187-gene phylogeny of protozoan phylum Amoebozoa reveals a new class (Cutosea) of deep-branching, ultrastructurally unique, enveloped marine Lobosa and clarifies amoeba evolution

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A B S T R A C T

Monophyly of protozoan phylum Amoebozoa, and subdivision into subphyla Conosa and Lobosa each with different cytoskeletons, are well established. However early diversification of non-ciliate lobose amoebae (Lobosa) is poorly understood. To clarify it we used recently available transcriptomes to construct a 187-gene amoebozoan tree for 30 species, the most comprehensive yet. This robustly places new genus Atrichosa (formerly lumped with Trichosphaerium) within lobosan class Tubulinea, not Discosea as previously supposed. We identified an earliest diverging lobosan clade comprising marine amoebae armoured by porose scaliform cell-envelopes, here made a novel class Cutosea with two pseudopodially distinct new families. Cutosea comprise Sapocribrum, ATCC PRA-29 misidentified as ‘Pesonella’, plus from other evidence Squammamoeba. We confirm that Acanthamoeba and ATCC 50082 misidentified as Stereomyxa ramosa are closely related. Discosea have a strongly supported major subclade comprising Thecamoebia plus Glycostyliida (suborders Dactylodopodina, Stygamoebina; Vannellina) phylogenetically distinct from Centramoebida. Stygamoeba is sister to Dactylopodina. Himatismenida are either sister to Centramoebida or deeper branching. Discosea usually appear holophyletic (rarely paraphyletic). Paramoeba transcriptomes include prokinetoplastid Perkinsia-like endosymbiont sequences. Cunea, misidentified as Mayorella, is closer to Paramoeba than Vexillifera within holophyletic Dactylopodina. Taxon-rich site-heterogeneous rDNA trees confirm cutosan distinctiveness, allow improved conosan taxonomy, and reveal previous dictyostelid tree misrooting.

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1. Introduction

Amoebozoa are one of three radically different major groups of amoeboid eukaryotes that had independent evolutionary origins from different flagellate ancestors: each belongs in a different one of the three eukaryote supergroups now recognised – Amoebozoa in scotokaryotes, Rhizaria in corticates, and Percolozoa in Eozoa (Cavalier-Smith et al., 2015a). Abounding in soil and all aquatic habitats, as well as including parasites of animals, and both aerobes and anaerobes, Amoebozoa have over a thousand species. Their higher classification was confused for two centuries until electron microscopy revealed some key features, whose importance sequence phylogeny confirmed (Cavalier-Smith et al., 2004; Smirnov et al., 2011). The purely amoeboid, entirely non-ciliate amoebozoan subphylum Lobosa (e.g. Ameoba, Acanthamoeba, Hartmannella; colloquially lobose amoebae) are the main focus of

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of special evolutionary significance. Thread-like extensions can also be produced from a broader typically lobose region in *Parvamoeba* (Cole et al., 2010; Kudryavtsev et al., 2011). Lobose amoebae with broad pseudopodia include those with shells or tests (testate amoebae, comprising probably unrelated orders – freshwater Arcellinida and marine Trichosida) and more diverse naked species called gymnamoebae (Page, 1987, 1988) that lack tests.

Largely in the past decade, naked amoebae (not as shapeless as often supposed) have been successfully grouped by their different characteristic pseudopodial forms during active locomotion (e.g. flattened, tubular, monopodial, multipodial, digitiform, fan-shaped, conical) combined with ultrastructural variations in their cell surface coats (e.g. thick, thin, scales, glycostyles) into 12 distinct orders, most of whose monophyly is well corroborated by 18S ribosomal DNA trees (Cavalier-Smith et al., 2004; Smirnov et al., 2005, 2011). Four naked orders are grouped with arcellinids as class Tubulinida, united by tubular pseudopodia with monaxial internal cytoplasmic flow. The other eight have flattened cells that by contrast exhibit multiaxial cytoplasmic flow or flow without a pronounced axis, and constitute class Discosa that at present also includes the unique and least well understood Trichosida.

Trichosida are phylogenetically obscure large multicellular marine amoebae that uniquely have tests with numerous pores through which hair-like non-locomotory pseudopods emerge. This multiporose test and their having both locomotory lobopodia and thin (perhaps sensory) dactylopodia set Trichosida apart from all other Amoebozoa (Schuster, 1976; Angell, 1975, 1976), so much so they were sometimes thought to be related to Foraminifera instead (Loeblich and Tappan, 1964). The sole available 18S rDNA sequence from ‘Trichosphaerium’ sp. seemingly confirmed that Trichosida are amoebozoa, but is so divergent from all others that it grouped in Conosa with the long-branch myxogastrid slime moulds. Actin plus rDNA jointly contradictorily put it within Lobosa with *Thecamoeba similis* (Tekle et al., 2008), whereas the trichosids have been tentatively classified (Smirnov et al., 2011), though a six-gene tree did put it in Conosa within slime moulds (Lahr et al., 2015). We argue here that this strain was misidentified and is not a *Trichosphaerium*, though is probably related, so establish a new genus *Atrichosa* for it.

Multigene trees support this subdivision of Lobosa into two monophyletic classes (Tubulinida and Discosa). However, even the broadest study to date using 17 Amoeboa sample relatively few orders; though resolution at the base of Discosa was weak because of a bush-like basal radiation and many orders being only singly represented by long unbroken branches, the trees hinted that its current classification into subclasses may be incorrect (Cavalier-Smith et al., 2015b). A parallel study provided partial transcriptome sequences for additional orders and two unclassified or unidentified strains (Grant and Katz, 2014); but their tree did not clarify amoebozoan relationships at all as it included only 13 Amoebozoa, used maximum likelihood only (combining 18S rDNA with 238 proteins, yet oddly using only 15,650 characters – roughly 60 amino acids per protein), and has some bizarre features compared with other published trees.

Therefore we now combine sequences from both studies and from Emé et al. (2014) and others now available in a more extensive analysis of 30 Amoebozoa using 187 concatenated protein genes (using 50,964 amino acids), now including most lobosan orders (8 of 11) plus five of the 16 conosan orders recognised here, with multiple representations of many of them. This more representative taxon sampling yields trees fully consistent with earlier ones (Cavalier-Smith et al., 2015b) but much stabler, yielding several new clear conclusions. This work was not straightforward since, in addition to some new transcriptomes being from then unidentified strains, it transpired that several were misidentified and three comprised obvious mixtures of two different eukaryotes.

This mixtures (Table 1) were revealed by the 187 single-gene trees that we routinely carry out to check purity and paragame uniformity. They showed that both Parvamoeba transcriptomes contain numerous genes also from obligate mutually related protokinetoplasm endosymbionts (parasomes, related to Perkinsina; Young et al., 2014) that together form a robust clade within Euglenozoa as sister to Metakinetoplastina. The other mixed culture was *Stygamoeba regulata*, a marine amoeba that uniquely for Lobosa has flat mitochondrial cristae (Smirnov, 1995/6) so has been of unclear phylogenetic position but was recently grouped with *Vermistella* as a separate order Stygamoebida within subclass Flabellinia of Discosa (Smirnov et al., 2011). Its transcriptome was heavily contaminated by genes from *Cunea* (‘Mayorella’ sp.), perhaps why Grant and Katz (2014) did not include *Stygamoeba* on their multiprotein tree. By phylogenetically separating *Stygamoeba* and *Cunea* sequences using the single-gene trees we were able to include *Stygamoeba* on multiprotein trees for the first time and demonstrate that it is sister to Vannellida/Dactylopodida.

### Table 1

| Amoeba name and strains with MMETSP transcriptomes (Keeling et al., 2014) whose proteins were analysed phylogenetically. |
| MMETSP name | Current name | Strain | Comment or reason for different name |
|-------------|--------------|--------|--------------------------------------|
| *Filamoeba nolandi* | *Filamoeba aff. nolandi* | ATCC 50430<sup>a</sup> | Undescribed marine species, not *F. nolandi* Page & Loeblich (1964) |
| *Neoparamoeba aequitoriana* | *Paramoeba aequitoriana*<sup>b</sup> | SoJalbtio B1-5/56/2<sup>c</sup> | Genera synonymised by Feehan et al. (2013) |
| *Paramoeba atlantica* | *Paramoeba atlantica*<sup>d</sup> | CCAP 1560/9<sup>e</sup> | Type culture (Kudryavtsev et al., 2011) |
| *Pessonnea sp.* | *PRA-29* | ATCC PRA-29<sup>f</sup> | Misidentified; not a *Mayorella* (18S rDNA) |
| *Saxoguluraria sp.* | *Saxoguluraria chionotageaenuse* | ATCC 59779<sup>g</sup> | New genus and species (Lahr et al., 2015) |
| *Stygamoeba regulata* | *Stygamoeba regulata*<sup>h</sup> | ATCC 50982<sup>i</sup> | Contaminated by ‘Mayorella’ sp. (*Cunea*) |
| *Trichosphaerium sp.* | *Atrichosa algivora* | Am-1-7 wt, ATCC 40318 | Misidentified; we describe new genus/species |
| *Vannella sp.* | *Vannella sp.* | DIVA3 317/6(12)* | Marine strain, Kudryavtsev and Pawlowski |
| *Veillifera sp.* | *Veillifera sp.* | JP, DIVA3 564/2<sup>j</sup> | From deep sea, Kudryavtsev and Pawlowski |

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<sup>a</sup> We are unaware of any published micrographs of these five strains.

<sup>b</sup> Illustrated in Fig. 1A of Tekle et al. (2008).

<sup>c</sup> Stated by Lahr et al. (2011); to be CHINC-5 isolate (presumably erroneously as Chinc5 is ‘Stereomyxa’) but not Sexangularia; their illustration labelled thus was not of this strain but erroneously of another unknown.

<sup>d</sup> Illustrated in Fig. 1d of Lahr et al. (2011).

<sup>e</sup> Illustrated in Fig. 1b–e of Lahr et al. (2011). It could be the same genus and species as *S. regulata* of Smirnov, but as no sequences are available for the type strain we cannot be sure that it is not a different but morphologically similar species. Of the seven ATCC strains this is the only one not obviously originally misidentified (*Stygamoeba regulata*); ATCC protist cultures have commonly been misidentified (see supplementary material).

<sup>f</sup> Transcriptomes include numerous genes from *Perkinsina*-like ichthyobodinid (prokinetoplasmid) endosymbiont endosymbionts (parasomes), so are a two-eukaryote mixture as was that of *Stygamoeba*. 
supporting all three taxa being now included in order Glycostyliida (Cavalier-Smith et al., 2004).

Misidentification of strain 'Mayorella sp.' (Grant and Katz, 2014) was confirmed by separate comprehensive 18S rDNA analyses of 227 Amoebozoa showing that it must be an undescribed species of Cunea (a paramoebid discosean recently discovered by Kudryavtsev and Pawlowski (2015)) that groups within order Dactylopodina as sister to other Paramoebidae, and is not a Mayorella. This rDNA analysis is the most comprehensive for Amoebozoa and first to use a site-heterogeneous nucleotide substitution model, generally accepted as superior (Lartillot et al., 2007; Brown et al., 2013) to site-homogeneous methods hitherto used for rDNA, and was also better in practice for particularly difficult groups where rDNA evolutionary rates vary dramatically as in Amoebozoa (Cavalier-Smith, 2015, where it yielded better trees for the even more challenging gregarines and Percolozoa). We critically compare the taxonomically more broadly sampled 18S rDNA trees with the genically far better sampled multigene trees.

Our most novel conclusion is that the two unidentified strains of Grant and Katz (2014), one now named Sapocribium chincoetaguense (Lahr et al., 2015), form a clade distinct from both Tubulinea and Discosea, here made a third lobosan class, Cutosea, because of its unique test with scale-like substructure. Cutosea must also include Squamamoeba (Kudryavtsev and Pawlowski, 2012) and constitute a novel type of enveloped lobosan. Cutosea are sister to Tubulinea plus Discosea (the two latter here united as a new superclass Glycopoda) and so the earliest lobosan lineage.

Next in broad significance concerns Atrichosa (formerly 'Trichosphaerium' sp.), whose rDNA diverges so extensively from all other Amoebozoa that it could not be clearly placed in Lobosa or Conosa (Tekle et al., 2008; Lahr et al., 2015). Earlier it had been either provisionally excluded from Amoebozoa or if included thought to be related to Tubulinea (Cavalier-Smith et al., 2004) or after rDNA showed it to be amoebozoan guessed to belong in Discosea (Smirnov et al., 2011). Atrichosa is unambiguously a deep branching member of Tubulinea, probably closer to the Nolandina/Amoebina (euamoebid) clade than to Echinamoebida. Thirdly, we show that Glycostyliida are holophyletic and sister to Thecamoebida, casting strong doubt on existing classification of Discosea into subclasses (Smirnov et al., 2011). We discuss the evolutionary and taxonomic significance of these and other phylogenetic discoveries and present a revised higher-level classification for Amoebozoa.

