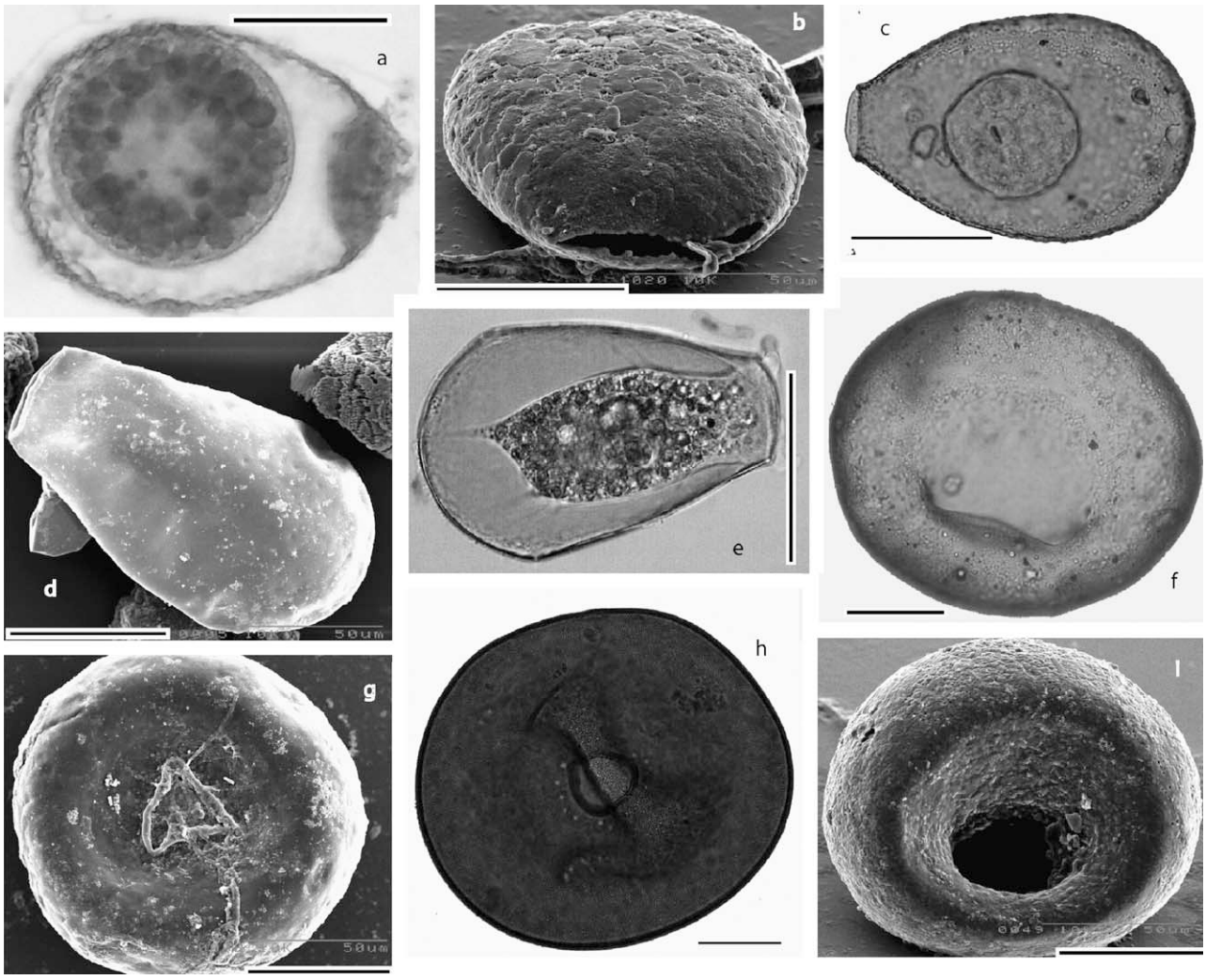

Figure 1. Micrographs of the seven testate amoebae taxa analyzed: **a, b:** *Heleopera sphagni* (small spheres inside the cyst are endosymbiotic algae); **c:** *Nebela tincta var. major*; **d, e:** *Hyalosphenia papilio*; **f:** *Bullinularia indica*; **g:** *Trigonopyxis arcula*; **h:** *Arcella artocrea*; **i:** *Centropyxis laevigata*. Scale bars indicate 50 μm. SEM pictures (b, d, g, and i) were taken at the University of Alaska Anchorage by Drs. J. Kudenov, E. Mitchell, and K. Kishaba.

