ACTA
PROTOZOOLOGICA

Acta Protozool. (2012) 51: 291–304
http://www.eko.uj.edu.pl/ap
doi:10.4467/16890027AP.12.023.0783

Ultrastructure of *Diplophrys parva*, a New Small Freshwater Species, and a Revised Analysis of Labyrinthulea (Heterokonta)

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Abstract. We describe *Diplophrys parva* n. sp., a freshwater heterotroph, using fine structural and sequence evidence. Cells are small (L = 6.5 ± 0.08 μm, W = 5.5 ± 0.06 μm; mean ± SE) enclosed by an envelope/theca of overlapping scales, slightly oval to elongated-oval with rounded ends, (1.0 × 0.5–0.7 μm), one to several intracellular refractive granules (~ 1.0–2.0 μm), smaller hyaline peripheral vacuoles, a nucleus with central nucleolus, tubulo-cristate mitochondria, and a prominent Golgi apparatus with multiple stacked saccules (≥ 10). It is smaller than published sizes of *Diplophrys archeri* (~ 10–20 μm), modestly less than *Diplophrys marina* (~ 5–9 μm), and differs in scale size and morphology from *D. marina*. No cysts were observed. We transfer *D. marina* to a new genus *Amphifila* as it falls within a molecular phylogenetic clade extremely distant from that including *D. parva*. Based on morphological and molecular phylogenetic evidence, Labyrinthulea are revised to include six new families, including Diplophryidae for *Diplophrys* and Amphifilidae containing *Amphifila*. The other new families have distinctive morphology: Oblongichytriidae and Aplanochytriidae are distinct clades on the rDNA tree, but Sorodiplophryidae and Althorniidae lack sequence data. Aplanochytriidae is in Labyrinthulida; the rest are in Thraustochytrida; Labyrinthomyxa is excluded.

Key words: Diplophryidae, fine structure, molecular genetics, Labyrinthomyxa, labyrinthulean taxonomy.

INTRODUCTION

Barker (1868), in a brief report to the Dublin Microscopical Club (1867), originally described *Diplophrys archeri* as an 'exceedingly minute, nearly orbicular or broadly elliptic' freshwater rhizopod bearing at 'two opposite points … a tuft of filiform pseudopodia', and containing within the body 'an oil-like refractive globule of an orange or amber color'. That was the first description of any *Diplophrys*, so *D. archeri* is the type species. Subsequent studies (e.g. Hertwig and Lesser 1874, Penard 1902) reported that this non-flagellate organism, containing a prominent refractive granule and emergent polar filopodia, occurred as small mounds of cells on submerged aquatic plants. The cells appeared to be enclosed by a thin, hyaline test, and for some time *Diplophrys*, though apparently non-phagotrophic, was considered to be a rhizopod, now belonging either to the cercozoan family Amphitremidae of filose testate amoebae with bipolar tufts of branching filopodia (e.g. Calkins 1926) or the foraminiferan family Allogromiidae, containing phagotrophic unicells enclosed by an

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organic test and having reticulose pseudopodia (e.g. Grassé 1953, p. 140). A second nominal species from the early literature, *Diplophrys stercorea* (Cienkowski 1876, Olive 1903), was later split off as a separate genus *Sorodiplophrys* because it makes multicellular fruiting bodies like a slime mold (Dykstra and Olive 1975), but its vegetative cells have the bipolar filopodial phenotype that led Cienkowski to put it in *Diplophrys*. Dykstra and Porter (1984), however, considered that both *Sorodiplophrys* and *Diplophrys* might be distantly related to labyrinthulids and thraustochytrids as all have tests of thin organic scales and naked thread-like absorptive projections and none of them are phagotrophs, in marked contrast to filose amoebae.

More recently, Dykstra and Porter (1984) isolated a new species (*Diplophrys marina*) from marine vascular plants and described its transmission electron microscopic fine structure and light microscopic morphology. They noted that its external envelope was not an organic membranous test characteristic of allogromids, but was composed of thin organic, overlapping scales, reinforcing their idea that *Diplophrys* and *Sorodiplophrys* are related to Labyrinthulea similar to *Labyrinthuloides* (*Labyrinthuloides* was later synonymized with *Aplanochytrium*: Leander and Porter 2000). Patterson *et al*. (2000), however, considered *Diplophrys* to have only one species, *D. archeri*, and as an amoeba of uncertain affinities. Leander and Porter (2001) presented the first molecular phylogenetic evidence, though with negligible bootstrap support, placing the non-flagellate *Diplophrys marina* amongst the labyrinthulids and thraustochytrids, previously shown to be a very deep-branching part of the chromistan Heterokonta (= stramenopiles) (Cavalier-Smith *et al.* 1994). Labyrinthulids and thraustochytrids mostly have flagellate zoospores and together constitute the heterokont class Labyrinthulea and subphylum Sagenista (Olive 1975, Cavalier-Smith 1986), of the most deeply branching heterokont phylum Bigyra, which otherwise consists predominantly of diverse phagotrophic flagellates (Cavalier-Smith 1997, Cavalier-Smith and Chao 2006). Labyrinthulea has two orders, Labyrinthulida (labyrinthulids) and Thraustochytrida (thraustochytrids), each with a single family (Cavalier-Smith and Chao 2006, Porter 1990). More comprehensive trees placed *D. marina* with very strong support firmly within Labyrinthulea (Cavalier--Smith and Chao 2006), but its position as sister to *Labyrinthula* was only weakly supported. However, a separate 18S rDNA tree suggested that an undescribed freshwater *Diplophrys* sp. deposited in the American Type Culture Collection (ATCC 50360) is genetically extremely distant from *D. marina* and does not group with it, but very weakly instead with an *Aplanochytrium*/*Labyrinthuloides* clade (Cavalier-Smith and Chao 2006); that cast doubt on whether strain ATCC 50360, for which there is no published morphology, really belongs to the same genus as *D. marina* and suggested that *Diplophrys*-like organisms had much greater phylogenetic depth than previously assumed.

To clarify more fully this putative deep diversity of *Diplophrys*-like species, we examined the light microscopic and fine structural morphology of strain ATCC 50360, isolated in 1992 from the intestinal tract of a goldfish (*Carassius auratus*) by S. A. Schaffer, and describe it as a new species, *D. parva*. We also carried out a more comprehensive phylogenetic analysis including 13 environmental 18S rDNA sequences related to *D. marina* or *D. parva*, and a comprehensive selection of other Labyrinthulea, using improved methods for 327 heterokonts, which reveals at least 15 genetically diverse *Diplophrys*-related species spread across two anciently diverged clades. Given their deep genetic divergence and ultrastructural differences, we transfer *D. marina* to a new genus *Amphifila* and establish separate new families: Diplophryidae for *D. archeri* and *D. parva*, and Amphifilidae for *Amphifila marina*. In the light of our 18S rDNA tree, we also conduct a broader taxonomic revision of Labyrinthulea at the family level to harmonize their classification better with deep genetic divergences revealed on this and other recent trees (Tsui *et al.* 2009, Lara *et al.* 2011) and with marked morphological differences across the tree that are insufficiently reflected in current family demarcations. Altogether, we establish six new families within Labyrinthulea, plus a seventh for *Labyrinthomyxa* (Labyrinthomyxidae) which because of limited data (Dubosq 1921) cannot be included in Labyrinthulea: we place it incertae sedis within the chromist subkingdom Harosa.