2. Materials and methods

Starting from the 187 gene alignments assembled by Cavalier-Smith et al. (2015a,b) we manually (using macgde http://macgde.bio.cnmich.edu/) added sequences of Copromyxa protea (family Hartmannellidae, order Euamoebida) from Eme et al. (2014) as well as many obtained by blasting against our alignments 11 Amoebozoa transcriptomes downloaded from the Marine Microbial Eukaryote Transciptome database (http://marinemicroeukaryotes.org/resources); this website is now discontinued but the data slightly less conveniently are in http://data.imicrobe.us/project/view/104 as ‘Marine Microbial Eukaryote Transciptome Sequencing Project (MMETSP)’. To check the purity of the downloaded transcriptomes we ran RAxML-MPI v.7.2.8 PROTGAMMALGF trees (4 gamma rates) with 100 fast bootstrap resamplings for all 187 genes prior to concatenating them using SCaFoS (Roure et al., 2007). This showed that three MMETSP transcriptomes were mixtures of two different eukaryotes, whose proteins we separated phylogenetically using the 187 single-gene trees, so could include both in our analyses. As we judged three cultures to be misidentified (one now recognised to have been by the original authors who have named it Sapocrobribum chincoetaguense (Lahr et al., 2015), Table 1 summarises the original names in MMETSP and those used here and also the nature of the detected mixtures.

Sequences for Stygamoeba regulata (strain ATCC 50892; MMETSP0447) were heavily contaminated by sequences from ‘Mayorella’ sp. (MMETSP0417; ? strain ATCC 50980, unannotated on website). This contamination was evident because for many genes our single-gene trees revealed two completely different sequences from the ‘Stygamoeba’ transcriptome, one identical to that for ‘Mayorella’ sp. and branching with Paramoeba within Dactylopodina and one greatly different and branching much more deeply. We removed all sequences identical to ‘Mayorella’ and treated the residue as authentic Stygamoeba regulata genes, enabling us to include this species on multigene trees. Neral isolated both cultures from salt marsh sediment, Hog Island, eastern shore of Virginia, 2001. We suspect that both came from the same sample and only the ‘Mayorella’ culture was fully purified, the Stygamoeba one retaining unnoticed sparse ‘Mayorella’ contaminants that multiplied greatly before cultures were frozen. Consistently with that inference, the imicrobe and ATCC websites oddly give the same strain identifier (BSH-02190019) for both ‘Mayorella’ sp. and Stygamoeba regulata, but different 18S rDNA sequences, which implies that some ATCC information is inaccurate. The 18S rDNA sequence shown for Stygamoeba is the published one (Tekle et al., 2008). Our comprehensive new amoebozoan 18S tree shows that ‘Mayorella’ is certainly not a Mayorella species (see Section 3.1), but is evidently an undescribed species of the recently discovered paramoebid genus Cunea (Kudryavtsev and Pawlowski, 2015).

We found for most genes from the type strain of Paramoeba atlantica (CCAP 1560/9) and Paramoeba (=Neoparamoeba) aestivalina transcriptomes (MMETSP0151_2 and MMETSP0161_2 respectively) that there were two even more radically different sequences. These divergent second sequences grouped within Euglenozoa, as sister to metakinetoplastids (bodonids and trypanosomatids), invariably with 100% support for this Kinetoplastida clade, and if available for both amoebae grouped together as a maximally supported clade. Clearly these euglenozoan sequences came from the parasome, an obligate prokinetoplastid endosymbiont of Paramoeba related to Ichthyobodo and Perkinsella, a long recognised shared character for Paramoeba/Neoparamoeba (Young et al., 2014). As prokinetoplastids (Ichthyobodoniidae) have not yet been placed on multigene trees we relabelled these prokinetoplastid sequences ‘ichthyobodonid from Paramoeba atlantica’ and ‘ichthyobodonid from Paramoeba aestivalina’ and included them in the multigene analysis as well as the two host Paramoeba sequences that invariably grouped within Lobosa, usually together. Because of the huge sequence distance between the taxa there was never even the slightest doubt which was a prokinetoplastid endosymbiont gene and which a host Paramoeba gene. As our gene selection protocol picked only the top hit against our alignments, sampling each member of the mixed transcriptomes was necessarily incomplete. That is probably why gene sampling was substantially lower for Stygamoeba than other MMETSP Amoeboza, why those for Paramoeba somewhat lower, and those for the highly divergent parasite genes immensely lower. More parasite genes should therefore be findable in these transcriptomes.

Transcriptome MMETSP0437 from strain ATCC 50979 was labelled Sexangularia on the website; however, as Lahr et al. (2011) explained, this strain was misidentified and is not Sexangularia but a non-testate then unnamed species (Grant and Katz, 2014 called it Eukaryota sp. JRG-2011); in error their illustration was not of ATCC 50979 but of another unknown strain having finger-like pseudopodia, unlike ATCC 50979. Lahr et al. (2015) formally described ATCC 50979 as Sapocribribum chincoetaguense, so we use this name instead of wrong Sexangularia. The transcriptome MMETSP0420 from strain ATCC PRA-29 was labelled Pessonella sp. on the websites and by Grant and Katz
Sequences from five other transcriptomes were added from http://marinemicroeukaryotes.org/resources, three arguably misidentified: Filamoeba aff. nolandii (class Variosea, order Varipodida, family Filamoebidae; ATCC 50430 MMETSP0413), a marine strain that is sister to the freshwater F. nolandii (Dyková et al., 2005) but sufficiently distinct genetically to be an undescribed species – we call it aff. nolandii to show its closest relative; ‘Stereomyxa ramosa’ strain Chinc5 (ATCC 50982) (MMETSP0439) is in inverted commas (as in Lahr et al., 2011) as it may be an undescribed genus perhaps not belonging in Stereomyxidae (see below); ‘Trichosphaerium’ sp. strain Am-I-7 wt (taxon 498012 ATCC 40318); MMETSP0405 (order Trichosida, family Trichosphaeriidae) – we perhaps not belonging in Stereomyxidae (see below); ‘strain that is sister to the freshwater Perkinsela-like symbionts

3.2. Eukaryote-wide 187-protein trees reveal a novel major amoebozoan clade and that Paramoeba transcriptomes contain related Perkinsella-like endosymbionts

The 121-taxan eukaryote-wide multiprotein tree (Fig. 2) shows in both CAT and LG trees a robust new lobosan clade (here called Cutosea) comprising Saprocribrum and PRA-29 probably misidentified as ‘Pessonella’. Cutosea are the deepest branching Lobosa on the consensus CAT tree, sister to the previously known lobosan classes Discosea and Tubulinea, all three maximally supported clades by CAT. However Cutosea were in this position on only one of the two PhyloBayes chains; the other put them within Conosa as sister to Variosea (1.0) and LG ML placed them weakly (60%) as sister to Tubulinea. Near the base of scotokaryotes the position of the sulcozoan planomonads was also unstable and contradictory between the chains, so four nodes (red in Fig. 2) have weak support because of these two non-convergences in topology. Both conflicts persisted after running chains much longer (burnin 7767; 22,919 trees summed), though 0.98 support for excluding Cutosea from Discosea and Discosea/Tubulinea increased to 1 and 0.99. CAT topology in the rest of the tree was identical in both chains and congruent with other recent multigene studies except for non-grouping of the long-branch parabasalian Histonomas with the other metamonads with which it groups (thick arrow) with insignificant (21%) support on the LG tree.

Fig. 2 confirms our inference from single-gene trees that the second highly divergent sequence found for most of the 187 genes in the Paramoeba atlantica and P. aestuarina transcriptomes is from the Ichthyobodo-related Perkinsella-like protokinetopl asteroid endosymbiont constituting the parasite. As Fig. 2 shows, both Perkinsella-like ichthyobodinid sequences group together with maximal support and this clade is within Euglenozoa as the maximally supported sister to metakinetoplastids, consistent with the strict congruence of the phylogeny of Perkinsella-like symbionts with their paramoebid hosts (Young et al., 2014).

This tree by both methods also shows that Amoebozoa are monophyletic for all 29 included species and divide into two clades, Lobosa and Conosa. With this very large taxon sample the two chains did not fully converge (maxdiff 1); the very distant outgroups might have distorted its internal phylogeny, so it is safest to use this tree simply to confirm the relatedness, distinctiveness, and euglenozan nature of the second gene set of the endosymbionts of Paramoeba, and to root the smaller Amoebozoa-only trees – the 30-taxan trees did converge.

3.3. Amoebozoa-only 187-protein trees confirm distinctiveness of Cutosea

The 29-taxan Amoebozoa-only tree (Fig. 3) with the same amoebozoan taxa as Fig. 2 had exactly the same topology, but also did not converge (maxdiff 1), though the nature of the non-convergence differed. In contrast to Fig. 2 the bipartition between Lobosa and Conosa was maximally supported by both chains and significantly supported by ML also. In both Cutosea are maximally
Fig. 1. 18S rDNA PhyloBayes CAT-GTR-C consensus tree for 227 Amoebozoa and 38 outgroup eukaryotes (all from the scotokaryote clade that includes Amoebozoa and their closest relatives; see Cavalier-Smith et al., 2015a). Two chains were run, which converged on the same topology (max diff. 0.222); 121, 286 trees were summed after removing the first 20% as burnin. Support values are posterior probabilities (left) and bootstrap percentages for 1000 resamplings for the corresponding ML GTR-C tree (right); black blobs signify maximal support by both methods (1.0, 100%). Taxa whose transcriptomes are in our multigene trees are in bold. Atrichosa algivora (='Trichosphaerium' sp.) was omitted as its 18S rDNA sequence is so divergent that it cannot be aligned for many of the 1470 nucleotide positions chosen for this analysis, making its position on previously published 18S rDNA trees largely meaningless. To reduce long-branch artefacts further, myxogastrid Mycetozoa (all with extremely long-branches) were omitted as were the longest branch vannellids (Clydonella, Ripella) and Archamoebae (Entamoeba, Pelomyxa); all 35 omitted longer-branch taxa except Atrichosa, Clydonella, and Ripella are present in a 300-taxon analysis with 262 Amoebozoa (Supplementary Fig. S1) that shows species names for all clades collapsed here to enable the tree to fit one page. Number of taxa in each collapsed lobosan branch is to the right of its name.
supported as the deepest branch in Lobosa. The corresponding ML tree also excluded Cutosea from Discosea but with only weak (63%) support and placed it insignificantly (43%) as sister to Tubulinea. Both methods had maximal support for Sapocribrum and PRA-29 being sisters.

Most branches on the CAT tree had maximal (one only 0.99) support except for the base of Discosea, which was a maximally supported clade with Himatismenida sister to Centramoebida (0.76) in one chain but in the other was paraphyletic with Himatismenida most deeply and Tubulinea sister to Centramoebida, contrary to Fig. 2. Atrichosa was weakly sister to Nolandella/Copromyxa (0.6; strongly 95% by ML) but maximally supported as a tubulinean by both methods. The monophyly of Glycostylida as in Fig. 2 and always maximally or well supported also by ML on both trees. Vannella simplex (most genes unsequenced) was excluded from Figs. 2 and 3, as excessive missing data can distort trees (Roure et al., 2013).

When V. simplex sequences (from Cavalier-Smith et al., 2015b) are added, the resulting 30-taxon tree converged well by PhyloBayes (maxdiff 0.1135) and also shows Cutosea as the deepest
branch in Lobosa with maximal support by both CAT and ML (Fig. 4). Adding *V. simplex* did not change the branching order of Glycostylica or reduce CAT maximal support for its monophyly and branching order and being sister to *Stenamoeba*; and had minimal effects on the non-maximal ML support values. *Atrichosa* remained in the same position within Tubulinea with similar support. In fact including *Vannella simplex* made tree topology identical by CAT and ML, unlike Figs. 2 and 3, but with exactly the same topology as in Figs. 2 and 3 CAT trees. This congruence may be an example of breaking long branches being more important than excluding taxa with missing data (Roure et al., 2013). Figs. 2 and 3 ML disagreed with CAT, placing Cutosea weakly as sister to Discosea plus Tubulinea (Fig. 2 60%; Fig. 3 0.43% support). As CAT never showed that with any taxon sample, 18S rDNA never showed it, and there is no morphological reason to suspect *Atrichosa* algivora *sp* was actually a *Vannella* sp, as originally misidentified by ‘Pessonella’ sp. This shows that the 18S rDNA tree as published by Kudryavtsev et al. (2013) significantly overestimates the monophyly of the *Atrichosa* group and supports the monophyly of *Vannella* sp. This is a conventional approach, but could be misleading due to the long-branch artefact of the 18S rDNA sequence. In a concatenated 18S rDNA and actin ML tree clade was absent (Kudryavtsev and Pawlowski, 2013). The original 18S rDNA grouping of PRA-29 with no support with *Vexillifera minutissima* (Tekle et al., 2008, wrongly labelled as Vaneliidiae [sic] and misgrouping with them) is an obvious long-branch artefact. So also was the placement of both a PRA-29/*Sapocribrum* moderately supported clade and *Trichosphaerium* with *V. minutissima* within Dactylopodida (Lahr et al., 2011); actin separately grouped PRA-29 and *Sapocribrum* with moderate support in a completely unresolved position (Lahr et al., 2011). In a concatenated 18S rDNA and actin ML tree clade Cutosea was moderately supported but there was no support for its apparent position within an incorrectly paraphyletic Dactylopodida (Lahr et al., 2015). On an ostensibly four-gene tree devoid of glycolectins (in which PRA-29 was probably represented by only those two genes) PRA-29 was within Conosa (Tekle et al., 2008). The almost total lack of basal amoeboid resolution of such 1–2 gene trees caused the phyletic distinctiveness of Cutosea to be previously entirely overlooked.