(PP of 1.00, BV of 92%; Fig. 3). All other well-supported amoebozoan clades present in the SSU rRNA trees are congruent with previous studies and will not be discussed here.

The actin phylogeny (Fig. 4) corroborates the results obtained with the SSU rRNA data. A strong relationship (PP of 1.00 and BV of 100%) is recovered between Arcellinida and members of the families Amoebidae and Hartmannellidae. However, the resolution between the different clades of lobose amoebae is poor, and the lack of actin sequences for the basal members of the Tubulina impedes more precise conclusions about the position of Arcellinida within this clade.

Discussion

Up to now, the Arcellinida remained one of the last higher taxa of eukaryotes for which no molecular data were available. The classical morphological approach failed to ascertain the evolutionary origin of this group so far (Meisterfeld 2002). The idea of placing testate lobose amoebae (order Arcellinida) within the group of lobose amoebae, class Lobosea Carpenter, 1861 was put forward by Deflandre (1953) and Loeblich and Tappan (1961, 1964), who united all amoebae with lobose pseudopodia. Results of our analyses clearly confirm that testate lobose amoebae are indeed

Table 1. Morphological characteristics of the seven Arcellinida taxa studied.

	Shell dimensions					Shell composition[a]	Position of aperture[b]	References for dimensions	Sampling location
	Diameter [μm]	Length [μm]	Breadth [μm]	Depth [μm]	Diameter of aperture [μm]				
Arcella artocrea Deflandre, 1928	184—216			46—64	36—42	S	V	Ogden and Hedley 1980	Creux de l'Epral (CH)
Bullinularia indica Penard, 1907	138—180		165—172	94—99	65—90	A	V	Ogden and Hedley 1980	Creux de l'Epral (CH)
Centropyxis laevigata Penard, 1902	70—135					A	V	Deflandre 1929	Anchorage (AK, USA)
Heleopera sphagni Leidy, 1874		80—145	50—120	42—51	40—45	A	T	Ogden and Hedley 1980	Anchorage (AK, USA)
Hyalosphenia papilio Leidy, 1875		90—175	60—115	21—25	32—40	S	T	Ogden and Hedley 1980	Anchorage (AK, USA)
Nebela tincta var. major Deflandre, 1936		90—120				R	T	Deflandre 1936	Creux de l'Epral (CH)
Trigonopyxis arcula Leidy, 1879	90—168			40—80	21—45	A	V	Ogden and Hedley 1980	Anchorage (AK, USA)

[a]S: secreted proteinaceous; A: agglutinate; R: recycled biosilica,
[b]T: terminal aperture; V: ventral aperture