MATERIALS AND METHODS

Light microscopy

Light microscopic observations were made on live cells using the following equipment: (1) a Zeiss Axioskop compound microscope equipped with an Optronics DEI-470 CCD camera, and (2) a Zeiss Axioplan compound microscope equipped with a Zeiss AxioCam digital camera. Images were captured electronically. Measurements were made from digital photographs.

Electron microscopy

Samples were prepared for ultrathin sectioning and direct observation of the surface scales using negative staining. Cultures of ATCC 50360 isolate (designated as *Diplophrys* sp.), maintained in ATCC medium 802: Sonneborn's Paramecium medium, were fixed for transmission electron microscopy as previously published (Anderson *et al.* 1997). The medium also contained *Aerobacter aerogenes* and mixed bacteria as prey. A suspension of cells placed in a 15 ml graduated conical centrifuge tube was mixed with an equal volume of TEM-grade glutaraldehyde (4% (w/v) in 0.2 M cacodylate buffer, pH 7.2), to yield a final fixative of 2% (w/v). After 20 min. at 3°C, the glutaraldehyde-fixed cells were gently spun down to form a pellet, the supernatant was removed by aspiration, and 2 ml of cold osmium tetroxide solution (2% (w/v) in 0.2 M cacodylate buffer, pH 7.2) were added and the pellet thoroughly dispersed in the fixative. After 1-h post-fixation at 3°C, the cells were again pelleted and the supernatant removed. The cells were enrobed in 0.4% (w/v) solidified agar. Small cubes (~ 1 mm) were cut from the agar block, washed in distilled water, dehydrated in a graded acetone/aqueous series, infiltrated with and embedded in low viscosity epon (Energy Beam Sciences, Agawam, MA), and polymerized at 75°C for 12–18 h. Ultrathin sections were cut with a Porter-Blum MT-2 ultramicrotome (Sorvall, Norwalk, CT) using a diamond knife, collected on uncoated copper grids, post-stained with Reynold's lead citrate, and observed with a Philips TEM-201 transmission electron microscope (Einthoven, Netherlands) operated at 60 kV accelerating voltage.

A portion of the glutaraldehyde-fixed suspension of intact cells and shed scales was prepared for negative staining. Fixed cells were gently sedimented to form a pellet, the supernatant was aspirated away, and distilled water added to resuspend the pellet. Small aliquots of suspended cells and free scales were deposited on carbon-coated formvar grids (200 mesh), excess liquid gently removed by placing a small segment of bibulous paper at the edge of the grid, and stained with 2% (w/v) ammonium molybdate adjusted to pH 6.8 with KOH solution. The air-dried grids were observed with the Philips TEM-201 transmission electron microscope (Einthoven, Netherlands).

Phylogenetic analysis

Based on the 18S rDNA alignment of Gómez *et al.* (2011), we added many more sequences manually (using MACGDE v. 2.4: Linton macgde.bio.cmich.edu), giving an alignment of 457 heterokont sequences, made preliminary distance trees and then selected for thorough phylogenetic analysis an additional 327 heterokont sequences giving a balanced and broad sampling of all lineages, plus 27 broadly representing the closest outgroups Alveolata and Rhizaria (rich and representative sampling of relatively close outgroups is important for correct rooting within Heterokonta; many published trees use so few outgroup sequences that the root position may be erroneous). We analyzed 1,654 unambiguously aligned nucleotide positions (more than any previous study) by ML using the rapid bootstrap option (1,000 resamplings) of RAxML v. 7.0.3 (Stamatakis 2006) with the GTRMIX model and evaluating the optimal tree by GTR + GAMMA with empirical base frequencies and 25 per site rate categories. To examine heterokont topology without outgroups, to see how taxon sampling affects the tree, we applied the same method to produce trees for the 327 Heterokonta alone (not shown) and for reduced data sets of 140, 188, 224, 268, 281, 300, 307, 327, 333, 342 and 354 sequences of Harosa (i.e. the chromist subkingdom comprising Heterokonta, Alveolata and Rhizaria: Cavalier-Smith 2010a), which removed most Ochrophyta and varying numbers of others by excluding more closely related sequences. To see which groupings were stable irrespective of method, we also ran neighbor joining (NJ) distance trees for these and other taxon samples using the F84 gamma model of Phylip v. 3.68 (Felsenstein http://evolution.genetics.washington.edu/phylip).

RESULTS

Light and electron microscopic morphology

The light microscopic morphology of the ATCC isolate 50360, here described as *Diplophrys parva* n. sp., exhibits typical features of the genus *Diplophrys* (Fig. 1), including the ovoid to ellipsoidal cell shape (~ 5–7 μm), emergent tufts of branching pseudopodia at one or two protruding portions of the cell periphery, and one to several prominent internal refractive granules (~ 1–2 μm) of unknown composition, but possi-

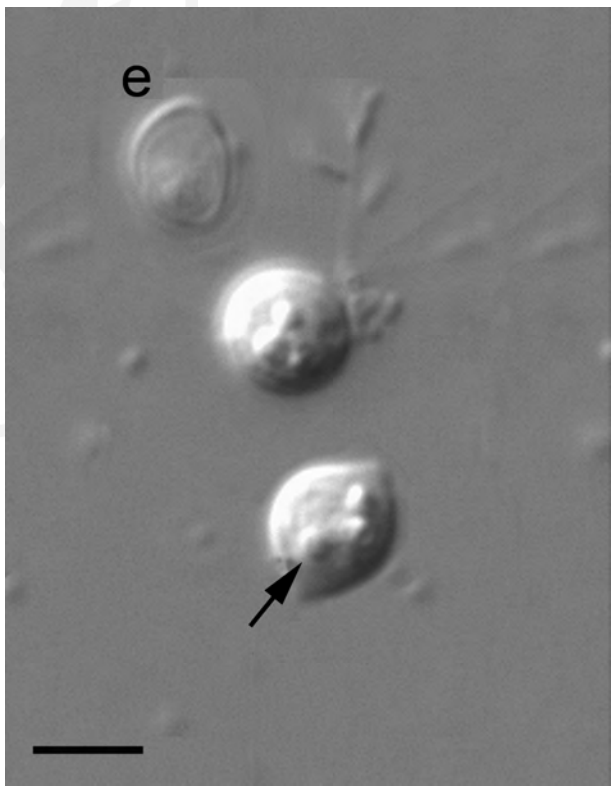

Fig. 1. Light micrographs of *Diplophrys parva* n. sp. Cells are ovate to rounded, containing one or more refractive granules (arrow). An empty cell envelope (theca: e) containing only a small fragment of cellular debris exhibits its thin composition. Bar: 5 μm.

bly lipid. Pseudopodia branch but do not anastomose. No evidence of cell aggregation, cysts, fruiting bodies, phagotrophy or cilia was seen, but the culture does contain bacteria. A small vacuole near the periphery of the cell, visible in light microscopic images particularly near the poles, appears to be a contractile vacuole.