The 239-gene ML tree of Grant and Katz (2014) agrees with ours in showing *Sapocribrum* plus PRA-29 as a maximally supported clade, but had too few other Amoebozoa (only one discosean, ‘*Mayorella*’ sp., actually a *Cunea* sp.) to show its true position. Cutosea is
### Table 2
Revised classification of phylum Amoebozoa and its two subphyla, seven classes and 28 orders.

| Subphylum 1. Lobosa (3 classes, 11 orders, 44 families) |
|--------------------------------------------------------|
| **Superclass 1. Cutoza Cavalier-Smith supercl. n.** |
| **Class Cutosea Cavalier-Smith cl. n.** |
| Order Squamocutida Cavalier-Smith ord. n. |
| Family 1. Squamamoebidae Cavalier-Smith fam. n. (Squamamoeba) |
| Family 2. Sapopiribidae Cavalier-Smith fam. n. (Sapocrillum) |
| **Superclass 2. Glycophoda Cavalier-Smith supercl. n.** |
| Class 1. Tubulinea Smirnov et al., 2005 em. (=<Lobosa Cavalier-Smith, 2004) |
| **Subclass 1. Neolobosa Cavalier-Smith subclass n.** |
| **Superorder 1. Eubloosa Cavalier-Smith superorder n.** |
| Order 1. Euanoeobiida Lepsi, 1960 em. (typical naked freshwater tubulinans) |
| Suborder 1. Amoeboa Cavalier-Smith subord. n. |
| Family 1. Amoeboidae (Ehrenberg, 1838) Page, 1987 (Amoebo, Chaos, Polychaos, Parachaos, Trichamoeba, Deuteramoeba, Hydramoeba) |
| Family 2. Hartmannellidae® Volkonsky, 1931 em. Smirnov et al., 2011 (Cashia, Copromyxa, Copromyxea, Glaseria, Hartmannella, Prolemeba, Saccamoeba) |
| **Suborder 2. Nolandia Cavalier-Smith subclass n.** |
| Family Nolandellidae Cavalier-Smith in Smirnov et al., 2011 (synonym Nolandellidae Lahr and Katz in Lahr et al., 2011) (Nolandella) |
| **Order 2. Arcelliidida Saville Kent, 1880. 3 suborders, 18 families, not listed (testate Lobosa)** |
| **Superorder 2. Trichosa Cavalier-Smith superorder n.** |
| **Subclass 2. Leptomysia Cavalier-Smith subclass n.** |
| **Order Leptomysida® (Pussard and Pons, 1976) Page, 1987** |
| Family 1. Leptomysidae® (Pussard and Pons, 1976) Page, 1987 (Leptomysia, Rhizamoeba) |
| Family 2. Flabellulidae Bovee, 1970 em. Page, 1987 (Habellula, Parafabellula) |
| Family 3. Cephalamoebidae Pussard and Pons, 1976 (Cephalamoeba) |
| **Subclass 3. Echinooesia Cavalier-Smith subclass n.** |
| **Order Echinooesia Cavalier-Smith in Cavalier-Smith et al., 2004 em. stat. n. Smirnov et al., 2011** |
| Family 1. Echinooesia Page, 1975 (Echinooesia, Micriumoea) |
| Family 2. Vermamoebidae Cavalier-Smith and Smirnov in Smirnov et al., 2011 (Vermamoeba) |
| **Order 3. Cutoesia Cavalier-Smith in Cavalier-Smith et al., 2004 em. Smirnov et al., 2011** |
| **Suborder 1. Vannellina® Smirnov et al. (2005) stat. n.** |
| Family Vannellinae Bovee, 1979 (Vannella, Odynella, Lingulamoeba, Ripella, Paravannella, Pessonella) |
| **Suborder 2. Dactylomysida® Smirnov et al. (2005) stat. n.** |
| Family 1. Paramoebidae Poche, 1913 em. Kudryavtsev et al., 2011, 2015 (Paramoeba, Cunea, Pseudoparamoeba, Janickina, Korotenevella) |
| Family 2. Vexilliferidae Page, 1987 (Vexillifera) |
| **Suborder 3. Stygomyesia® Smirnov and Cavalier-Smith in Smirnov et al., 2011 stat. n.** |
| Family Stygomyesia Smirnov and Cavalier-Smith in Smirnov et al., 2011 (Stygamoeba, Vermistella) |
| **Order 2. Dermamoebida Cavalier-Smith in Cavalier-Smith et al., 2004 em. Smirnov et al., 2011** |
| Family 1. Dermamoebidae Cavalier-Smith and Smirnov in Smirnov et al., 2011 (Dermamoeba, Paradermamoeba) |
| Family 2. Mayorellidae Schaeffer, 1926 (Mayorella) |
| **Order 3. Thecoamyida Smirnov and Cavalier-Smith in Smirnov et al., 2011** |
| Family 1. Thecoamyidae Schaeffer, 1926, em. Smirnov et al., 2011 (Thecoamyia, Pseudothecamoeba, Thecochaos) |
| Family 2. Stamamoebidae Cavalier-Smith fam. n. (Sapphio, Stamamoeba) |
| **Order 4. Centramoebida® Rogerson and Patterson, 2002** |
| Family 1. Acanthamoebidae Sawyer and Griffin, 1975 (Acanthamoeba, Proacanthamoeba) |
| Family 2. Balamuthiidae Cavalier-Smith in Cavalier-Smith et al., 2004 (Balamuthia) |
| **Order 5. Himatiosmidena Page, 1987** |
| **Suborder 1. Tectifera Cavalier-Smith and Smirnov in Smirnov et al., 2011** |
| Family Cylindricalidae® De Saedeleer, 1934 (Cylindricalium, Ceropodium, Ovalopodium) |
| **Suborder 2. Pellitina® Smirnov and Cavalier-Smith in Smirnov et al., 2011 stat. n. Cavalier-Smith. Diagnosis as for Pellitida em. in Kudryavtsev et al., 2014, p. 226** |
| Family 1. Pellitidina Smirnov and Kudryavtsev, 2005 (Pellita) |
| Family 2. Goeceidae Cavalier-Smith and Smirnov in Smirnov et al., 2011 em. Kudryavtsev et al., 2014 (Goeceia, Paragoeceia, Endostelium) |
| **Suborder 3. Parameoebida Cavalier-Smith in Smirnov et al., 2011** |
| Family Parameoebidae Cavalier-Smith and Smirnov in Smirnov et al., 2011 (Parameoba) |
| **Subphylum 2. Conosa Cavalier-Smith, 1998 (4 classes, 17 orders, 42 families)** |
| **Infraphylum 1. Semiconosa Cavalier-Smith, 2013** |
| **Superclass 1. Variosa Cavalier-Smith supercl. n.** (diagnosis as for Variosea in Cavalier-Smith et al., 2004 p. 43) |
| **Class Variosea Cavalier-Smith in Cavalier-Smith et al., 2004 em.** |
| **Order 1. Holomastigida Lauterborn, 1805 (multiciliate)** |
| Family Multiciliidae Poche, 1913 (Multicia) |
| **Order 2. Phalansteriida® Hibberd, 1983 em. (7 uniciliate families)** |
| Family 1. Phalansteridae Saville Kent, 1880–1881 (Phalansterium) |
| Family 2. Dictyamoebidae Cavalier-Smith fam. n. (Dictyamoeba) |
| Family 3. Arboramoebidae Cavalier-Smith fam. n. (Arboramoeba) |
| Family 4. Schizoplasmodiidae Cavalier-Smith, 2013 (Schizoplasmodium, Nematoselidium, Ceratomyxea) |
| Family 5. Rhizomonoasidae® Cavalier-Smith in Cavalier-Smith and Soble, 2013 (Rhizomonas) |
| Family 6. Rigidomastigidae® Cavalier-Smith in Cavalier-Smith and Soble, 2013 (Rigidimastix) |
| Family 7. Trichonemidae® Cavalier-Smith fam. n. (Trichonema, Mitophorus) |
| **Order 3. Artodiscida Cavalier-Smith, 2013 (putatively multiciliate)** |
| Family Artodiscidae Cavalier-Smith, 2013 (Artodiscus, Tetracilia) |
| **Order 4. Varipodida® Cavalier-Smith in Cavalier-Smith et al., 2004 (non-uniciliate)** |
| Family 1. Filamoebidae Cavalier-Smith in Cavalier-Smith et al., 2004 (Filamoeba, Heliamoeba) |
| Family 2. Flamellidae Cavalier-Smith fam. n. (Filamoeba, Telamoeba) |
| **Order 5. Protostelida® Olive, 1967 ex Olive and Stoianovitch, 1966 em. (multiciliate, several kinetids or non-uniciliate)** |
| Family 1. Protostelidae Olive, 1962 em. (Protostelium, Planoprotostelium) |
| Family 2. Soliformovidae Cavalier-Smith, 2013 em. (Soliformovum, Grellamoeba) |
maximally supported as a clade on the 151-gene LG ML tree of Katz and Grant (2015) but wrongly placed (no support given, therefore according to their figure legend <70%) as sister to the also weakly supported artefactual ‘Trichosphaerium’ [Arirchosa]/[Mayorella] [Cunea] sp. clade criticised above. On this ill-sampled tree neither Lobosa nor Conosa is a clade; Lobosa and Conosa are also mixed up on their supplementary non-converged 150-gene 235-taxon CAT tree where Cutosoa was wrongly sister to Myctozoa plus Archamoebae. The 207-gene ML tree of Katz and Grant (2015) was seriously distorted at the base of scotokaryotes by long-branch attraction of anaerobic protozoan clades to distant bacterial outgroups; weakly sampled Amoebozoa were wrongly shown as polyphyletic, branching in three different places; nonetheless Cutosoa were a maximally supported clade, insignificantly (46%) sister to Filamoeba. Neither Grant and Katz (2014) nor Katz and Grant (2015) specified the genes used, but as the 150-gene analysis included 34,991 amino acids whereas the 239-gene analysis had only 15,650 the two sets were presumably very different despite assembly by the same pipeline. Taken together, our taxonomically much better sampled, genically better sampled (187 genes, 50,964 amino acids) and more thoroughly analysed trees strongly contradict all thes by confidently showing Cutosoa to be the most divergent lobosan lineage of all. Previously 185 rDNA trees grouped PRA-29 ‘Pessonne’ sp. with Squamamoeba japonica with maximal support (Kudryavtsev and Pawlowski, 2013). Their site-homogeneous covarion Bayesian tree much better sampled, genically better sampled (187 genes, 50,964 amino acids) and more thoroughly analysed trees strongly contradict all their by confidently showing Cutosoa to be the most divergent lobosan lineage of all.

Family 3. Actinophryidae Smirnov et al. (2008) (Actinophrya)

Order 6. Ramamoebida Cavalier-Smith ord. n. (multikinetid or non-ciliate)

Family 1. Cavasteriidae olive, 1964 em. Cavalier-Smith, 2013 (Cavasteria, Schizoplasmodiospiroid, Tychosporium)

Family 2. Angulamoebidae Cavalier-Smith fam. n. (Angulamoeba)

Family 3. Trichiidae Cavalier-Smith fam. n. (Trichiidae, Mastigamoeba, Darbyshirella)

Superc class 2. Myctozoa de Bary, 1859 ex Rostafinski, 1873

Class 1. Stelamoebida Cavalier-Smith in Cavalier-Smith et al., 2004 em.

Subclass 1. Exosporae Rostafinski, 1873 stat. n. Smith, 1938 em. Cavalier-Smith

Order 1. Protosporangida Cavalier-Smith ord. n.