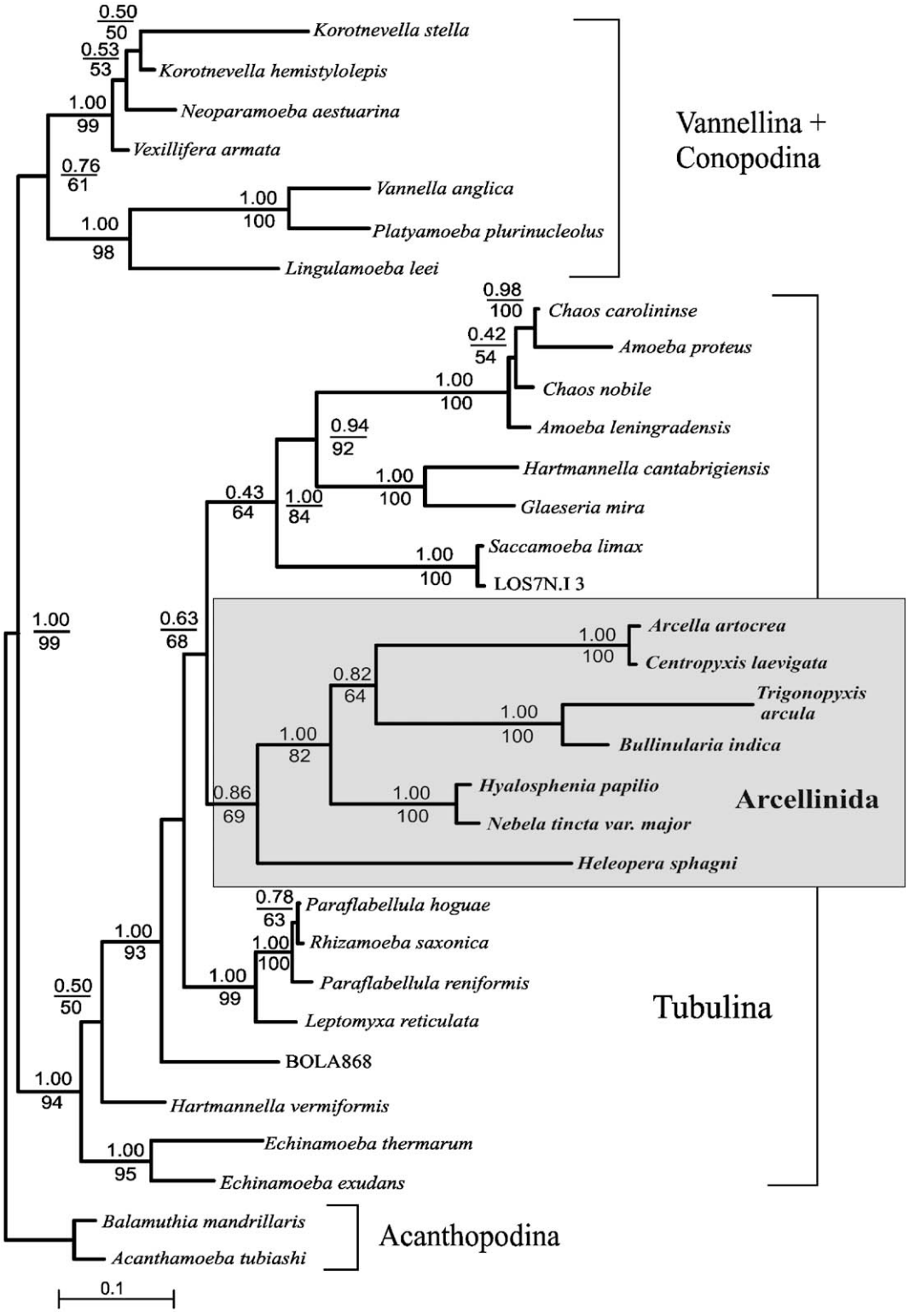

Figure 2. MrBayes tree of partial SSU rRNA genes showing the monophyly of Arcellinida. Numbers at nodes indicate posterior probabilities for MrBayes analysis (upper) and bootstrap values for ML analysis (down). The scale bar indicates 0.1% sequence divergence.