Images of ultrathin sections show fine structural details characteristic of the genus, including the surrounding theca (envelope) of overlapping scales and characteristic refractive bodies within the cell (Fig. 2). The overall features of a section through a cell (Fig. 2) show the loosely arranged organic scales (arrow) forming the theca. The prominent nucleus contains a somewhat denser nucleolus (Fig. 3). Mitochondria with tubular cristae are scattered throughout the peripheral cytoplasm, and patches of convoluted smooth endoplasmic reticulum are sometimes observed in the vicinity of the nucleus (Fig. 3). Pseudopodia emerge from the cell surface as electron dense conical projections, possibly sagenetosomes (also known as bothrosomes) and become longer tubular extensions. At first they may be contained within the surrounding envelope of scales, but eventually penetrate through the scales at one or a few places and emerge, becoming less electron dense. The peripheral cytoplasm also contains rounded vacuoles with less electron dense deposits of unknown composition. The characteristic refractive granules appear to begin development as less-enlarged, irregularly shaped membrane-bound spaces (Fig. 2), often near electron-dense deposits that appear to be lipid. Eventually, they become much enlarged and more rounded (Fig. 4). The prominent Golgi apparatus contains multiple flattened saccules (≥ 10) that are inflated at the periphery where Golgi-derived vesicles are secreted (Fig. 4). The surface scales, when viewed with negative staining, are variable in shape; but typically are elongated and oval (Fig. 5), approximately 1 μm × 0.5 to 0.7 μm. Other scales are somewhat more broadly oval or become deformed, appearing rounded or angular when dried on the grid in contiguity to one another (Fig. 5, inset). These scales are approximately the same size or somewhat smaller than the more elongated oval scales (Fig. 5).

Molecular phylogenetic evidence

On a previously published distance tree, the *Diplophrys marina* 18S rDNA sequence was weakly sister to *Labyrinthula* (Cavalier-Smith and Chao 2006); but that tree did not include the partial sequence of *Diplophrys parva* in case it distorted the topology. We have, therefore, done a new analysis using maximum likelihood, which can place partial sequences more accurately, including it and a much larger number of labyrinthulean sequences, both cultured ones of known phenotype and environmental DNA sequences, in order to further clarify the position of *D. parva*. Figure 6 shows the branching order of the Labyrinthulea part only of the heterokont tree: the three main heterokont branches (Ochrophyta, Pseudofungi, Opalozoa) are collapsed as their internal branching order is irrelevant to this paper and would make the tree too large to fit on the page. The internal branching order of the three collapsed branches on this same comprehensive tree is shown in a separate paper describing a novel opalozoan flagellate (Cavalier-Smith and Scoble in press). Figure 6 gives strong support (98%) to the holophyly of Labyrinthulea including both *D. parva* and *A. marina*, but shows that *D. parva* is extremely distant from both *A. marina* and Labyrinthulida. *Diplophrys parva* is sister with 100% bootstrap support to an environmental sequence from a peat bog as first noted by Lara *et al.* (2011), and this clade is sister in turn to a much more deeply branching marine sequence of unknown phenotype (GU823043). The three together form a robust clade (labeled Diplophryidae) that is at least as deeply diverging from the clade containing *A. marina* as are Thraustochytriidae *sensu stricto*. By contrast *A. marina* is sister with 84% support to a freshwater environ-

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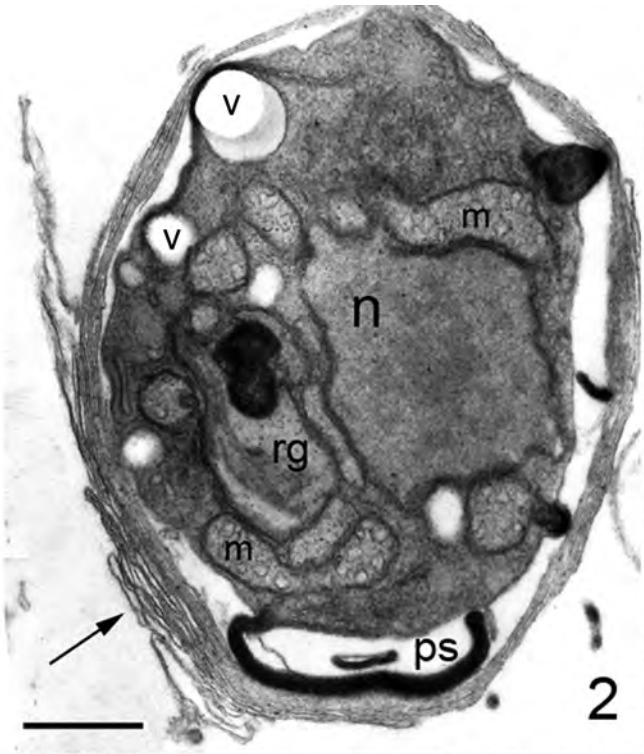
Figs 2–5. Fine structure of ultrathin sections of *Diplophrys parva* n. sp. **2** – Cell section showing the nucleus (n) with undulating to angular margin, mitochondria (m) with tubular cristae, segment of a "refractive granule" (rg), small peripheral vacuoles (v), emergent pseudopodium (ps) with characteristic electron dense cytoplasm, but typically becoming more granular and translucent after emerging through the scale coat, and profile of the multi-lamellated scales (arrow) forming the envelope. Bar: 0.5 μm; **3** – nucleus (n), with a more electron dense nucleolus (nu), and nearby profiles of convoluted smooth endoplasmic reticulum (er), and a longitudinal section through an elongated mitochondrion (m) with prominent tubular cristae. Bar: 0.5 μm; **4** – peripheral region of a cell showing the Golgi body (g) with multiple stacks of cisternae, mitochondria (m), and a prominent section through a membrane-enclosed "refractive granule" (rg). Bar: 0.2 μm; **5** – negatively stained preparations of scales (arrow) that typically appear elliptical (~ 1 × 0.5–0.7 μm) or occasionally much more irregular in shape (inset) from polygonal to broadly ovate (arrow, inset). Bars: 0.5 μm.



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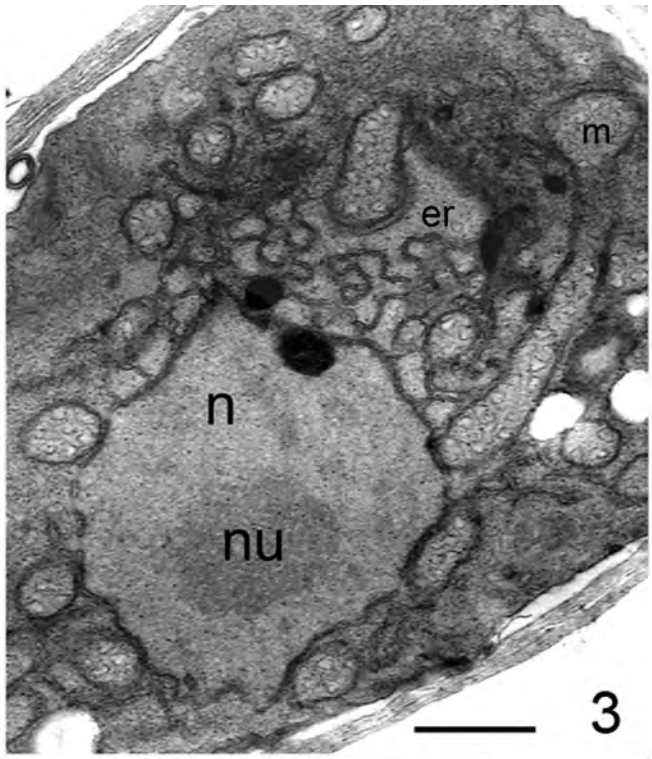