Family Protosporangiidae Cavalier-Smith fam. n. (Protosporangium, Clastostelum)

Order 2. Ceratomyxida Martin, 1961 ex Farr and Alexopoulos (1 family: Ceratomyxida)

Subclass 2. Dicytostelia Cavendish, 1975

Order Dicytostelida Lister, 1909 or Olive, 1970 (2 families, e.g. Actinostelia, Dicytostelium, Polyphryclidium)

Class 2. Myxogastrea Fries, 1829 stat. n. Cavalier-Smith, 1993 (syn. Myxomycetes Link, 1883)

Superorder 1. Luciporida Cavalier-Smith, 2013

Order 1. Luciporida Jahn, 1928 (5 families, e.g. Licea, Cribridia, Lindbladia, Reticularia)

Order 2. Trichidiida Macbride, 1922 (4 families, e.g. Trichia, Ar cryia, Minakatella, Di anema)

Superorder 2. Columellida Cavalier-Smith, 2013

Order 1. Echinostelida Cavalier-Smith, 1993 (2 families, e.g. Echinostelium, Clasterodema)

Order 2. Fusciporida Cavalier-Smith, 2013 (4 families, e.g. Lomproderma, Stenomis, Amaurochaete, Badhamia, Physarum)

Infra phylum 2. Archaemoebida Cavalier-Smith, 1993

Class Archamoebida Cavalier-Smith, 1993 stat. n. 2004

Order 1. Mastigamoebida Frenzel, 1897a

Family 1. Mastigamoebidae Goldschmidt, 1907 (Dinamoeba, Mastigamoeba, Mastigina, Phreatamoeba)

Family 2. Endolomicae Cavalier-Smith in Cavalier-Smith et al., 2004 (Endolimax, Iodamoeba, Endamoeba)

Order 2. Pelobiontida Page, 1976 em.

Family 1. Pelomyxidae Schultze, 1877 em. (Pelomyxa, Mastigella)

Family 2. Tricholimacidae Cavalier-Smith, 2013 (Tricholimax)

Order 3. Rhizostomatida Dalloff, 1916 em. Cavalier-Smith, 2013

Family Rhizostomatidae Cavalier-Smith in Cavalier-Smith and Scoble, 2013 (Rhizostomatax)

Order 4. Entamoebida Cavalier-Smith, 1993

Family Entamoebidae Chatton, 1925 (Entamoeba)

Amoebozoa incertae sedis: Family Stereomyxidae Grel, 1966 (Stereomyxa, Corallomyxa)
grouped this clade weakly with the himatismenid Cochliopioidae, but ML put it as sister to the dactylopodid Vexillifera with no bootstrap support. Though no rDNA sequence is available for Sapocribium and no proteins for Squamaamoeba, an actin/18S rDNA ML tree with missing data grouped Sapocribium as sister to PRA-29 ‘Pesssonella’ sp. plus Squamaamoeba (72% BS) and a six-gene ML tree (including only rDNA for Squamaamoeba) put Squamaamoeba as sister to Sapocribium/PRA-29 ‘Pesssonella’ sp. with maximal support (Lahr et al., 2015). Thus Squamaamoeba is a robust member of the Sapocribium/PRA-29 cutoosean clade and we can safely use ultrastructural features of both Sapocribium and Squamaamoeba for defining morphologically this newly recognised major lobosan group, even though such oligogenic trees did not clarify its position: the two-gene tree put it as sister to Vexillifera with no support and the six-gene tree with Cochliopioidium also with no support, but neither tree had any basal resolution.

3.4. Phylogeny and probable holophyly of Discosea on Amoebozoa—only 187-protein trees

Irrespective of whether Vannella simplex is included (Fig. 4) or excluded (Fig. 3) Cunea is sister to Paramoeba and this clade sister to Vexillifera, forming a Dactylopodina clade with maximal support by both methods for every branch in the clade. Likewise Stigmaamoeba is sister to Dactylopodina with maximal support by CAT and 92/93% support by ML (1/96% in Fig. 2). This clade is sister to Vannellida with maximal CAT and moderate (71/67%) ML support; the joint clade corresponds to order Glycostylicina as revised below and is sister to Stereomyxa (Thecamoebida) with maximal CAT and high (98/99%) ML support (100% in Fig. 2). Thus all our multigene trees strongly support Stereomyxa being sister to Glycostylicina, contradicting the previous subdivision of Discosea into subclasses. Stereomyxa should have been sister to Centramoebida were Longamoebida (i.e. Dermamoebida, Thecamoebida and Centramoebida) a clade (Smirnov et al., 2011). We therefore do not use subclass Longamoebia in our revised classification (Table 2). Both methods equally strongly exclude Himatismenida from the Glycostylicina clade showing that including them in a broadened Flabellinia (Smirnov et al., 2011) was incorrect so we do not use subclass Flabellinia either in Table 2. Multiprotein trees for more Discosea should enable a sounder subclass division.

Discosea themselves were a weakly supported clade on all ML trees; though maximally supported on two CAT trees (Figs. 2 and 4) in Fig. 3 that was only true for one chain, the other showing Discosea as paraphyletic as noted above. More likely than not Discosea are a clade, but this single discrepancy makes more extensive taxon sampling, especially of Dermamoebida, Paramoeba, and Pellitina, vital to decide if Discosea are a clade or ancestral to Tubulinea. It is also uncertain whether Himatismenida are sister to Centremoebida (Figs. 2 and 4, and one Fig. 3 chain; we informally call this potential clade Centramoebia as both orders have centrosomes) or all other Discosea plus Tubulinea (only shown on the other Fig. 3 chain).

All trees agree in putting ‘Stereomyxa ramosa’ as sister to Acanthamoeba with maximal support, in agreement with the sparsely sampled ML trees of Grant and Katz (2014) and Katz and Grant (2015). If strain Chinc5 (ATCC 50982) really were ‘Stereomyxa ramosa’, that would support including both Stereomyxidae and Acanthamoebidae in Centramoebida (Rogerson and Patterson, 2002). Cavalier-Smith et al. (2004) and Smirnov et al. (2011) doubted that relationship so excluded Stereomyxidae (and Dictyosteliidae whose inclusion by Rogerson and Patterson was obviously mistaken). Table 2 leaves Stereomyxidae incertae sedis, as the only published micrographs of Chinc5 (Lahr et al., 2011) do not show nearly such extensively branched pseudopodia as in Stereomyxa or obvious anastomoses but show a lamellipodium.
not seen in Stereomyxa (Grell, 1966; Benwitz and Grell, 1971); this strain is probably misidentified and actually an undescribed genus.

Additional representation of three previously sequenced orders or suborders in Fig. 4 compared with Cavalier-Smith et al. (2015b) confirms their monophyly. Both Vannella group together with maximal support (suborder Vannellina), as do both Vexillifera, both Paramoeba, and both Filamoeba (order Varipodida, now with three species, remains maximally supported).

3.5 New genus Atrichosa is a tubulinid distinct from Trichosphaerium

Trichosphaerium is an unusual giant multinucleate marine amoeba with a thick test-like cuticle composed of calcite spicules (discovered by Schneider, 1878) that led some to place it in Foraminifera (Loeblich and Tappan, 1964) rather than Amoebozoa, or in Testacealobosa (Schuster, 1990, not in Arcellinida as Tekle et al. (2008) wrongly wrote). However unlike Foraminifera it has numerous lobose pseudopodia, leading others to include it in Lobosa (Sheehan and Banner, 1973), though Möbius (1889); placed it in its own group Trichosa. Cavalier-Smith et al. (2004) suggested that its lobopodia hinted that Trichosphaerium may belong in Tubulinea; but because of early reports that T. sieboldi had a complex multiphasic life cycle with a biciliate swarmer stage (Schaudinn, 1899) suggesting an affinity with Rhizaria, they provisionally excluded it from Amoebozoa until sequence evidence became available. Schaudinn also found similar giant amoebae lacking spicules; though rarely present, he thought they were stages in the life cycle of T. sieboldi variously called gamonts or sporonts, postulated to come from the spiculate form named schizonts and to regenerate spiculate schizonts in an alternation of generations.

Polne-Fuller (1987) isolated into axenic culture a non-spiculate trichosid with a thinner smooth test that fed on macroscopic seaweeds (Polne-Fuller et al., 1990). It could grow much larger (up to 1 mm) than non-spiculate forms associated with T. platystyrum (Angell, 1976) and unlike them could undergo multiple fission into small amoebae with only few nuclei when less well fed and binary fission when well fed. The presence of both lobopodia and dactylopodia identify it as a trichosid, but similarity in pseudopodia and its large size and multinuclearity was insufficient reason to identify it as Trichosphaerium, which was done primarily because of Schaudinn's uncorroborated theory that naked and spicular trichosids are stages in one life cycle. Tekle et al. (2008) obtained an 18S rRNA sequence for Polne-Fuller's non-spiculate strain (ATCC 40318) included on our multiprotein trees (under our new name Atrichosa algivora), which they stated to be ‘very similar to’ an unpublished one of the ‘smooth stage of Trichosphaerium sieboldi CCAP 1585/2’ studied by Pawlowski and Fahrni (2007). Their 95% identity is enough to put them in the same family, but not close, and means they must be separate species; however, in the absence of experimental proof of the three-phase life cycle postulated by Schaudinn, there is no evidence for the Pawlowski strain being T. sieboldi rather than a separate genus. Though referred to only as dactylopodia by Tekle et al. (2008), most pseudopodia in their Fig. 1E are lobose, only one dactylopodium being evident. The Tekle et al. sequence is so divergent from all other Amoebozoa that it is hard to align and initially grouped with the equally longbranch myxogastrids, but as this seeming relationship disappeared when faster evolving nucleotides were removed Tekle et al. (2008) considered it a long-branch artefact.

That reasoning is shown to be correct by our firm evidence using 41,675 amino acids from 149 ‘Trichosphaerium’ sp. genes that it is part of the Tubulinea clade, thus not a conosan. Our trees more weakly put it as sister to Copromyxa plus Nolimdella, as on 18S rDNA trees are Arcellinida and Leptomysida (Smirnov et al., 2011; Kudryavtsev et al., 2014). We need transcriptome or genome sequences from these two orders to be sure that Atrichosa (‘Trichosphaerium’) is not specifically related to Testacealobosa which Schuster (1990) grouped with Trichosida in subclass Testacealobosa, not an entirely distinct clade as morphology makes likely.

We are unconvinced that strain ATCC 40318 is a Trichosphaerium, for reasons elaborated in the supplementary material. In essence there is no evidence that spicule-free ‘Trichosphaerium’ amoebae are life cycle stages of the original spiculate Trichosphaerium, rather than contaminants of non-clonal cultures. Biphasic alternation between spiculate ‘schizonts’ and smooth ‘gamonts’ (Schaudinn, 1899) is unproven; even Page (1983) who thought that the non-spiculate Pontifex maximus of Schaeffer (1926) was just a Trichosphaerium phase admitted that was uncertain. As strain ATCC 40318 entirely lacks spicules it was safest to make this strain of non-spiculate trichosid-like amoebae a new genus and species, Atrichosa algivora.

As, apart from absence of spicules Atrichosa are similar to Trichosphaerium, including having similar mitosis with intranuclear spindle and metaphase plate (compare Schuster, 1976; Angell, 1976), we assign Atrichosa to Trichosphaeridae, but until sequences are available from a spiculate Trichosphaerium we cannot be sure they belong in the same family or even order. Nonetheless having both lobose pseudopodia and hairlike subpseudopodia emanating from them and passing through pores in a multiporose test is unique for Amoebozoa; these features are unlikely to be convergent and thus are synapomorphies for Trichosida. Sheehan and Banner (1973) thought that lobopodia were entirely within the test, but Angell (1975, 1976) noted that they can penetrate through pores to contact the substratum; unless they do they cannot cause locomotion, which even Sheehan and Banner considered their function.

Previously an actin tree did not group ‘Trichosphaerium’ with the arcellinid Arcella or with any Tubulinea, but placed it without statistical support in an apparently paraphyletic Discosea, and on a four-gene tree Trichosphaerium grouped strongly with PRA-35 (later renamed Parvamoeba mononora: Cole et al., 2010) (Tekle et al., 2008), which led Smirnov et al. (2011) to assume that it probably belonged in Flabellinia within Discosea despite lacking obvious discosean morphology. Though Parvamoeba is not on our multiprotein trees, Fig. 1 shows that by rDNA it does not group within Tubulinea as Atrichosa (‘Trichosphaerium’) robustly does on our protein trees, which therefore robustly contradict the grouping with Parvamoeba that is attributable to insufficient data plus the 18S rDNA long-branch bias. With six genes ‘Trichosphaerium’ again wrongly grouped with myxogastrids, not PRA-35 – still wrongly called ‘Thecamoeba sp.’ (Lahr et al., 2015). Another actin tree grouped ‘Trichosphaerium’ with the tubulinid Vermamoeba with 4% support but far away from the well-supported main Tubulinea clade (Lahr et al., 2011), confirming that actin sequences have too little information for reconstructing deep eukaryote phylogeny (see also Cavalier-Smith, 2015).