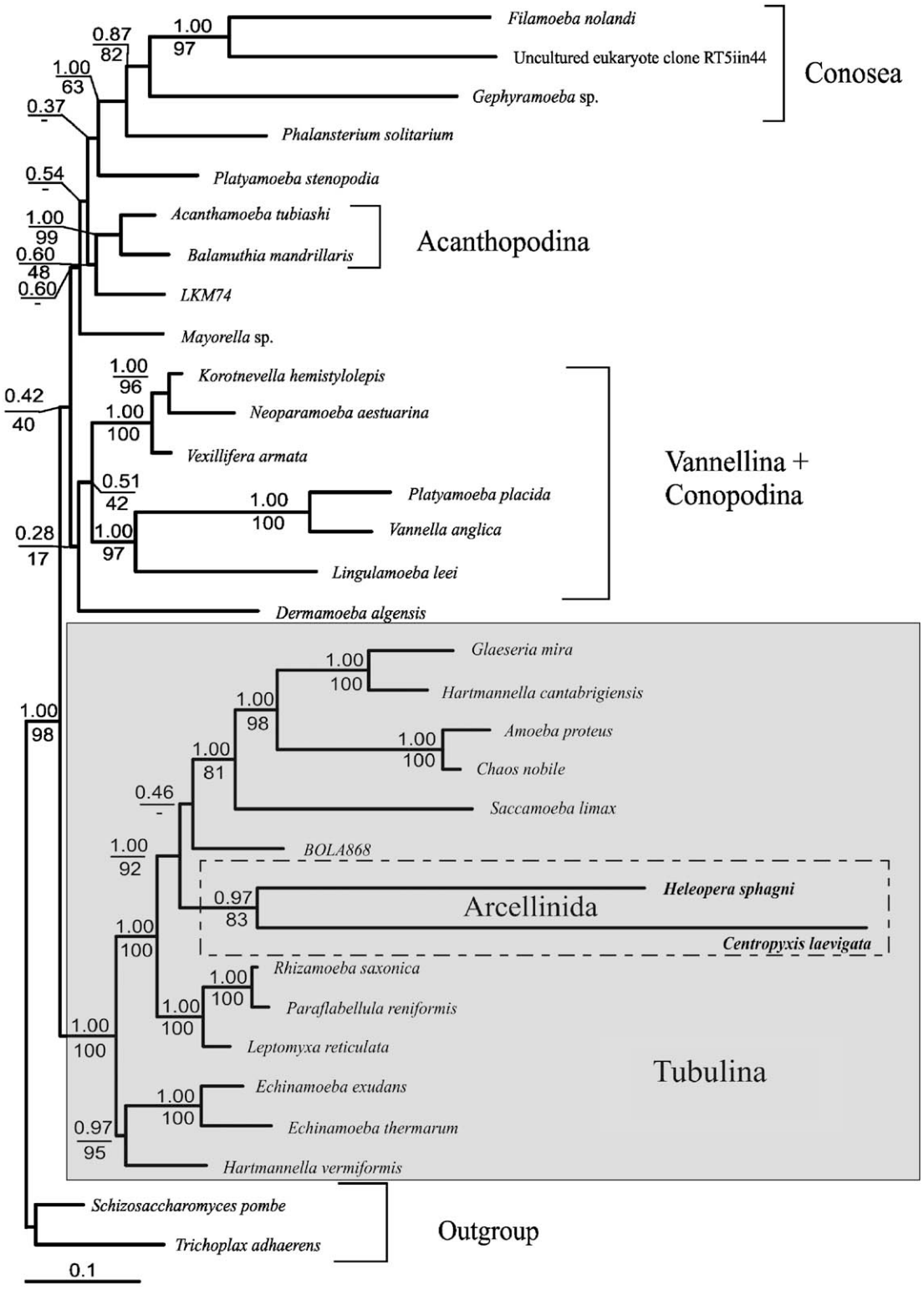

Figure 3. MrBayes tree of full length SSU rRNA genes indicating the phylogenetic position of Arcellinida among Amoebozoa. Numbers at nodes indicate posterior probabilities for MrBayes analysis (upper) and bootstrap values for ML analysis (down). The scale bar indicates 0.1% sequence divergence.