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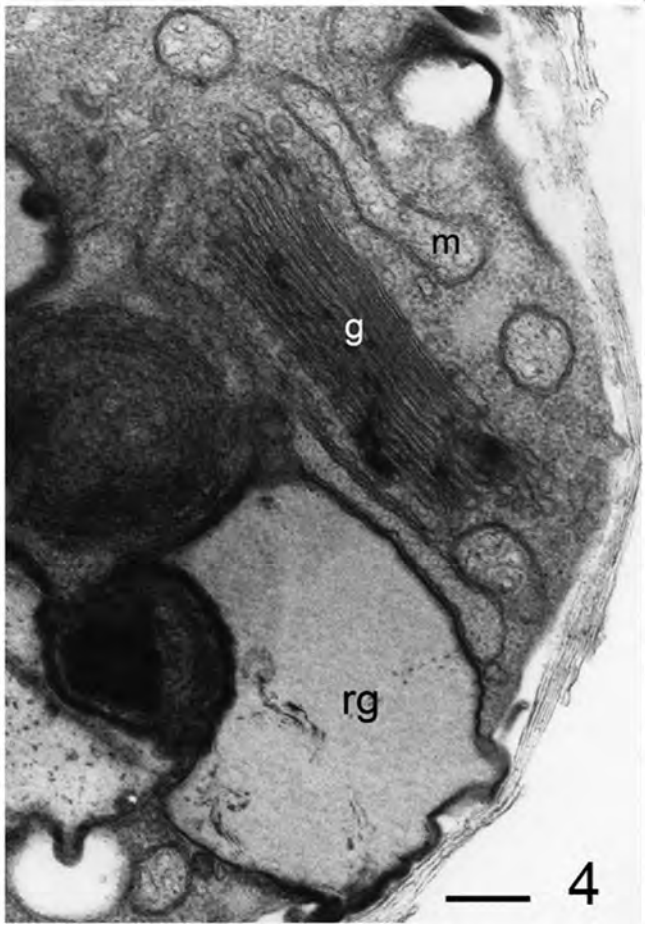

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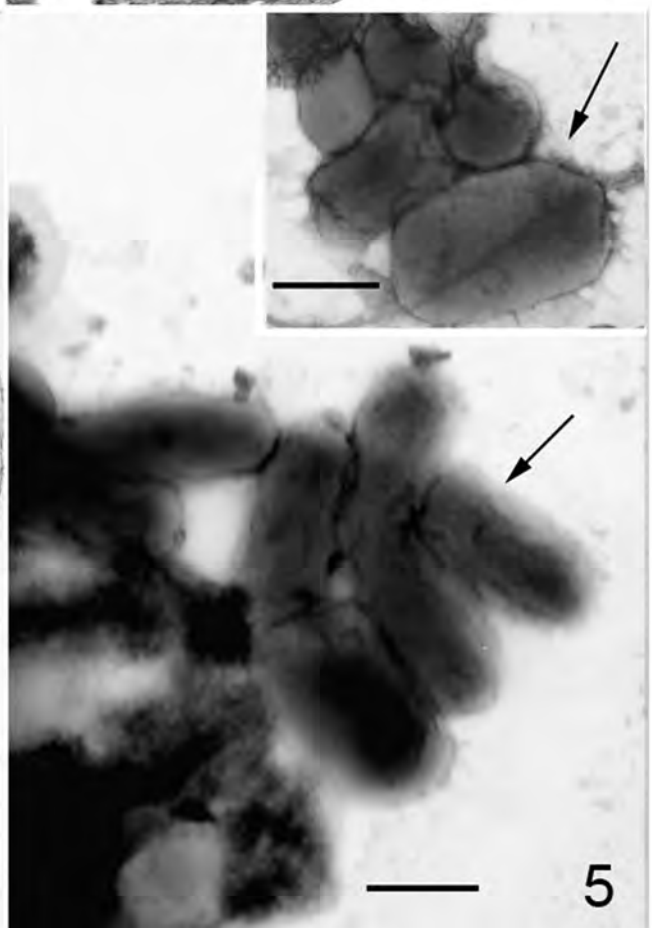

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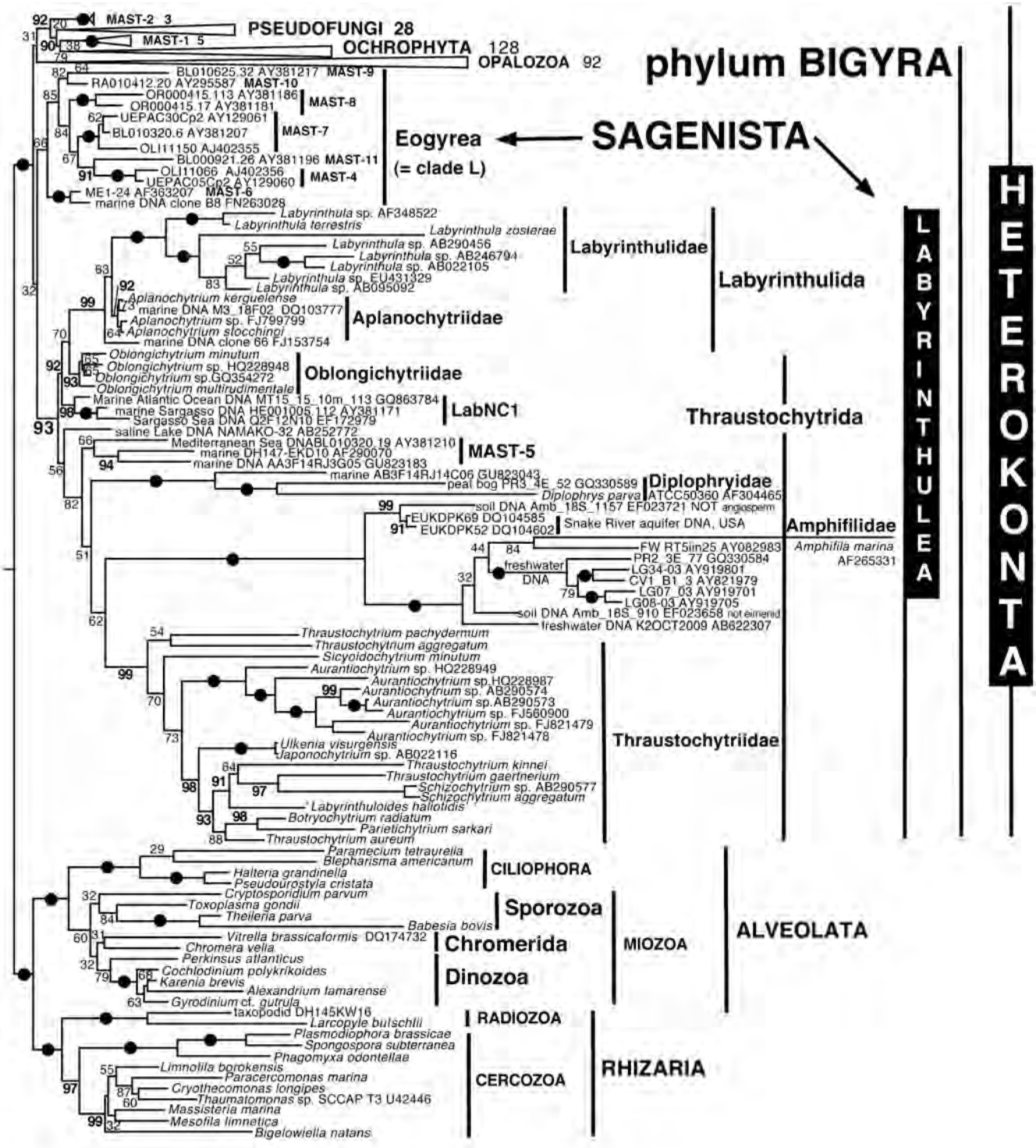