Bizarrely, the eukaryote-wide ML tree of Grant and Katz (2014) based on 238 proteins plus 18S rDNA grouped ‘Trichosphaerium’ with myxogastrids with 89% support. However, it was limited to just 13 Amoebozoa and only two other Tubulinea. That tree had another peculiar feature: placing breviates as sister to metamonads and grouping that presumably artefactual clade with Discoba, in complete contradiction to other multigene trees that using ML robustly place breviates as sister to apusomonads and this clade as sister to opisthokonts or if using PhyloBayes CAT place them as sister to opisthokonts plus apusomonads (Brown et al., 2013; Cavalier-Smith et al., 2014, 2015a,b). We once noted a similar Breviceptae/Metamonada grouping when using many too few genes and amino acid positions (about a third of those in our published 187–192-gene papers: Cavalier-Smith et al., 2014, 2015a,b), and note that when Trichozoa are included in our multigene alignments
they sometime do branch as sister to obazoa (opisthokonts, apusomonads, breviates) but only on CAT trees (Cavalier-Smith et al., 2015b). Contradictory features of the Grant and Katz tree might also have something to do with the usual automated pipeline they used perhaps excluding too many informative characters, or even including shared LGTs between the anaerobic Breviates and Metamonada. We especially do not understand why even though it uses 238 proteins (51 more than our 187) it included only 15, 560 characters (rDNA plus protein) little more than a third of the amino acids that we were able to align (50,964). Burki et al. (2012) using 258 genes included only slightly fewer amino acids than we did, so the Grant–Katz pipeline discarded vast amounts of reliable data. Grant and Katz did not use single-gene trees for quality control, essential for reliable multigene phylogeny. Virtual checking such trees can reveal problems missed by automated scripts. As ‘Trichosphaerium’ would have to cross eight maximally supported nodes on our trees to group within Mycetozoa to be sister to myxogastrids, and has no morphological or ecological supported nodes on our trees to group within Mycetozoa to be Dactylopodina, and splits to Vexilliferidae, as was Cutosea alone (no support) on a 151-taxon contradicted by the 187-gene trees) was insignificantly (13%) sister to obazoa with Cochliopodiidae (18%) and this ‘clade’ (false, as totally eage. However, the corresponding ML tree wrongly grouped Cutosea (‘Trichosphaerium’) is a tubulinean as we unambiguously show.

3.6. A 26S-taxon amoebozoan 18S rDNA site-heterogeneous (CAT) tree is more accurate than ML

_Atrichosa_ was omitted from this tree (Fig. 1) because its 18S rDNA is so divergent and hard to align that its position is unreliable; including it risks distorting other branching patterns. Representatives of all other significant amoebozoan lineages are included except the also long-branch myxogastrids (included in Fig. S1 with a few other long-branch taxa in a 300-taxon tree). In several respects this first amoebozoan tree using an evolutionarily realistic site-heterogeneous model is more congruent with our multiprotein trees and morphologically based classification (Table 2) than is the algorithmically oversimplified site-homogeneous ML tree. Here ML agrees with CAT PhylоБayes in grouping _Cunea_ with other paramoebids (77%), unlike previous trees where MrBayes moderately supported this clade but ML support was <50% (Kudryavtsev and Pawlowski, 2015). Unlike the CAT rDNA tree and multiprotein trees, the ML rDNA tree for Fig. 1 alignment wrongly places Cutosea and _Cochliopodium_ within Dactylopodina, and splits _Ovalopodium_ away from _Cochliopodium_, making _Cochliopodidae_ wrongly appear polyphyletic in strong contradiction to multiprotein trees; _Cochliopodidae_ is correctly a clade (low 0.5 supporting) in Fig. 1.

_Cutosea_ are a deeply divergent robust clade (0.98 100% sister to Tubulinea plus Conosa, and do not group with any discosean lineage. However, the corresponding ML tree wrongly grouped Cutosea with _Cochliopodidae_ (18%) and this ‘clade’ (false, as totally contradicted by the 187-gene trees) was insignificantly (13%) sister to Vexilliferidae, as was Cutosea alone (no support) on a 151-taxon tree (Kudryavtsev and Pawlowski, 2013). This confirms that rDNA has insufficient information to place Cutosea; rDNA is consistent with 187-gene trees in Cutosea being very early diverging.

Like many earlier site-homogeneous Amoebozoa-wide trees (e.g. Cavalier-Smith et al., 2004; Smirnov et al., 2011; Lahr et al., 2011; Zadrožníková et al., 2015) Fig. 1 does not show Conosa, Lobosa and Variosea as clades, as Archamoebae group with Tubulinea (seen also by Zadrožníková et al., 2015) and Discosea appear paraphyletic (by CAT and ML, but no support). Though some published trees omitting many of the more problematic branches we included show clade Conosa (Kudryavtsev and Pawlowski, 2013; Kudryavtsev et al., 2014; Berney et al., 2015), our results confirm previous conclusions that rDNA has too little information to resolve basal amoebozoan branches (Smirnov et al., 2011; Cavalier-Smith et al., 2015b), which site-heterogeneous trees cannot be expected to improve. Nonetheless Fig. 1 is more informative than most site-homogeneous trees for evolutionary important branches in Conosa, with important taxonomic implications for Variosea and Mycetozoa especially; as they are still severely undersampled by multiprotein trees, we summarise them below.

In Mycetozoa, Fig. 1 shows a robust clade comprising protosporangids and ceratiomyxids (0.99, 84%) – first found for 18S rDNA by Berney et al. (2015) and Zadrožníková et al. (2015) with strong support; and by Lahr et al. (2015) on 18S rDNA/actin trees (negligible support). With ML this clade is weakly (37%) sister to dictyostelids as in Berney et al. (2015) who also omitted Myxogastrea, and in Zadrožníková et al. (2015) and Lahr et al. (2015) who both included Myxogastrea which did not branch with Amoebozoa but is oddly sister to Apusozoa with insignificant support (0.49 Fig. 1). This curious position is not because we omitted myxogastrids, as when they are present Protosporangida/Ceratiomyxida remain with Apusozoa, and myxogastrids are sister to Filamoebidae within Variosea (Fig. S1). Even when long-branch Ceratiomyxida (plus some other longer branches) are excluded in a 248-taxon tree, Protosporangium is oddly within Apusozoa (0.47, insignificant support). Though Protosporangium is not a particularly long branch its sequence is rather divergent from other Amoebozoa in places, which might cause an unidentified model incongruency with PhylоБayes alone, giving this unexpected position. Despite lack of agreement between ML and CAT, these trees are consistent with protosporangids alone among major protosteloid lineages lacking the V7 18S rRNA synapomorphy for Variosea (Berney et al., 2015) and with our revised classification excluding them from Variosea and placing them within mycetozoan subclass Exosporeae (Table 2). Neither our trees nor those of Berney et al. (2015) or Zadrožníková et al. (2015) support an earlier extremely weak grouping of protosporangids with protostelids (Shadwick et al., 2009) that this V7 synapomorphy contradicts; instead they are consistent with Lahr et al. (2011) who included only protosporangids, and found them strongly sister to dictyostelids, not Myxogastrea.

Traditionally plasmodial _Ceratiomyxa_ was considered related to Myxogastrea not dictyostelids (e.g. Calkins, 1926), though put in a separate group Exosporeae, continued by Cavalier-Smith et al. (2004) in their own order. After discovering protostelids, Olive (1975; see also Hutner, 1985; Spiegel, 1990) included them in Protostelida. When first sequenced _Ceratiomyxa_ branched immediately below Myxogastrea/dictyostelid bifurcation on 18S rDNA ML trees; branching order of these three clades using MrBayes was unresolved (Fiore-Donno et al., 2009); _Ceratiomyxa_ did not group with _Cavostelium_, the only protosteloid then sequenced. As _Ceratiomyxa_ grouped with Myxogastrea on an 18S rDNA/EF1α tree (Fiore-Donno et al., 2009) later classifications kept the Exosporeae/Myxogastria grouping (Cavalier-Smith, 2013; Ruggiero et al., 2015), though an 18S rDNA/actin tree put Exosporeae as sister to Dictyostelida (Lahr et al., 2013). Our study is the fourth to show _Ceratiomyxa’s_ relationship with Protosporangium; none contradicts it, so we added Protosporangida to Exosporeae (Table 2). All studies including both exosporean orders show a weak relationship with Dictyostelida (Berney et al., 2015; Lahr et al., 2015;
Zadroblíková et al., 2015) as did our ML trees (whether or not Myxogastrea are included), and none with myxogastriods; our CAT trees including Myxogastrea did not group them with either Exosporae or Dictyostelida or further clarify the branching order of these three myctezoan groups. In the absence of sequence phylogeny or other evidence for a specific grouping of Exosporae, we transfer thus expanded Exosporae to Stelamoeboza, grouping them with dictyostelids, which their unicellular fruiting body structure favours (Table 2). Following Cavalier-Smith (1993a,b), Cavalier-Smith et al. (2004), and Ruggiero et al. (2015) for Myxogastrea, we abandon the misleading synonym Myxomycetes Link, 1883 that wrongly implies they are fungi.

Except for excluding Angulamoeba and including Dictyostelida (neither significantly supported – merely 0.32 and 0.46 so unresolved), CAT shows Variosea as a clade (Fig. 1). The corresponding ML tree correctly included Angulamoeba in the Variosea clade but also included Dictyostelida (trivial 6% BS support). Our trees are therefore consistent with, but do not demonstrate, the idea that Variosea, redefined as those Conosa sharing a characteristic 18S rDNA V7 synapomorphy (Berney et al., 2015), are a clade distinct from Mycetozoa and Archamoebae and not paraphyletic. All variosean families, and all orders in Table 2 are clades in Fig. 1 except for morphologically coherent Ramamoebida that is a clade on the Variosea-only tree of Berney et al. (2015). For the first time, our trees show that Heliamoeba is sister to Filamoebida (0.9, 35%) so belongs in Filamoebidae. Teleaepolella (Lahr et al., 2012) as Berney et al. (2015) found is robustly sister to Flanelia with which it shares a fan-shaped locomotive form unique in Varipodida and almost so in Variosea (Solfiformovum expulsum can be fan-shaped: Shadwick et al., 2009), the basis for new varipodid family Flamellidae distinct from Filamoebidae (Table 2). Acramoebida, groups with Solfiformovum and Grellamoebida, so Acramoebidae is now transferred from Varipodida to Protostelida and Grellamoebida from Acramoebidae to Solfiformovidae. This grouping is consistent with locomotory phase morphology and agrees with the site-homogeneous Amoebozoa-wide tree of Berney et al. (2015) but not their contradictory Variosea-only tree that showed a morphologically unreasonable grouping of Acramoebida with Multicilia. As the Solfiformovidae/Acramoebida clade is weakly sister of Protostelidae we retain Solfiformovum in Protostelida, but former protosteloids that consistently do not group with Protostelidae are placed in other orders (Table 2, see Section 3.7).

Basal phylogeny of cellular slime moulds (Dictyostelida) in site-heterogeneous rDNA trees (e.g. Fig. 1) strongly contradicts the earlier conclusion that the typically small-spored Group 1 dictyostelids are the most divergent branch (Schaap et al., 2006). Our most comprehensive 300-taxon CAT tree (Fig. S1) and corresponding ML tree both show the dictyostelid root between two large clades: Group 1/Group 2/Dictyostelium polycephalum and Groups 3/4/Dictyostelium polycarpum and Groups 3/4/Dictyostelium polycarpum. Group 1 is not the most divergent, but a very long-branch sister to Group 2 protostelids (0.97; this clade is sister to Dictyostelium polycephalum with maximal support) or to Group 4 plus D. polycarpum (ML 69%; only 64% support for it branching below the D. polycarpum/Group 2 last common ancestor). Fig. 1 analyses omitted this potentially confusing long-branch clade.

An unexpected feature of Fig. 1 is that by CAT Thecamoebida does not group with Sappinia/Stenamoebida, but with Protacanthamoebida within Centranoebida with remarkably high support at two nodes (0.99, 0.97). However, the 300-taxon CAT tree places Thecamoebida instead weakly (0.4) as sister to the Sappinia/Stenamoebida clade (Fig. S1), showing Thecamoebida as a clade in agreement with site-homogeneous trees, so this topology, not the apparent polyphyly of Thecamoebida in Fig. 1 is probably correct. As both site-heterogeneous trees show a statistically supported Sappinia/Stenamoebida clade, we establish a new family for it: Stenamoebidae Cavalier-Smith fam. n. Diagnosis: flattened uninnucleate or binucleate, non-ciliate elongate, smoothly monodidal, lobose amoebae without prominent longitudinal folds (unlike Thecamoebidae); subsuperpseudopodia absent; thin amorphous surface coat without scales or glycosylates, continuous and close to plasma membrane (Stenamoebida) or somewhat separated from membrane by discrete vertical elements and variable from continuous to a fragmented layer (Sappinia); aerobic; branched, tubular mitochondrion cristae. Type genus Stenamoeba Smirnov et al., 2007. Other genus Sappinia Dangeard, 1896.