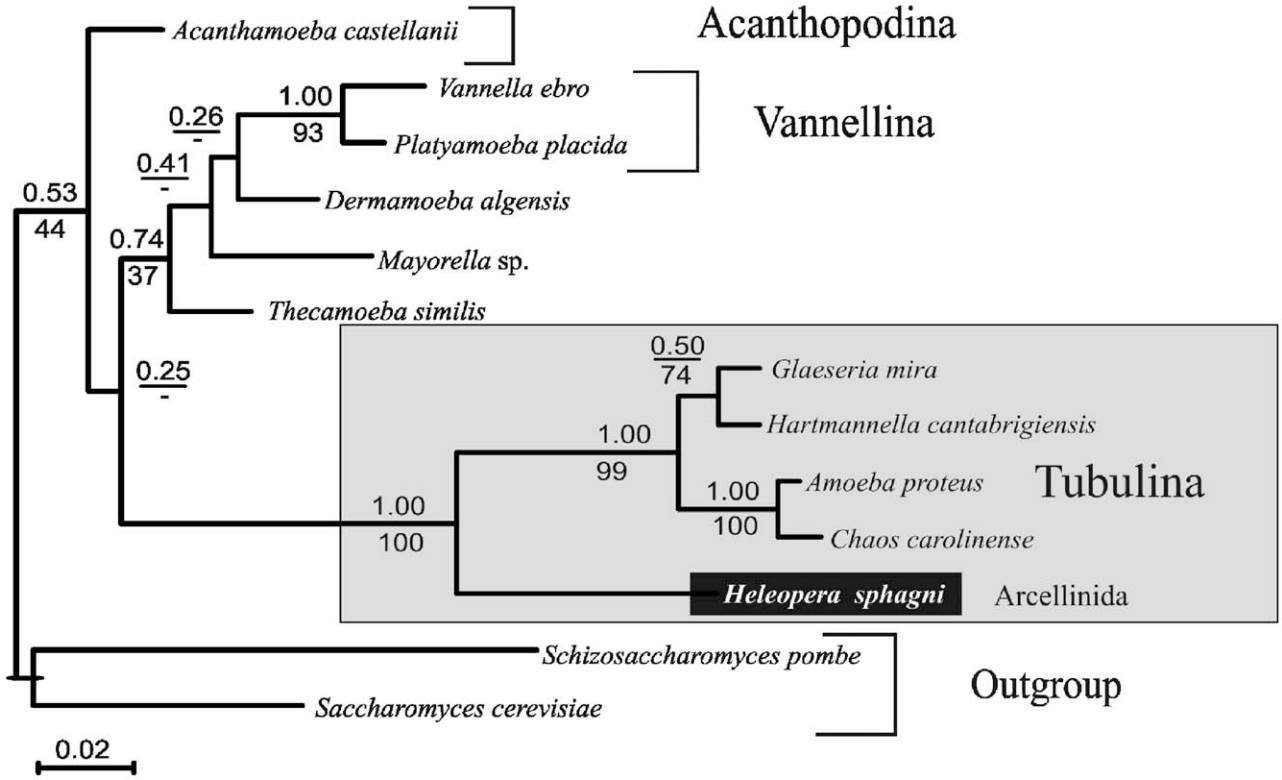

Figure 4. MrBayes tree of actin genes indicating the phylogenetic position of Arcellinida among Amoebozoa. Numbers at nodes indicate posterior probabilities for MrBayes analysis (upper) and bootstrap values for ML analysis (down). The scale bar indicates 0.02% sequence divergence.

related to naked lobose amoebae. The Arcellinida belong to the Amoebozoa, a position supported by both SSU rRNA and actin gene data (Figs 2—4). Members of the Arcellinida possess branching tubular mitochondrial cristae (Meisterfeld 2002), as do other lobose amoebae, which were named ramicristate amoebae by Rogerson and Patterson (2002). This suggests that the shape of mitochondrial cristae might be a valid derived feature of the Amoebozoa.

Both SSU rRNA and actin gene data support a close relationship between testate lobose amoebae and the families Amoebidae and Hartmannellidae. This is in agreement with the hypothesis, based on the shape of lobopodia, that the Arcellinida are related to typical large lobose amoebae such as *Amoeba proteus*, with polypodial or monopodial locomotion, and possessing classical fingerlike, non-eruptive pseudopodia, cylindrical in cross-section (Smirnov et al. pers. commun.).