Fig. 6. Maximum likelihood tree for 327 heterokonts emphasizing Labyrinthulea and Eogyrea of phylum Bigyra and the non-heterokont outgroups. Internal branches for the five clades at the top of the tree are collapsed, but are being published separately (Cavalier-Smith and Scoble in press); the numbers to their right indicate how many sequences were included in each. Bootstrap supports for bipartitions are based on 1,000 resamplings using GTRMIX option of RAxML. Black bullets indicate 100% bootstrap support. The sequence attributed to '*Labyrinthuloides haliotidis*' might be from a thraustochytrid contaminant rather than from *Aplanochytrium* (=*Labyrinthuloides*) *haliotidis* (Leander and Porter 2001).

mental sequence that is part of a very robustly supported, apparently ancestrally freshwater, clade of 12 diverse sequences, which is sister with weak support to Thraustochytriidae, not Diplophryidae. Our tree suggests with 82% support that the clades containing *Diplophrys* and *Amphifila* are both more closely related to Thraustochytriidae than to Labyrinthulida.

As previously suspected (Cavalier-Smith and Chao 2006), Thraustochytrida are probably paraphyletic and ancestral to Labyrinthulida, because Oblongichytriidae are robustly sister to Labyrinthulida (92% support), not Thraustochytriidae. Eogyrea comprise 7 distinct subclades MAST-4, MAST-6-11 of marine pico-nanoeukaryotes, which previously had not so clearly been shown to be all related; MAST-4 at least has a cilium, is phagotrophic and lacks plastids (Massana *et al.* 2006), so Eogyrea may all be zooflagellates. They are the closest phagotrophic relatives to Labyrinthulea on our tree, though with less support for being sisters to Labyrinthulea than on our earlier tree, where they were then simply called clade L (Cavalier-Smith and Chao 2006), as it was then unclear whether they were deep branching Labyrinthulea or a distinct phenotype, as is now evidently the case.

TAXONOMY

New species description

Diplophrys parva n. sp. Anderson and Cavalier-Smith

Diagnosis. Cells ellipsoid, or ovate to round (L = 6.5 ± 0.08 μm, W = 5.5 ± 0.06 μm, means ± S.E., N = 50) (Fig. 1), enclosed by thin envelope of overlapping scales (Fig. 2); sometimes rounded to broadly oval, but typically elongated oval with rounded ends (1.0 × 0.5–0.7 μm) (Fig. 5), one to several intracytoplasmic refractive granules (1–2 μm) and smaller round hyaline vacuoles near cell periphery (Fig. 2). Pseudopodia emanate from cell surface, appearing initially as an electron-dense conical protrusion (sagenetosome or bothrosome), eventually elongating and penetrating through the extracellular envelope of scales, either at one pole of the cell or two (two apparently always in *Diplophrys archeri*). Tubulocristate mitochondria; prominent Golgi with numerous (10 or more) stacked saccules located at the periphery of the cytoplasm (Fig. 4). No cysts observed. *D. archeri* is about twice as large and *A. marina* has more obvious, more densely branched filopodial tufts.

Etymology: Species epithet, *parva*, refers to the small size of this species.

Type source: Intestinal tract of a freshwater fish (*Carassius auratus*) isolated by S. A. Schaffer in 1992, Baltimore, MD.

Type material: Cryopreserved culture (ATCC 50360), American Type Culture Collection, Manassas, VA, USA. Type 18S rDNA sequence: AF304465.

Revision of Labyrinthulea

We first establish three new families to include all non-ciliate Labyrinthulea whose vegetative cells have polar tufts of extremely slender branching filopodia: (1) Diplophryidae to contain the genus *Diplophrys*; (2) Amphifilidae to contain the species *D. marina*, now assigned to the new genus *Amphifila*, including the numerous environmental sequences that group with it on Fig. 6; and (3) Sorodiplophryidae for *Sorodiplophrys* (Cienkowski 1876*)*, because unlike the other two it has an aggregative sorocarp-bearing fruiting mode, uniquely within Labyrinthulea. Secondly, to rationalize current classification of Labyrinthulea, which lags behind recent evidence for much greater deep diversity than hitherto realized (Lara *et al.* 2011, Tsui *et al.* 2009, Yokoyama and Honda 2007, Yokoyama *et al.* 2007), we establish three other new families. Two (Aplanochytriidae, Oblongichytriidae) each correspond with a well-supported clade on the rDNA tree and are morphologically distinct from the classical Thraustochytriidae, but were traditionally included in Thraustochytrida. The third is Althorniidae for *Althornia*, which unlike other Labyrinthulea is planktonic not benthic, unlike both thraustochytrids and labyrinthulids lacks sagenetosomes, and unlike Labyrinthulida lacks an ectoplasmic net (Jones and Alderman 1971). Finally we establish a new family for the net-forming *Labyrinthomyxa*, which we do not accept as a labyrinthulid and exclude from Labyrinthulea.

All these taxa are treated here under ICZN, not IBN, as none of them is a fungus or alga and they belong in the purely heterotrophic plastidless phylum Bigyra. Their closest relatives are the phagotrophic zooflagellate subphylum Opalozoa (recently revised to include Bicoecea: Cavalier-Smith and Scoble in press), and it is best to treat all Bigyra under ICZN for uniformity across the whole phylum and because ICZN does not intrusively recommend suffixes like -phyceae, -mycetes or -mycota that wrongly suggest that Labyrinthulea are algae or fungi.

Six new families of Labyrinthulea

1. Diplophryidae Cavalier-Smith fam. n.

Diagnosis: Non-ciliated spherical, unicellular heterotrophic protists with scaly theca; without zoospores; with one or two polar tufts of sometimes branching but not anastomosing ectoplasmic threads stemming from a sagenetosome-like structure – if two, passing through the scaly theca on opposite sides of cell but with an offset; with a large (or few smaller), refractive golden yellow or amber lipid drop beside the nucleus. Glide slowly without obviously moving filopodia. Type genus *Diplophrys* Barker, 1868, p. 123.

Comment: As both have filopodia stemming from two pores *Diplophrys* was once grouped with *Amphitrema* in Amphitremidae Poche, 1913, or in Amphistomina (Wailes 1915; Calkins 1926). We agree with Wailes (1915) that they are probably unrelated; *Amphitrema* is not scaly but has an agglutinated test like the undoubtedly cercozoan filose amoeba *Pseudodifflugia*, so Amphitremidae (without *Diplophrys*) is now included in the order Tectofilosida of the cercozoan class Tectofilosea (Howe *et al.* 2011a). Amphistomidae is invalid, not being based on an included genus and preoccupied by a family or subfamily including the fluke genus *Amphistoma* Rudolphi, 1801 or *Amphistomum* Rudolphi, 1814. *Diplophrys* is not a foraminiferan, so cannot be kept in Allogromiidae, thus making a new family is essential as absence of zoospores and strong divergence on our tree prevent inclusion of *Diplophrys* in Thraustochytriidae.