3.7. Revised higher-level taxonomy for Amoebozoa

In light of our new rDNA and multiprotein trees and also of many ultrastructural and sequence phylogenetic studies published since the last gymnozean classification was prepared for Smirnov et al. (2011), Table 2 presents an updated classification that treats Conosa in more detail than before, especially to integrate numerous new variosean genera (Berney et al., 2015). Diagnoses are below with comments clarifying some innovations:

**Diagnosis of new superclass Cutosida** Cavalier-Smith, new class Cutosia Cavalier-Smith, and new order Squamocutida

Cavalier-Smith: uninnucleate amoebae bounded by a continuous thin, somewhat flexible, envelope (not closely attached to plasma membrane) having oval scale-like substructure within a denser matrix; one or many small pores penetrate the envelope, allowing pseudopodia to protrude for very slow, occasional locomotion; locomoting cells flattened, oval or round; cilia or centrosome absent; radiate floating forms absent. *Etymology:* cutis L. skin; *squama* L. scale; names chosen because cell envelopes are flexible like skin, but part of a mechanically continuous structure with scaliform substructure, morphologically and presumably mechanically separate from the cell body – like a recently moulted snake skin not yet sloughed off. Separate names are used for class and order to avoid confusion should cutoseans meritting a second order be found.

**Squamamoebidae** Cavalier-Smith fam. n. Diagnosis: as for Cutosia, plus: scale-like test structures have central projecting filament; locomotion involves numerous tiny mamiliform pseudopodia fixed to substratum as cell moves forward; stationary forms can be strongly branched. Type genus *Squamamoeba* Kudryavtsev and Pawlowski, 2013. *Comment:* ATCC PRA-29 ‘Pessonella’ sp. probably also belongs in this family if its bosses protrude through a multiporose test overlooked in the light microscope, but is excluded pending ultrastructure, needed to see if it is an undescribed *Squamamoeba* or new genus. Its statistically unsupported grouping with *Squamamoeba* rather than *Saprocrubinum* on a 2-gene tree (Lahr et al., 2015) fits its greater morphological similarity, but its deep branching suggests PRA-29 may be an undescribed sister genus.

**Sapocrubidae** Cavalier-Smith fam. n. Diagnosis: as for Cutosea, plus: scale-like test structures lack central projecting filament; one or sometimes two thin contractile filose pseudopodia longer than cell body; stationary forms rounded, unbranched. Type genus *Saprocrubinum* Lahr et al., 2015.

As Tubulinea and Discosea differ radically in surface structure from Cutosea and group together as a strongly supported clade we group them as new lobosan superclass Glycopoda.

**Diagnosis of superclass Glycopoda** Cavalier-Smith. Non-ciliate aerobic amoebae typically exhibiting active locomotion by usually lobose broad pseudopodia ancestrally and usually covered by a thick glycoalyx; mitochondrial cristae tubular, usually branched. Unlike Cutosa, without porose scaliform cell envelope distinct from plasma membrane. *Etymology:* glyco Gk sweet, refers to glycoalyx; *podos* Gk foot, to the locomotory pseudopodia.
3.7.1. New taxa in Tubulinea

Diagnosis of new genus *Atrichosa* Cavalier-Smith: Multinucleate algivorous marine amoebae with broad, short lobose pseudopodia; calcite spicules, cilia, and dactylopodia absent. Hair-like filopodia emanate from hyaline lobopodial zone and pass through pores in thin test that envelops most of the cell. **Etymology:** A Gk without; *trich* Gk hair, referring to the absence of spicules, unlike *Trichosphaerium*. Type species *Atrichosa algivora* Cavalier-Smith sp. n. **Diagnosis** as for genus, plus cells broadly oval, length 10–1000+ μm. Type culture ATCC 40318; type sequences EU273464-71; type illustration Fig.1E of Tekle et al. (2008).

**Comment.** This is the species whose transcriptome was sequenced by Katz and Grant (2015) under the name *Trichosphaerium* and shown in our multigene trees (Figs. 2–4). We considered the possibility that this is the same species or genus as *Pontifex maximus* (Schaeffer, 1926), but Shaeffer believed his amoeba was naked. If he was right they are not the same. If he was wrong and it was testate as Page (1983) assumed, it is possible that they are congeneric, but it is extremely hard to decide whether or not the *Pontifex* micrographs of Page or other observations/records of *Pontifex* are the same species as ATCC 40318. The identity or otherwise of *Atrichosa* and *Pontifex* may be undecidable; only when a large number of morphologically similar strains are sequenced will it be possible to evaluate the phylogenetic diversity of such pheno-types. As for other poorly studied amoeboid groups like Variosea (Berney et al., 2015) it may be much greater than Page realised; nomenclatural clarity and unambiguity is probably best served by attaching a new name to a sequenced and microscopically defined strain with type culture available. Page (1983) thought *P. maximus* the same as *Amoeba tentaculata* (Gruber, 1881). *Atrichosa* is morphologically highly dissimilar from any true *Amoeba*, and if did belong to that genus it would have grouped with *Cromopryxa* on our trees which it did not. One cannot logically claim simultaneously that *Amoeba* is a more valid name for *Pontifex* (as did Page, 1983) and the same genus as *Atrichosa*.

Addition of Trichosida makes Tubulinea distinctly more heterogeneous, so it is now advisable to subdivide them into three new subclasses, separated at points of maximal phenotypic disparity:

1. **Neolobosia** Cavalier-Smith: **Diagnosis:** uninucleate or multinucleate aerobic amoebae; branched tubular mitochondrial crista; locomotory shape monopodial or multipodial with cylindrical cross section pseudopodia, not strongly flattened; anastomoses or spine-like subsupopodia absent. **Etymology:** Neo Gk new refers to this clade originating substantially later than the ancestral lobosan. Includes two new superorders: *Eulobosia* Cavalier-Smith (**Diagnosis**): amoebae without dactylopodia or tests of calcite spicules. **Etymol:** Eu- Gk well; *lobose* E. lobed, referring to the well-developed cylindrical lobe-like pseudopods of these classical lobose amoebae, comprising the naked order Euamoebida and testate Arcellinida; and **Trichosia** Cavalier-Smith (**Diagnosis**): giant multinucleate naked marine amoebae with fibrous multinucleate test through which non-locomotory filopodia protrude; locomotory lobopodia vary from a single broad major hyaline zone to numerous short mammiform lobes). Sole order Trichosida.

The very close branching of *Nolandella* and *Cromopyxa* (much closer than any other two lobosan orders) and rather small phenotypic differences between them lead us to transfer Nolandellidae into Euamoebida as a new suborder and to establish a suborder for the traditional euamoebids:

**Suborder 1. Amoebina** Cavalier-Smith subord. n. **Diagnosis** as for order Euamoebida (Smirnov et al., 2011, p. 565)

**Suborder 2. Nolandina** Cavalier-Smith subord. n. **Diagnosis** as for order Nolandida (Smirnov et al., 2011, p. 565). Lahr et al. (2013) wrote ‘Hartmannella aberratensis stands out as an immediate candidate to be transferred to Nolandella’, unaware that Cavalier-Smith and Smirnov (in Smirnov et al., 2011, p. 561) had already done that and Smith & Cavalier-Smith had established order Nolandida for this taxon (Smirnov et al., 2011, p. 565) independently of synonymous Poseidonida of Lahr et al. (2011).

2. **Leptomyxida** Cavalier-Smith: **Diagnosis:** naked Lobosa; locomotory form varies from flattened expanded or reticulate, if slowly moving, to subcylindrical and monopodial especially in rapid movement; adhesive uroid; uni- to multi-nucleate; glycolcalyx amorphous. **Etymol:** named after sole order Leptomixida.

3. **Echinamoebia** Cavalier-Smith: diagnosis and etymology as for its sole order Echinamoebida (Smirnov et al., 2011, p. 565).

3.7.2. New families and orders of Variosea

**Ramamoebida** Cavalier-Smith ord. n. **Diagnosis:** uninucleate, often elongated, sometimes reticulose, amoebae with branched filose pseudopodia or subsupopodia that generally move too slowly for motility to be observable in the light microscope; phylogenetically more closely related to Cavosteliidae than to Filamoeba. Unlike Varipodida, cilia (in multiple kinetids) and a stalked spore-bearing fruiting stage sometimes present. **Etymol:** ramus L. branch, denoting their often branched, non-reticulose pseudopodia + amoeba (the cell body also is often branched). Includes the protosteloid Cavosteliidae and two new families with unstalked cysts, each a strongly supported rDNA clade: *Lobosa* Cavalier-Smith fam. n. **Diagnosis:** non-fruited, non-ciliate amoebae with often branching filopodia; distinguished from Filamoebidae and Acramoebidae by narrow elongated or narrowly branched cell body and closer genetic relationship to Cavosteliidae; cysts round, oval or bean-shaped, unstalked. Type genus *Lobosia* Geisen, Bass, and Berney in Berney et al., 2015. Other genus *Darbyshirella* Berney, Bass, and Geisen in Berney et al., 2015.

**Angulamoebida** Cavalier-Smith fam. n. **Diagnosis:** non-fruited, amoebae with often branching filopodia, markedly less elongate cell bodies than *Lobosa*; distinguished from *Acramoebia* by cell body often being tripily branched and by eating fungi not bacteria, and in at least one species by a multiciliate stage with more than one kinetid; cysts spherical to oval, unstalked. Type genus *Angulamoeba* Bass, and Geisen in Berney et al., 2015.

The sometimes bicipitate (Ceratomyxella only) protosteloid Schizophlasmodiidae (Nematostellida, Ceratomyxellida, Schizoplasmomodium) also do not group with Protostelida on rDNA trees but weakly with the non-amoeboid unciliate Phalansteridium (Shadwick et al., 2009). Two highly reticulose, non-fruited (i.e. non-protosteloid), extremely slow-moving amoebae (Arbramoeba, Dictyamoeba), having a novel reticulose morphotype (sensu Smirnov et al., 2011) for Amoebozoa, also group weakly with this clade (Berney et al., 2015). We establish new families within Phalansteridi for these previously unclassified genera: *Arboramoebidae* Cavalier-Smith fam. n. **Diagnosis:** non-ciliate, reticulose amoebae moving too slowly to be seen by real time microscopy; no obvious main cell body; protoplasmic networks grow to >600 μm, filopodia anastomosing when older; fine pointed pseudopodia predominantly at anterior front; double-walled cysts, not on stalks. Type genus *Arboramoeba* Geisen, Bass and Berney in Berney et al., 2015.

*Dictyamoebidae* Cavalier-Smith fam. n. **Diagnosis:** non-ciliate, reticulose amoebae moving too slowly to see by real time microscopy.
microscopy; multiply branched main cell body, anastomosing, giant networks up to several mm; fine pointed pseudopodia near tips; unicellular cysts not on stalks. Type genus Dictyamoeba Berney, Bass, and Geisen, 2015.

Our site-heterogeneous rDNA trees (Fig. 1) confirm that both genera, despite apparent absence of cilia (presumably independent losses), form a weakly supported clade with the non-amoeboïd uniciliate Phalansterium plus the biciliate amoeboïd, protosteloid Schizoplasmodiidae, as weakly suggested by Fig. 1 of Berney et al. (2015, but contradicted by their Fig. 2 that put Phalansterium slightly deeper than the five amoeboïd genera, without support). We therefore transfer Schizoplasmodiidae from Prostomelida to Phalansteriida and also add Arbormoeba and Dictyamoeba to Phalansteriida; as all five amoeboïd genera now in Phalansteriida are reticulose this was likely the ancestral condition for Phalansteriida, lost by Phalansterium alone.

Our site-heterogeneous rDNA trees (Fig. 1) strongly confirm previous evidence (see Section 3.6) that Protosporangium plus Clastostelium are a clade entirely distinct from Prostomelida sensu stricto, so we make this clade a new order: Protosporangida Cavalier-Smith. Diagnosis: non-ciliate amoeboïae with separable ciliate swarm cell phase with one or more unequally biciliate kinetids; fruiting stage with one or two spores borne on a single stalk; unlike Prostomelida and other variousian protosteloids, lack the variousian-specific highly conserved nucleotide sequence motif with the consensus GCGUGAAG in the ascending stem, and UGGAUCCU in the descending stem of the unpaired region at the base of helix E43_2 in the V7 region of 18S rRNA.

Etymol: proto Gk first; sporangium E.; to emphasise that the singly stalked uni- or bisporous sporangium is more primitive than other Mycetozoa.

Protosporangidae Cavalier-Smith fam. n. Diagnosis: as for Protosporangida. Type genus Protosporangium Olive and Stoaianovitch, 1972. Other genus Clastostelium Olive and Stoaianovitch, 1977.