Based on morphological characters alone, it was supposed that Arcellinidae species with a simple membranous shell have a different origin from the species with foreign bodies and scales incorporated in the shell (Kudo 1954). Our analyses of partial SSU rRNA sequences including seven species of testate lobose amoebae belonging to two out of the three suborders and seven out of the 13 families of Arcellinida indicate that the order Arcellinida is monophyletic (Fig. 2). However, our results show that the character of the composition of the shell does not reflect the relationships within Arcellinida: in our analysis, species with proteinaceous (*Hyalosphenia and Arcella*) and agglutinated (*Nebela, Trigonopyxis, Centropyxis, Bullinularia, and Heleopera*) shells are mixed (Fig. 2). Our molecular phylogeny of the Arcellinida suggests that primitive members of the order probably started building shells by agglutinating organic and/or mineral debris from the environment (xenosomes), while some more evolved taxa (e.g. *Nebela*) later started producing shells by recycling biosilica plates synthesized by their prey, such as filose testate amoebae from the order Euglyphida, or

produced proteinaceous shells (e.g. *Hyalosphenia and Arcella*). The independent branching of *Hyalosphenia* and *Arcella* provides evidence that the evolution towards more resistant proteinaceous shells took place more than once within the Arcellinida.

Given the topology shown in Figure 2, other morphological characters seem to make sense regarding the internal relationships of testate lobose amoebae. The phylogenetic position of the seven studied taxa suggests that all taxa with a ventral aperture (*Arcella, Centropyxis, Bullinularia* and *Trigonopyxis*) share a common ancestry regardless of the shell composition (PP of 0.82 and BV of 64%, Fig. 1). In contrast, the taxa that produce shells with a terminal aperture form two successive lineages (*Heleopera* and *Hyalosphenia+Nebela*) at the base of the Arcellinida. The latter lineage shares very similar shell morphologies (Fig. 1), despite the different material used for shell construction. Based on these morphological similarities, these two genera were grouped in the family Hyalospheniidae, and this grouping is strongly supported by our results (PP of 1.00, BV of 100%). In contrast, *Heleopera* species use xenosomes to build their shells and in this respect they resemble some of the taxa with ventral aperture included in this study (*Centropyxis, Bullinularia*, and *Trigonopyxis*).

Our data provide some support to a hypothetical phylogeny based on morphology (Bonnet 1964), suggesting that a test with a terminal aperture is a primitive trait within the Arcellinida, while a test with a ventral aperture is a derived trait. Although more data are clearly needed to confirm this observation, it also would make sense in light of the probable aquatic origin of the Arcellinida. Indeed, a ventral aperture, especially when combined with a flat shell, allows the amoeba to remain active in relatively dry conditions as long as a capillary water film is present on the substrate. All other things being equal, taxa with a ventral aperture can reach a larger volume and biomass under hydric stress than taxa with terminal apertures. In support of this idea, large *Nebela* species are only found in the wettest micro-sites in *Sphagnum*-dominated peatlands, while small taxa such as *N. militaris* and *N. tincta* are common in dry habitats such as hummocks and non-*Sphagnum* mosses, in places where much larger taxa with a ventral aperture such as *Trigonopyxis arcula* and *Bullinularia indica* occur regularly (Booth 2002; Charman and Warner 1992; Lamentowicz and Mitchell in press; Mitchell et al. 1999).

Methods

Testate amoebae were obtained from *Sphagnum* samples collected at two *Sphagnum*-dominated peatlands: Bicentennial park, Anchorage, Alaska (samples taken in fall 2003; 61° 06′ N, 149°44′ W) and Le Creux de l'Epral, in the Swiss Jura Mountains (samples taken on September 15th 2004; 47° 12′ N, 4° 37′ E) (Table 1, Fig. 1). The specimens were extracted from the mosses and picked using a narrow diameter pipette under the dissecting microscope. Identifications were confirmed under the microscope at 400 × magnification.