2. Amphifilidae Cavalier-Smith fam. n.

Diagnosis: Non-ciliated spindle-shaped, thecate unicellular heterotrophic protists; without definite sagenetosome or zoospores; with two tufts of sometimes branching but not anastomosing filopodia emanating though two pores situated on opposite acute points of scaly theca or wall; with a large (or few smaller), refractive lipid drop beside the nucleus. Glide with filopodia preceding cell. Type genus *Amphifila* Cavalier-Smith gen. n. Diagnosis as for family. Type species *Amphifila marina* Cavalier-Smith comb. n. Basionym *Diplophrys marina* Dykstra and Porter (1984, p. 627).

Etymology: *Amphi* Gk both; *filum* L. thread, as filopodia extend from both ends of cell.

Comment: *D. archeri* Barker (1868), the type species, was a spherical freshwater organism. Although not then figured, the description and plates in Wailes (1915) and the photograph on p. 82 of Patterson (1992) correspond closely with Barker's original description of *D. archeri* and with a bloom of freshwater *Diplophrys* observed by TCS in South Africa. In marked contrast, *A. marina* was spindle-shaped with pointed ends, so we do not accept the opinion that they are the same species (Patterson 1989). Figure 6 shows that freshwater *Diplophrys parva* is exceedingly distantly related to the type strain of *A. marina*; each belongs to a separate genetically highly diverse clade. Patterson (1989) did not say whether the strain depicted in his Figs 19.17–19 was from freshwater or marine samples, but his light micrograph fits his identification as *D. archeri* (different in shape from *A. marina*). His electron micrographs show a thinner wall than in *A. marina* and it is unclear if it is composed of scales; unlike in *A. marina,* the wall has no conical point where filopodia exit. His Fig. 17 shows many more filopodia in each tuft than in *A. marina* (or *D. parva*) and no ectoplasmic swelling as in *A. marina* and Thraustochytriidae. As *Amphifila* and *Diplophrys* are somewhat different morphologically and very distinct in sequence, naming spindle-shaped marine protists *D. archeri* (Vørs 1992; following Patterson 1989) was incorrect. Their marine habitat and spindle shape suggest they were *A. marina*; the cell labeled '*D. archeri* after Vørs 1992' in Patterson *et al.* (2000) also is probably *A. marina* and misleading as to the *Diplophrys* phenotype. The marine Arctic cells of Vørs (1993, Fig. 40F), more oval than *D. archeri* and less pointed than *A. marina*, are probably misidentified as *D. archeri* and could be a third species. The Antarctic cells of Tong *et al.* (1997, Figs 5F, 6N) are probably also misidentified as *D. archeri*, being broadly spindle-shaped with unusually short and unbranched filopodia (not stated whether the protists illustrated were marine or freshwater), probably a fourth species. These previously overlooked subtle differences in morphology are consistent with our tree showing that there must be over a dozen undescribed species related to *Diplophrys* or *Amphifila* and thus likely to have a broadly similar two-tufted phenotype; contrary to Patterson *et al.* (2000) there is not just one species – as in other formerly overlumped taxa with relatively minor light microscopic variation (e.g. glissomonads: Howe *et al.* 2009, 2011b) there could be many.

3. Sorodiplophryidae Cavalier-Smith fam. n.

Diagnosis: Coprophilic non-ciliated, unicellular heterotrophic protists with filopodial gliding motility; without definite sagenetosome or zoospores; with predominantly polar tufts of highly branched, sometimes anastomosing filopodia emanating at opposite points of cell wall composed of thin scales, often with lamellipo-

dium at the base that may extend round the sides of the cell. On starvation, vegetative cells aggregate to form a stalked, golden yellow sorocarp containing numerous elliptical sorocytes, analogously to dictyostelid slime molds. Sorocytes with contractile vacuole and refractive yellow bodies; vegetative cells ovoid to elliptical, with small colorless granules instead of yellow bodies. Glide at 30 μm/min. with filopodia at both ends, anterior ones shortening as they progress. Type genus *Sorodiplophrys* Cienkowski, 1876.

Comment: We agree with Dykstra and Olive (1975) that *Sorodiplophrys* cannot be included in Labyrinthulidae or Thraustochytridae, and with their conjecture that it may nonetheless be related to both. The more elongated nature of its cells than in *Diplophrys*, its tendency for filopodial anastomosis and marked mobility suggest that it is evolutionarily closer to Amphifilidae than Diplophryidae. Therefore, we group Amphifilidae and Sorodiplophryidae together as superfamily Amphifiloidea Cavalier-Smith fam. n.

Diagnosis: Vegetatively unicellular, non-ciliate typically elongated osmotrophic heterotrophs, with scaly walls and two opposite tufts of highly branching, sometimes anastomosing, filopodia; sagenetosome not obvious.

4. Aplanochytriidae Leander ex Cavalier-Smith fam. n.

Diagnosis: Marine saprophytic or parasitic heterotrophic protists forming scaly walled sporangia that release crawling non-flagellate gliding cells and or biciliate zoospores; unlike Labyrinthulidae vegetative cells not spindle-shaped, often typically spherical or nearly so and mobile upon an ectoplasmic net that does not completely enrobe them as it does in Labyrinthulidae; sagenetosome single unlike *Labyrinthula*.

Comment: Alderman *et al.* (1974) wrote that *Aplanochytrium* should be removed from Thraustochytriidae as a new family; Leander and Porter named this Aplanochytriaceae but only in Leander's 2001 PhD Thesis (Georgia University, USA), so the name was not validly published; it did not appear in their journal article (Leander and Porter 2001). Type genus *Aplanochytrium* Bahnweg and Sparrow, 1972. The gliding motility of vegetative cells (unlike Thraustochytriidae) and 100% BS support for *Aplanochytrium* being sisters to Labyrinthulidae justify placement within Labyrinthulida as a new family.

5. Oblongichytriidae Cavalier-Smith fam. n.

Diagnosis: Thraustochytrids that have slender oblong zoospores, not squat oval ones as in Thraustochytriidae; their 18S rRNA sequences do not group with those of Thraustochytriidae but near the base of the labyrinthulean clade. Type genus *Oblongichytrium* Yokoyama and Honda (2007, p. 2002). Their non-grouping with Thraustochytriidae *sensu stricto* (Table 1) is robustly on all our trees and the 3- and 4-gene trees of Tsui *et al.* (2009).

6. Althorniidae Cavalier-Smith fam. n.

Diagnosis: Floating thraustochytrids with laterally biciliate zoospores but no ectoplasmic net or sagenetosomes. Type genus *Althornia* Jones and Alderman, 1971.

Table 1. Revised classification of Labyrinthulea (phylum Bigyra, subphylum Sagenista).

Class Labyrinthulea (Lister 1891) Olive ex Cavalier-Smith 1986

Order 1. Thraustochytrida Sparrow 1973

Family 1. Thraustochytriidae Sparrow ex Cejp 1959 (*Thraustochytrium*, *Ulkenia*, *Schizochytrium*, *Japonochytrium*, *Aurantiochytrium*, *Sicyoidochytrium*, *Parietichytrium*, *Botryochytrium*)
Family 2. Oblongichytriidae Cavalier-Smith fam. n. (*Oblongichytrium*)
Family 3. Althorniidae Cavalier-Smith fam. n. (*Althornia*)
Family 4. Diplophryidae Cavalier-Smith fam. n. (*Diplophrys*)

Superfamily Amphifiloidea Cavalier-Smith superfam. n.