3.8. Stygamoeba and the unity of order Glycostylida

Our trees provide the first strong evidence that Stygamoebidae are sister to Dactylopodida. Dactylopodida are not sisters to Vannellida as assumed when class Flabellinea was proposed for them (Smirnov et al., 2005). Instead Vannellida are sister to Stygamoebida/Dactylopodida. These three orders share the common feature of flattened shape with polyaxial flow of endoplasm and rather thick glycostyly, but are sharply differentiated into three rather different phenotypes: Vannellida (fan-shaped, often with glycostyles forming the cell coat), Stygamoebida (flattened irregular long worm shape, with pyramidal glycostyles in Vermistella but none in Stygamoeba), and Dactylopodida [finger-like pseudopodia (dactylopodia) formed from a frontal hyaline area and a cell coat of usually glycostyles (boat-shaped scales in Korotnevella only)]. Collectively they correspond closely (not quite exactly) with the earlier order Glycostylida (Cavalier-Smith et al., 2004) assumed ancestrally to have had glycostyles. As ordinal rank should be reserved for taxa that differ rather substantially in phenotype from their closest relatives we have retained the now phylogenetically well substantiated, more comprehensive order Glycostylida emended by the exclusion of Multicilia (which is phylogenetically a variosean (Fig. 1); glycostyles that led to its former erroneous inclusion in Glycostylida presumably came from its vannellid food) and Mayorella, and inclusion of Stygamoebidae that were unknown when Glycostylida was established; the three glycostyle-bearing orders of Smirnov et al. (2011) become suborders (Table 2). By contrast subclass Flabellinia sensu Smirnov et al. (2011) is clearly not a clade as neither Histamismenida, nor Trichosida group with glycostylids on multiprotein trees, so we abandon Flabellinia as a taxon, though use it informally in Figs. 3 and 4 in a revised sense to label a robust, often flabellate, clade. Table 2 places the only other former flabellinian order, Pellitida, in Histamismenida as a suborder. Kudryavtsev and Pawlowski (2013) showed Pellitia to be related to Goceviidae and so transferred Goceviidae from Histamismenida to Pellitida. Taxonomic simplicity is better favoured by instead transferring Pellitia to Histamismenida, and grouping it with Goceviidae as new suborder Pellitina with the same composition as Pellitida sensu Kudryavtsev et al. (2014). Pellitina is now put in Centramoebida (see Section 5).

Absence of glycostyles from Stygamoeba may be secondary loss as it is in a few vannellids (Smirnov et al., 2007). However, in Fig. 1 Stygamoebidae is weakly paraphyletic with glycostyle-bearing Vermistella only being sister to other glycostylids (insignificantly different paraphyly in Fig. S1). Though our protein trees make it likely that this non-grouping of Stygamoeba is an incorrect consequence of low basal resolution by rDNA trees, if multiprotein trees confirm that Vermistella is even more closely related to other glycostylids than is Stygamoeba, absence of glycostyles in Stygamoeba could be the ancestral condition for the glycostyly clad and it would be appropriate to segregate Vermistella into a separate family (see Table 2 footnote b). In our trees the sister to Glycostylida is Stenamoeba (Thecamoebida, all with elongate monopodial cell bodies without dactylopodia), which indicates that their common ancestor had an undivided flattened cell shape without discrete finger-like pseudopods (dactylopodia) and that dactylopodia are a derived condition for suborder Dactylopodina alone. Differences in body form between Thecamoebida and Glycostylida are no greater than between the three glycostylid suborders, so they might reasonably be grouped one day as a superorder, whose common ancestor almost certainly had a flat cell body and no dactylopodia, these pseudopods evolving later in an ancestor of Dactylopodina.

The only discosean order not now represented on multigene trees is Dermamoebida comprising Dermamoebidae and Mayorellidae. Both have a very thick glycoalyx like Glycostylida with a complex substructure in marked contrast to Thecamoebida with a simple thin glycoalyx. Dermamoebida could be sisters to Glycostylida, even closer than Thecamoebida; on unresolved Figs. 1 and S1 all three are equidistant.

3.9. Distinctive features of Cutosea, the deepest branching Lobosa

We call this novel amoebozoan clade Cutosea, because Sapocribrum and Squamamoeba share a unique skin-like cuticle or envelope surrounding the whole cell except for rare small breaks or pores through which pseudopodia protrude (Kudryavtsev and Pawlowski, 2013; Lahr et al., 2015). The envelope comprises a dense matrix in which irregularly arranged oval structures about 150 nm (Sapocribrum) or 125 nm (Squamamoeba) in length are embedded. Though the embedded ovals were initially called a ‘cell coat’ of ‘scales’ (Kudryavtsev and Pawlowski, 2013; Lahr et al., 2015), the ovals are not discrete separable scales like true scales of Discosea (Cochliopodium, Dactylopodina) or chromists such as Paraphysomonadida, Thaumatomonadida or Haptista, but internal specialisations of a continuous, mechanically coherent–envelope. Consequently we refer to the ovals embedded in the cutosean envelope matrix not as scales but as scale-like (as on a crocodile or snake skin). In Sapocribrum and Squamamoeba the envelope is clearly separate from the plasma membrane, like the test of testate amoebae, not directly attached to it as is the carbohydrate-rich surface coat of animal cells or the much thicker but still flexible cell coats of many Discosea (Pellita, Dermamoebida – called cuticle in Mayorella, Glycostylida). Lahr et al. (2015) noted that the Sapocribrum ‘coat’ appears more plastic and deformable than a typical test and so refrained from calling it a test, which because of its clear mechanical distinctness from the plasma membrane might
be thought more appropriate than cell-coat or cuticle. Its deformability during specimen drying might just be because it is rather thin and unmineralised like the Atrichosa test. Though tests of Arcellinida are uniporous, those of Trichosida are multiperforate like that of Squamamoeba, and being multiperforate is therefore no reason not to call the Squamamoeba enveloping structure a test. However, the locomotory mechanism of Squamamoeba (Kudryavtsev and Pawlowski, 2013) implies a plastic envelope that can deform as cytoplasm flows past the ventrally anchored mamilloform pseudopodia, not a rigid test. As testa originally meant shell, implying rigidity, we agree that the scaliform outermost layer of Cutosea is best not called a test, and refer to it as an envelope as, like an ordinary letter envelope, it is distinct from its contents and completely encloses them except for some tiny pores. Cutosea are a previously unrecognised third group of lobosan armoured, non-naked amoebae additional to the testate Arcellinida (uniporous) and Trichosida (multiperforate).

Cutosean test structure is unique not only in Amoebozoa but for all protists. Squamamoeba locomotive forms have a hyaline anterior and produce small mamilloform pseudopodia as rounded bulges that fix to the substratum as the cell protoplasm slides past them, unlike most pseudopodia of Tubulinea, but probably rather like Atrichosa. These pseudopodia somewhat resemble those of the discosean Mayorella, which also has a dense membrane-adherent cuticle that is thicker, but with no oval scale-like substructure. Unlike in Squamamoeba the Mayorella naked subpseudopodia do not protrude through cuticle breaks. Pellita also has multiple small subpseudopodia that emerge through cuticular breaks and mediate a similar locomotion, but they are smaller than in Squamamoeba and not mamilloform bulges; their coat substructure is multilayered with an outer layer of glycostyles and inner layer of small discrete subunits directly attached to the plasma membrane, and lacks scale-like ovals. Moreover Pellita has centrosomes (unlike Cutosea) and therefore fits better in Discosea like centrosome-bearing Himatismenida and Centramoebida. Sapocribrum has much thinner and longer pseudopodia, i.e. filopodia more like those of the conosan Filamoeba (but proportionally thinner) than any Lobosa except for the filiform subpseudopodia of Trichosphaeriidae. Usually there is only a single pseudopod, longer than the cell body, postulated to contract to pull the cell forward, but sometimes there are two joined together near their base that emerges though the single envelope pore (Lahr et al., 2015); a terminal web of subpseudopodia also mentioned might possibly help locomotion similarly to Atrichosa.

The fundamental ultrastructural similarity of Squamamoeba and Sapocribrum, unique in protists, and grouping of both with PRA-29 on oligogenic trees (Lahr et al., 2015), together with the firm exclusion by our 187-gene trees of Cutosea from Discosea, shows that including Squamamoeba in Dactylopodida (Kudryavtsev and Pawlowski, 2013) was incorrect, being based only on poorly resolved 18S rDNA trees, a flattened body form, and slight pseudopodial similarity to Mayorella subpseudopodia. Likewise including Sapocribrum in Flabellinia (Lahr et al., 2015) was incorrect. Given their unique multiperforate flexible envelopes comprising linked scale-like plates (like ancient Chinese and medieval Japanese body armour) and deep divergence from all other Amoebozoa, we ranked Cutosea as a third lobosan class with single order, Squamocutida but two families to emphasise their marked pseudopodial and envelope differences.

3.10. Evolutionary diversification and origin of Lobosa

Our evidence for early divergence of Cutosea illuminates the ancestral state for Lobosa. Their distinctive perforated dense envelope somewhat resembles the thick amorphous pellicle of Pellita (in Discosea) in that breaks in it are necessary for locomotion and also the multiperforated flexible theca of the tubulinean Trichosphaeriidae, but differs in not being integrated with the cell membrane. Thus one deep branching clade in both the other lobosan classes has a perforated flexible envelope-like morphology, structurally and functionally similar but not identical to that of Cutosea. Did this morphotype evolve independently three times in slightly different ways from ancestral naked amoebae? Or did the ancestral lobosan have a perforated envelope morphology like that of Cutosea and tubulinean and discosean ‘gymnamoebae’ evolve nakedness independently by modifying it into an even more flexible cell coat? We think it somewhat more likely than not that the common ancestor of Lobosa had cells enveloped by a thick coherent envelope, theca or pellicle that needed occasional breaks to allow pseudopodial extrusion. These coherent envelopes evidently rather limit active amoeboid locomotion; such amoebae are much less speciose than other Lobosa that have liberated their pseudopodia from this rather rigid constraint, which we postulate occurred in contrasting ways in five independently evolving lobosan subgroups.

First, Cochliopodiidae and Goecevidae restricted the thick layer to the dorsal surface as a tectum (probably independently, judging from rDNA trees: Kudryavtsev et al., 2014) and thus could develop the whole ventral surface for locomotion in a limpet-like manner, retaining the tectum as a dorsal protective shell. What the tectum protects against is an important question; though it might offer some protection against predation by large protists, we are sceptical of this and suggest that it may mainly protect against viruses invading from the supernatant medium through the plasma membrane and/or from ultraviolet radiation. UV-protection is important in many shallow aquatic habitats and in uppermost soil layers. The first author noted that almost all protozoa he found living on a sandy beach in the very clear waters of Lake Baikal (Siberia) had marked light-aversive behaviour, hiding below sand grains and only occasionally very briefly emerging; he once watched a Baikal beach Gocevia (Pellittina) in a Petri dish crawling among sand grains carrying a layer of tiny dense granules on its back – as it progressed these accidentally fell off, but remained coherent – presumably by mucilage, and the amoeba turned round, crawled under the granule layer and once it was reinstated on its back proceeded onwards with its dorsal cargo. Such behaviour arguably indicates that the granules are important to and controlled by it. We suggest that they and other dorsal structures like the large scales of Cochliopodium may offer UV-protection amongst other benefits.

Secondly Glycostylida/Dermamoebida evolved a more flexible but still thick glycoalyx, possibly ancestrally composed of
glycocalyces which allow the coat to stay with the plasma membrane during pseudopodial motion, unlike in Pellita and Cutosea and to evolve fan-like, irregularly vermiciform or dactylopodial locomotory forms. Thirdly, by contrast, Stenamoeba the sister group to Glycordermia achieved flexibility by retaining surface coherence by greatly thinning the ancestrally thick glycocalyx so that the cell surface can be easily deformed (often wrinkled) during monopodial locomotion.

In contrast to those three ways of achieving flexibility during multiaxial endoplasmic flow in Discosia, Tubulinea evolved tubular near-cylindrical pseudopodia with a thick cortical gel and central uniaxial flow. Many larger Tubulinea have a thick amorphous glycocalyx (not so obviously multilayered as in many Flabellinia). Its thickness might be the ancestral condition for Tubulinea and derived from that of the earliest flattened common ancestor of Tubulinea and Discosea; if so the real novelty for Tubulinea was greater flexibility allowed by novel tubular pseudopodia. Were Tubulinea ancestrally monopodial like Echinamoebidae, the most divergent order, so the multipodal condition evolved independently in Amoebina and Leptomyxida? Or are unipodial forms derived from multipodal ones? Given that Cutosea include both multipodial and predominantly unipodial genera, and Discosea are predominantly unipodial with only a few (e.g. Dactylopodina and Pellitina) essentially multipodal, it is hard to decide whether the ancestral state for Amoebozoa was multipodal or unipodial. A reasonable scenario, given that the sulcozoan ancestors of Amoebozoa (see Cavalier-Smith, 2013 and Cavalier-Smith et al., 2014) have very irregular pseudopodia variable in numbers, is that this may also have been true of the first Amoebozoa, and unipodial Lobosa evolved by multiple divergent simplifications. As Echinamoebida are the most divergent Tubulinea, the pointed subpseudopodia of Echinamoeba only within Tubulinea might be a relic of an ancestral condition for Amoebozoa, as those of Conosa were probably ancestrally pointed. Whether Leptomyxida, which likely diverged before the common ancestor of the most typical Tubulinea (subclass Neolobosia), and which adopt a more flattened, sometimes anastomosing, morphology unlike other Tubulinea, are secondarily atypical or retained a flattened form from ancestral Lobosa is uncertain. Multinuclearity clearly evolved at least twice in Tubulinea – in Trichosida and in some giant Amoebina.