DNA was extracted using the DNeasy Plant Minikit (Qiagen, Basel, Switzerland) or with guanidine buffer (Chomczynski and Sacchi 1987). Partial SSU rRNA genes were amplified for *Arcella artocrea* (550 bp), *Hyalosphenia papilio* (999 bp), *Nebela tincta var. major* (670 bp), *Trigonopyxis arcula* (1762 bp), and *Bullinularia indica* (1076 bp), using the universal primers s12.2 (5′-GAT(CT)AGATACCGTCGTAGTC-3′) and sB (5′-TGATCCTTCTGCAGGTTCACCTAC-3′). Complete SSU rRNA gene sequences were obtained for *Heleopera sphagni* (2379 bp) and *Centropyxis laevigata* (2133 bp). The partial sequences amplified with s12.2 and sB were extended in the 5′ direction using reverse primers specifically designed for each species paired with the forward universal primers sA (5′-ACCTGGTTGATCCTGCCAGT-3′) and s6 (5′-CNGCGGTAATTCCAGCTC-3′). A partial fragment (1093 bp) of the actin gene of *H. sphagni* was amplified using the forward primers Act-F1 (5′-CNGARGCDCCATTRAAYC-3′) and Act-N2 (5′-AACTGGGA(CT)GA(CT)ATGGA-3′) and the reverse primer Act-1354r (5′-GGACCAGATTCATCATAYTC-3′). The fragment contains an intron of 311 bp and the amplified coding region is 782 bp long. PCR amplifications were carried out in a total volume of 50 µl with an amplification profile consisting of 40 cycles with 30 s at 94 °C, 30 s at 50 °C, and 2 min at 72 °C, followed by 5 min at 72 °C for the final extension. The amplified PCR products were purified using the High Pure PCR Purification Kit (Roche, Rotkreuz, Switzerland), then ligated into pGEM-T Vector System (Promega, Wallisellen, Switzerland), cloned in XL-2 Ultracompetent Cells (Stratagene, Basel, Switzerland), sequenced with the ABI-PRISM Big Dye Terminator Cycle Sequencing Kit, and analyzed with an ABI-3100 DNA sequencer (Perkin-Elmer, Rotkreuz, Switzerland), all according to the manufacturers' instructions. The sequences obtained in this study have been submitted to GenBank

Table 2. Taxonomic position, species names and GenBank accession numbers of all SSU rRNA and actin gene sequences used in this study.

Taxonomic position	Species name	GenBank accession numbers
New sequences presented in this study		
SSU rRNA gene		
Arcellinida	*Heleopera sphagni*	AY848964
	Nebela tincta var. major	AY848968
	Hyalosphenia papilio	AY848966
	Bullinularia indica	AY848970
	Trigonopyxis arcula	AY848967
	Centropyxis laevigata	AY848965
	Arcella artocrea	AY848969
Actin gene		
Arcellinida	*Heleopera sphagni*	AY848971
Other sequences used in our analyses		
SSU rRNA gene		
Tubulina	*Echinamoeba exundans*	AF293895
	Echinamoeba thermarum	AJ489268
	Hartmannella vermiformis	M95168
	Uncultured eukaryote clone BOLA868	AF372795
	Leptomyxa reticulata	AF293898
	Paraflabellula reniformis	AF293900
	Rhizamoeba saxonica	AY121847
	Paraflabellula hoguae	AF293899
	Hartmannellidae sp. LOS7N/I	AY145442
	Saccamoeba limax	AF293902
	Glaeseria mira	AY294196
	Hartmannella cantabrigiensis	AY294147
	Amoeba leningradensis	AJ314605
	Chaos nobile	AJ314606
	Amoeba proteus	AJ314604
	Chaos carolinense	AJ314607
Vannellina+Conopodina	*Lingulamoeba leei*	AY183886
	Vannella anglica	AF099101
	Platyamoeba placida	AY294150
	Platyamoeba plurinucleolus	AY121849
	Vexillifera armata	AY183891
	Neoparamoeba aestuarina	AY121848
	Korotnevella hemistylolepis	AY121850
	Korotnevella stella	AY183893
Acanthopodina	*Balamuthia mandrillaris*	AF019071
	Acanthamoeba tubiashi	AF019065
Other Amoebozoa	*Dermamoeba algensis*	AY294148
	Mayorella sp.	AY294143
	LKM74	AJ130863
	Platyamoeba stenopodia	AY294144
	Phalansterium solitarium	AF280078
	Gephyramoeba sp.	AF293897
	Uncultured eukaryote clone RT5iin44	AY082989
	Filamoeba nolandi	AF293896
Opisthokonta	*Trichoplax adhaerens*	L10828
	Schizosaccharomyces pombe	X58056