Family 1. Amphifilidae Cavalier-Smith fam. n. (*Amphifila*)
Family 2. Sorodiplophryidae Cavalier-Smith fam. n. (*Sorodiplophrys*)

Order 2. Labyrinthulida Doflein 1901

Family 1. Labyrinthulidae Cienkowski 1867 (*Labyrinthula*)
Family 2. Aplanochytriidae Leander ex Cavalier-Smith fam. n. (*Aplanochytrium*)

Incertae sedis

***Labyrinthomyxa*; a possibly unrelated net-forming protist**

A heterotrophic net-forming protist superficially similar to labyrinthulids is *Labyrinthomyxa* (Dubosq 1921) with an anteriorly directed single cilium and a single amoeba phase; as its spindles do not move within the net, which in some respects is more like that of *Leukarachnion* (Grant *et al.* 2009), and there is no evidence for a laminate (or other) theca we exclude it from Labyrinthulea and establish a separate family (Labyrinthomyxidae), here placed incertae sedis in Harosa as it is unclear whether it belongs in Heterokonta (possibly Leukarachnida; unlike *Leukarachnion* not known to be phagotrophic) or Cercozoa (possibly Endomyxa).

Labyrinthomyxidae Cavalier-Smith fam. n.

Diagnosis: Filoplasmodial heterotrophs whose spindle-shaped cells with bipolar projections form linear, branching, anastomosing rows and parasitize solenocysts of the brown alga *Laminaria*; with uninucleate amoeba or uniciliate phases; cyst or theca unknown. Type genus *Labyrinthomyxa* (Dubosq 1921).

Another protist with similarities to labyrinthulids is *Chlamydomyxa labyrinthuloides* (Archer 1875), but its taxonomy is confused by probable later misidentifications. Unless Archer conflated multiple organisms, we support his interpretation of the original *Chlamydomyxa labyrinthuloides* as probably a labyrinthulid (distinct enough to merit its own family), unlike subsequent authors who questioned that or described other probably unrelated '*Chlamydomyxa*' species (Geddes 1882, Hieronymus 1898, Lankester 1896, Pascher 1930, Pearlmutter and Tumpano 1984, Penard 1904) – clonal cultures more similar to those of Archer are needed to check this. In particular we consider the non-reticulose, filose amoeboid heterokont alga identified as *Chlamydomyxa labyrinthuloides* by Wenderoth *et al.* (1999), whose 18S rDNA places it in Picophagea within the phylum Ochrophyta (Cavalier-Smith and Chao 2006), was misidentified and is really a new species in an undescribed genus – to be established elsewhere.

DISCUSSION

Our most striking conclusion is that Labyrinthulea includes two genetically extremely divergent clades of non-ciliated protists with two polar tufts of filopodia, which are so similar in the light microscope that some have thought they were just one species (Patterson *et al.* 2000). Overall, based on available morphological evidence, we conclude that the ATCC 50360 strain is a new species in the genus *Diplophrys*, but *Amphifila* is only remotely related.

Novelty of *Diplophrys parva*

The ATCC 50360 isolate that we name *Diplophrys parva* n. sp., differs substantially in size, shape, and/or fine structural features from published descriptions of *D. archeri* and *A. marina.* It is essentially the same length as *A. marina,* but only about half the size of *D. archeri* that typically has a much larger refractive granule (often filling over half the diameter of the cell) and larger cell size (10 μm or larger) compared to *D. parva* (5–7 μm). However, the original description of *D. archeri* stated only that it was exceedingly minute and gave no size measurement or illustration. Archer, as described in Barker (1868), first stated that its average size was 1/2000 inch, i.e. 12.7 μm. As Archer was present at the meeting the previous year where *D. archeri* was first shown in public, described and its name published, we consider that this should be accepted as the average size of *D. archeri*. *D. parva* is about half the size of *D. archeri*, a sufficiently large difference to make it unwise to treat them as one species. As many different species have probably been lumped under that name and most descriptions may relate to others, different sizes given in some later studies should not be attributed to *D. archeri*. Despite their somewhat similar size and general appearance, there is no possibility of confusing *D. parva* and *A. marina*. One is marine and the other freshwater; as they have ultrastructurally different scales and their rDNA sequences are radically different, two genera are merited.

Contrast between *Diplophrys* and *Amphifila marina*

Conservation of the name *Diplophrys* for *D. parva* rather than *A. marina* merits discussion. Until an authentic culture of *D. archeri* is sequenced, we cannot be sure that retention of the generic name *Diplophrys* for the *D. parva* rather than the *Amphifila* clade is correct, but a decision one way or the other had to be made. We picked *D. parva* for three reasons: first because its more rounded, less pointed shape, is more like *D. archeri* than is the spindle-shaped *A. marina*. The consistent phylogenetic contrast between the elongated *Oblongichytrium* and round Thraustochytriidae *sensu stricto* (Yokoyama and Honda 2007, and Fig. 6) shows

that small differences in cell shape can have surprisingly deep phylogenetic significance in Labryrinthulea. Second are the filopodia: in *D. parva* and *archeri* they are branched but non-anastomising, and both show only minimal cell motility if any – no locomotion was mentioned in the original descriptions of *D. archeri* (Barker 1868). By contrast *Amphifila* locomotes by active gliding and shows fine filopodial anastomoses, both characters shared with *Sorodiplophrys*, but not *D. archeri*. Thirdly, *D. archeri* and *D. parva* are both from freshwater, whereas *Amphifila* is marine, and conservatism of freshwater versus marine habitat is pronounced in many protists (Cavalier-Smith and Chao 2012), and also shows a non-random distribution across Labyrinthulea. One can argue that Labryrinthulea were probably ancestrally marine. However, most lineages of the clade to which *Amphifila* belongs are freshwater (or soil, ecologically cognate), so that clade was probably freshwater for most of its evolutionary history, and the ancestor (or ancestors) must have made one relatively recent switch into the oceans, perhaps accompanying the sea grasses with which it is commensal. The sequence closest to *D. parva* comes from European peat bogs and *D. archeri* was from Irish moors, both consistent with the morphological evidence that *D. parva* and *archeri* are mutually closer than to *Amphifila*.

Scale ultrastructure, often good phylogenetic indicators (Cavalier-Smith and Chao 2012, Howe *et al.* 2011a), strongly supports this; we found that *D. parva* has oval to elongated scales (~ 1 μm) decidedly different in size and shape from the round scales (~ 2 μm) of *A. marina*. A second ultrastructural difference is that *D. parva* has an obvious dense structure somewhat resembling a sagenetosome, whereas no evidence for a sagenetosome was seen in *Amphifila*, in which respect also it resembles *Sorodiplophrys* (Dykstra and Olive 1975).