Table 2. (*continued*)

Taxonomic position	Species name	GenBank accession numbers
Other sequences used in our analyses		
Actin gene		
Tubulina	*Chaos carolinense*	AY294157
	Amoeba proteus	AJ314604
	Hartmannella cantabrigiensis	AY294158
	Glaeseria mira	AY294155
Vannellina	*Platyamoeba placida*	AY294153
	Vannella ebro	AY294151
Acanthopodina	*Acanthamoeba castellanii*	V00002
Other Amoebozoa	*Thecamoeba similis*	AY294154
	Mayorella sp.	AY294152
	Dermamoeba algensis	AY294159
Opisthokonta	*Saccharomyces cerevisiae*	L00026
	Schizosaccharomyces pombe	Y00447

(see Table 2 for the species names and accession numbers of all sequences used in this study).

The partial SSU rRNA gene sequences from *H. sphagni, N. tincta var. major, H. papilio, B. indica, T. arcula, C. laevigata, and A. artocrea* were manually fitted to an general alignment of eukaryotic SSU rRNA gene sequences, based on a universal model of eukaryotic SSU rRNA secondary structure (Van de Peer et al. 2000). Preliminary analyses indicated that all sequences of Arcellinida belong to the phylum Amoebozoa (data not shown). Our alignment was thus reduced to 32 sequences for the phylogenetic analyses, including the 7 sequences of testate lobose amoebae and 25 sequences from other Amoebozoa (all fast-evolving members of Amoebozoa that are clearly not closely related to testate lobose amoebae according to the preliminary analyses, such as archamoebae and mycetozoans, were omitted to allow the use of a maximum amount of nucleotide positions that was increased up to 606 bp). A second alignment of complete SSU rRNA gene sequences was prepared, including the sequences of *H. sphagni* and *C. laevigata*, 28 sequences from other Amoebozoa, and 2 sequences from opisthokonts used as outgroup. In the analyses 1623 positions were included. Finally, the partial actin sequence of *H. sphagni* was manually fitted to an alignment of 13 eukaryotic actin gene sequences, including 10 sequences of lobose amoebae and 2 sequences of Fungi used as outgroup. In the analyses 255 amino acid positions were included. Actin and SSU rRNA alignments are available upon request from the authors.

Maximum likelihood (ML) analyses were performed with PhyML, version 2.4 (Guindon and Gascuel 2003), using the GTR model of evolution (Lanave et al. 1984; Rodriguez et al. 1990) for all SSU rRNA gene analyses and the JTT model of evolution (Jones et al. 1992) for the amino acid alignment of actin gene sequences, taking into account a proportion of invariables sites, and a gamma distribution of the rates of substitution for the variable positions, with 4 rate categories. As starting trees, BIONJ trees were used. Bayesian analyses (BA) were performed with MrBayes (Huelsenbeck and Ronquist 2001) using the same models of evolution. The program was run for 1,000,000 generations, sampled every 100 generations, with 4 simultaneous chains. The trees sampled before the chains reached stationarity were discarded as a burn-in.

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