Increased diversity of *Diplophrys*-like protists

Only two previously described species were recently accepted as *Diplophrys*: *Diplophrys archeri* and *Diplophrys marina* (here moved to *Amphifila*. *Diplophrys stercorea* described by Cienkowski (1876) was reassigned to a separate genus *Sorodiplophrys* (Dykstra and Olive 1975), with *stercorea* the type species. It is a sorocarp-producing protist, thus sharply distinct from *Diplophrys* and *Amphifila*, despite having sufficiently similar vegetative cells to *A. marina* (net-like filopodia) to make a relationship plausible. The sorocarp (stalk-borne fruiting body) is a product of cellular aggregation as occurs among some slime molds, but as its vegetative cell structure is dissimilar from slime molds, and cell aggregation is well known as a polyphyletic character, it should not be placed in Mycetozoa. Though its ultrastructure remains unpublished, Dykstra and Olive (1975) stated that it lacks sagenetogens and has thin scales. *Sorodiplophrys* vegetative cells crawl using contractile non-granular filopodia, whose contractility makes them perhaps more similar to those in the cercozoan superclass Ventrifilosa (Cavalier-Smith and Karpov 2012), comprising the filose amoeboid classes Imbricatea and Thecofilosea (Howe *et al.* 2011a), than to *Diplophrys*. As Imbricatea often also have scales, it is possible that *Sorodiplophrys* belongs in that class, which includes a variety of amoebae and flagellates with similar contractile, non-granular branching filopodia (Howe *et al.* 2011a, Cavalier-Smith and Chao 2012). Moreover, the testate Amphitremidae, with bipolar filopodia analogous to, but more robust than, those of *Diplophrys,* is currently assigned to Thecofilosea (Cavalier-Smith and Chao 2012). However, we have adopted the more conservative stance of retaining *Sorodiplophrys* within Labyrinthulea, for two reasons. First, Dykstra and Porter (1984) noted thin scales of *Sorodiplophrys* resembling those of Labyrinthulea. If the scales had been more like any of the diverse siliceous scales of the scaly taxa now placed in Imbricatea, they would probably have mentioned that and even more strongly doubted its affinity with Labyrinthulea. Thus *Sorodiplophrys* is probably not an imbricate. Secondly, they stressed that *Sorodiplophrys* is osmotrophic and not phagotrophic, also making it unlikely that it is a scaly imbricate cercozoan amoeba (all have siliceous scales, not unmineralized organic ones like Labyrinthulea).

In contrast to *Amphifila* and *Sorodiplophrys*, both currently recognized species of *Diplophrys* present diagnostic features of the genus, i.e. ellipsoidal to ovoid cells, non-aggregating cells, enclosed by a thin envelope (shown to be imbricated scales by fine structure analysis) with pseudopodia emerging typically from two poles of the cell, forming a branching rhizopodial fan toward the periphery; there is at least one intracytoplasmic refractive granule, presumed to be lipid. In *D. archeri*, the refractive granules (one or more) are typically very prominent, yellowish in color, and occupy a large portion of the cell volume when viewed by light microscopy. Published images of *D. archeri* are typically in the range of 10–15 μm or somewhat larger (e.g. Barker 1868, Kudo 1977, p. 568). *D. marina* cells (3.7–5.9 × 5.1–8.5 μm) are ovoid with round Gol-

gi-derived scales (1.5–1.9 μm). The *Diplophrys*-like phenotype had only three named species (*D. archeri*, D. *marina*, and *Sorodiplophrys stercorea*) prior to this publication. Their placement now in three separate genera and families better reflects their evolutionary diversity and should stimulate further research on this unique protist type – neither a rhizopod nor a fungus but a very distinctive, albeit neglected, osmotrophic phenotype. Many understudied protists are not in culture (e.g. *D. archeri*), impeding molecular genetic analyses, but many more could probably be cultured with even a modest effort. Recent light micrographs of *D. archeri* with accurate diagnostic size and morphology for this species as described by Barker (1868) (e.g. http://starcentral.mbl.edu/microscope/portal.php?pagetitle=assetfactsheet&imageid=9704) show that *D. archeri*, and no doubt many genetically distinct look-alikes, can be isolated from the natural environment. Without a targeted study of *Diplophrys*, currently with only two verifiable species (*archeri* and *parva*), it is premature to judge whether its taxonomic diversity is really limited to the three sequences that branch robustly together in Fig. 6, or is much more extensive. However, given the small size of *D. parva*, and its broad similarity to the genetically very distant *Amphifila*, it is likely that many additional cryptic species will be discovered. The clade containing *Amphifila* is currently more speciose. Possibly one of the two distinctly deep-branching soil lineages in that clade is related to the dung-dwelling *Sorodiplophrys*, as dung dwellers are most likely to have evolved from soil biota; if that could be confirmed, it would make that quite speciose clade equivalent to the new superfamily Amphifiloidea. More intensive research on this microscopically distinctive but remarkably conservative morphotype is warranted.

Large-scale evolution in Labyrinthulea

We can now conclude that there are not just two broad phenotypes in Labryinthulea, but three. The thraustochytrid-like condition appears to be ancestral; i.e. scaly thecate vegetative cells with a single aperture from which a sagenetosome emits slender branching, but not anastomosing, filopodia used not for locomotion but presumably to increase surface area for absorbing dissolved organic molecules; biciliate zoospores mediate dispersal. Secondly are the net-like Labyrinthulida, with cells stationary in the net (Aplanochytriidae) or self-propelling within it (Labyrinthulidae). The third major phenotype is the *Diplophrys*-like one with two polar tufts of filopodia and no zoospores. Our trees show that Labyrinthulida and the *Diplophrys*-like phenotype are both derived from thraustochrytrid-like ancestors, but independently: both *Diplophrys*-like clades are unambiguously closer to Thraustochytriidae than to Labyrinthulida.

Our trees also raise the possibility that the *Diplophrys*-like phenotype evolved twice independently in Diplophryidae and Amphifiloidea. Such evolution involves only two things: loss of the zoospore, a very common evolutionary event in protists, and evolution of a second pore through the theca, which also is probably not difficult; so we should not be concerned that Diplophryidae and the clade including *Amphifila* are not sisters on our tree. But 18S rDNA clearly lacks the resolution to prove an independent origin of a second polar pore, though that would be consistent with the ultrastructural differences we found between *D. parva* and *Amphifila*, but these also do not establish an independent origin, either. Probably sequences from many genes will be needed for a firmer conclusion. A better supported instance of convergent evolution is filopodial anastomosis, which seemingly created a net-like absorptive surface independently in Labyrinthulida and Amphifiloidea, also probably not difficult to evolve twice; net-like pseudopodia also involved independently in Rhizaria (their ancestral state), Amoebozoa (e.g. leptomyxids) and elsewhere in Heterokonta in Chrysomonadea (e.g. *Leukarachnion*). However, they are all phagotrophs. We have used the word filopodium for the threadlike extensions of Labyrinthulea, but should stress that they are probably not homologous with filopodia in rhizopods and have no phagotrophic function; they appear to be purely absorptive like the microvilli of the mammalian intestine and it is open to debate whether the term filopodium is somewhat misleading, especially in most thraustochytrids where it lacks a locomotory function and is not in any sense a foot. This emphasizes that Labyrinthulea are protists *sui generis* that should not be slotted unthinkingly into conventional textbook categories.

Acknowledgements. We thank Dr. Robert Molestina (American Type Culture Collection, Manassas, VA) for providing the light micrograph from the ATCC collection. We also appreciate information provided by Dr. Tom Nerad on the history of the cultures and some details of light microscopic observations made at ATCC of isolate 50360. This is Lamont-Doherty Earth Observatory Contribution No. 7610. TCS thanks NERC for past grant support and Josephine Scoble for help with old literature and agreeing to the inclusion of part of the tree stemming from our joint work on heterokonts.

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Received on 1st June, 2012; revised on 1st September, 2012; accepted on 9th September, 2012