The above evolutionary synthesis emphasises that tubular pseudopodia of Amoeba proteus, the prototype for early ideas about amoeboid locomotion (e.g. Mast, 1926; Pantin, 1923) are not the archetypal lobosan state, still less that of Amoebozoa as a whole. As the sulcozoan ancestor of Amoebozoa had a dorsal submembrane theca, ventral pseudopodia and posterior ciliary gliding (Cavalier-Smith, 2013), all three were lost during or immediately prior to the evolutionary transition to the ancestral lobosan. It would not be surprising if (as suggested above) the ancestral lobosan evolved an alternative extracellular flexible envelope to replace the lost stabilising function of the sulcozoan dorsal intracellular pellicular thickening, allowing retention of the ancestral sulcozoan ventral multiple pseudopodial condition during the radical changes that made Amoebozoa. The above interpretation of an enveloped lobosan common ancestor makes the transition from sulcozoan to amoeboid body plan much more gradual and less dramatic, therefore more evolutionarily comprehensible, than previously (Cavalier-Smith, 2013); it is much more plausible than would be the conversion of a sulcozoan in one step to a classical neolobosan amoeba. We need information about comparative chemistry and biogenesis of these varied envelopes and their distribution in more Cutosea and other ill-studied early lineages before we can judge whether this envelope-first scenario for early lobosan evolution is really preferable to the only slightly less plausible standard naked-amoeba-first assumption implying polyphyletic origins of envelopes/coats.

Whichever is correct it is entirely clear that Amoeba is not a primitive organism, contrary to a centuries-old misconception, but a highly derived specialised one. After a century and a half, the idea that eukaryotes and cells generally evolved from an amoebo-oid ancestor (Haechel, 1886), which Cavalier-Smith (1987) dubbed the ‘moneran myth’ is finally defunct. Both prokaryotes and eukaryotes ancestrally had rigid cell surfaces and the transition between them was mediated by loss of the eubacterial cell wall and consequential origin of phagocytosis, cilia, and a largely rigid cell surface supported by cross-linked actin microfilaments and microtubules (Cavalier-Smith, 2014). Cavalier-Smith (1981) argued that amoebo-oid locomotion is clearly advanced not primitive, rejecting Haechel’s idea of the first eukaryote being an amoeba as the Zoological Myth. We are now left with semi-rigid biciliate phagotrophic zooflagellate reality as the only well-corroborated idea of our first eukaryotic ancestor (Cavalier-Smith, 2000, 2013, 2014).

3.11. Variosean diversity and basal evolution of Conosa

The ancestral conosan would have had two cilia and multiple ventral pointed pseudopodia or subsuspseudopodia when it evolved from the sulcozoan ancestor of Amoebozoa by losing dorsal ciliary gliding, as Cavalier-Smith (2013) explained. For a much better understanding of their origins we need multigene trees for all lineages of Variosea and Mycetozoa, as previous 18S rDNA trees suggested that protosteloids (non-social amoeba whose resting cysts are born on stalks and thus are called spores by analogy with those of slime mould fruiting bodies, and which were formerly lumped in class Protostelea) and Variosea are phylogenetically partially overlapping (Shadwick et al., 2009). Prior to the present paper Variosea comprised four morphologically distinct orders, three ciliated: uniciliate Plalanteriida (Plalanteriidae, Rhizomasonia) and three other largely sedentary and non-amoebo-oid genera: Cavalier-Smith, 2013; Cavalier-Smith and Scole, 2013; Holomastigida, (the only minimally amoebo-oid Multicia); and putatively multiciliate but not highly mobile Artodiscida (no sequences available). The non-ciliate Varipodida are unfortunately the only variosean order with available transcriptomes, making it very hard to establish their relationships and to decide whether Variosea are the paraphyletic ancestors of all Conosa or the earliest diverging clade or clarify phenotypic evolutionary patterns within the class.

Though no transcriptomes are available for ‘Protostelea’, the recent discovery of several new genera of non-fruited Variosea, their placement on rDNA trees, and the recognition that some protosteloid lineages share an apparent molecular synapomorphy in the V7 region of 18S rDNA with them (Berney et al., 2015) enabled us to realign the boundary between Variosea and Mycetozoa, radically revise Protostelea, and establish new conosan orders Ramamoebida and Protosporangida, yielding a conosan classification more consistent with sequence phylogeny and morphological diversity. For the first time this assigns the plethora of recently discovered variosean genera (Berney et al., 2015) to families and orders. We followed Berney et al. (2015) in regarding all protosteloids possessing the variosean V7 synapomorphy as members of class Variosea. Table 2 formally transfers them into class Variosea, but their deep genetic diversity compared with Varipodida made it inappropriate to include them in that order.

Previously all conosan protosteloids were in order Protostelida (Olive, 1967) arranged in four morphologically and genetically distinct families (Cavalier-Smith, 2013). We removed from Protostelida all protosteloids except the two families whose amoebae have a simple non-branched and non-reticulose cell structure (Protostelidae, Soliformovidae), which form a weakly supported
clade on some 18S rDNA trees (Berney et al., 2015; and our distance trees, not shown), but not on others (Shadwick et al., 2009). Cavosteliidae, a strongly supported rDNA clade with sculptured spore walls and often a ciliated stage, do not group on rDNA trees with Protostelida sensu stricto (Shadwick et al., 2009; Berney et al., 2015); instead they either group weakly with Dictyostelida (Shadwick et al., 2009) or form a weakly supported clade (Ramamoebida) with three recently established variosean genera of amoebae with branched pointed pseudopodia that move too slowly for motility to be observable in the light microscope (like Acramoeba) and which are not known to form aerial spores (thus not protostelids) (Berney et al., 2015). One of these non-fruiting genera (Angulamoeba) has branched cells sometimes with cilia in multiple kinetids as in Cavostellium. Though the filose subspeu- dopodia of Cavostelidiaceae are typically shorter than those of the non-fruiting amoebae of this putative clade, they are sufficiently similar to be grouped in a new, mostly soil-dwelling, presently entirely non-marine, order Ramamoebida.

Protosporangids are robustly sister with near maximum support to Ceratiomyxa in published site-homogeneous rDNA trees and in our Fig. 1 site-heterogeneous tree. We therefore placed Protosporangida within subclass Exosporeae of Mycetozoa, the probable sister to dictyostelids. Exosporeae as thus expanded all have non-ciliate trophic cells and short duration swarming cells with one or more biciliate kinetids formed just after germination of smooth-walled stalked spores.

The new phylogeny and taxonomic revision of Variosea emphasi- ses that cilia have been lost independently by Varipodida and several members of other orders. One large order (Phalansteriida) retains the ancestral single kinetid (reduced to one cilium in Phalansterium but not Ceratiomyxella), but four orders (e.g. Proto- stelida) comprise or include families that multiplied kinetids per cell. Thus kinetid multiplication is not restricted to Multicilia as once thought (Cavalier-Smith et al., 2004) but is a pervasive evolutionary phenomenon in Variosea. Multigene trees including all these orders are necessary to establish the currently unclear basal branching order of Variosea and determine how many kine- tid multiplications have occurred; present evidence suggests multiplication was independent in mycetozoan protosporangids and Variosea.

3.12. Protosteloid polyphyly and the origin and diversification of Mycetozoa

The discovery of many new Variosea with unstalked cysts, some related to protosteloids with stalked cysts/spores (Berney et al., 2015), throws new light on the origins of protosteloids and Variosea. Berney et al. suggested that an ancestor of Variosea related to protosteloids with stalked cysts/spores (Berney et al., 2015). One of these non-fruiting amoebae of this putative clade, they are sufficiently similar to be grouped in a new, mostly soil-dwelling, presently entirely non-marine, order Ramamoebida.

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There is no convincing argument for the variosean ancestor having had aerial spore dispersal. The idea that all Amoebozoa ancestrally had fruiting bodies that were lost many times (Shadwick et al., 2009) is extremely unlikely. The isolated positions of Copromyxa in Tubulinea (Fig. 2) and the protosteloid Protoste- liopsis in Vannellida (Fig. S1) make it virtually certain that both evolved from non-stalked ancestors and the protosteloid state evolved independently in Protostelespis and variosean proto- steloids. The protosteloid phenotype also evolved twice independently in Discosea (Endostellum, and an acanthamoebid: Shadwick et al., 2009), so it probably arose about 10 times in Amoebozoa. We agree with Shadwick et al. (2009) that Eumyceto- zoa should no longer be used as a taxon name for all Amoebozoa with fruiting bodies; we abandoned it before then, correctly considering Copromyxa as not mycetozoan (Cavalier-Smith et al., 2004). The close relationship of the protosporangid protosteloids to Ceratiomyxa makes it unlikely that their common ancestor (the ancestral exosporean) was a protosteloid in the sense of having single stalked spores (cysts), but there is no convincing evidence from sequence trees that Exosporeae are related specifically to any variosean protosteloid lineages; exosporean stalks probably evolved independently from them. As the assumption that Amoebozoa ancestrally had fruiting bodies is devoid of evidence, we strongly oppose the suggestion that it could be appropriate to replace the name Amoebozoa by Eumetazoa or any other merely because stalked cysts are more widely present in Amoebozoa than previously suspected (Shadwick et al., 2009). Such pointless replacement would be highly confusing.

Fiore-Donno et al. (2009) called dictyostelids, myxogastrids, and Ceratiomyxa collectively Macromycetozoa, made a superclass by Cavalier-Smith (2013). However, the strong evidence that unicellular protosporangids are within this clade makes the name descriptively less appropriate. Moreover, it becomes an unneces- sary synonym of Mycetozoa now Protosteles is abandoned, so Table 2 discontinues its use. That protosporangids are now included and acrasids excluded is no obstacle to continued use of the historic name Mycetozoa for this taxon and clade, whose last common ancestor probably did have aerial spore dispersal. Until multigene trees are available for additional variosean orders it will not be clear what are the closest relatives of Mycetozoa. Our site- heterogeneous rDNA trees do not help resolve the branching order amongst the four conosan classes; on past homogeneous rDNA trees the long-branch Myxogastrea often branched weakly with or near Archamoebae which contain two especially long branches (Entamoeba, Pelomyxa) (Shadwick et al., 2009; Kudryavtsev and Pawlowski, 2013), but when these are excluded (Fig. 1) there is no tendency for Archamoebae to group with shorter branch Mycetozoa – thus Fig. 1 neither contradicts nor supports the possible holophyly of Semiconosia (Variosea plus Mycetozoa), which previous multigene trees did not unambiguously resolve (Cavalier-Smith et al., 2015b).

Our rDNA trees are also important for mapping character evolution onto dictyostelid cellular slime mould phylogeny, as they convincingly disprove the idea that small-spored Group 1 Dictyostelium (e.g. D. antarcticum, D. stellarum) are the most diver- gent clade (Schaap et al., 2006). Schaap et al. (2006) based that on two-gene trees with codon second nucleotides of α-tubulin given twice the weight of codon 1 nucleotides (codon 3 nucleotides omitted) plus 18S rDNA nucleotides (their supplementary Fig. S1C) with only nine outgroup species: three Variosea; six
Discosea. That tree did not even group Dictyostelida with Variosea as our multigene trees robustly show to be correct, but weakly (0.7, 54%) with Thecamoeba similis, which our Fig. 1 shows is like Dictyostelida an extremely long branch, and thus a bad outgroup choice. Most likely the long-branch Group 1 Dictyostelida were drawn by the Thecamoeba sequence to the base of the dictyostelid clade by long-branch attraction artefacts, just as all dictyostelids were wrongly pulled towards it. In fact, their Fig. S3 two-gene analyses where Thecamoeba was omitted and three Variosea, one tubulinine, and four green plants were outgroups (more balanced), and their homogenous Bayesian tree weighting both tubulin codon positions and rDNA nucleotides equally, found the same topology as we did (with 0.85 support). Curiously they did not mention that, possibly because their ML and LogDet trees showed the same topology when tubulin second codon positions were single weighted as well as double weighting. Their two-gene trees using α-tubulin amino acids (generally accepted as superior to using nucleotides) also had the same topology as we found (whether weighted the same, twice or thrice as rDNA nucleotides) so it is a mystery why their text entirely ignored that topology.

Their single-gene α-tubulin tree rooted on one protostelid and three myxogastrid (Physarum) sequences yielded a third topology with Group 2 the deepest branch (no support shown, so <50%). If one roots that tree between Groups 1/2 and Groups 3/4, as our trees show to be correct. Group 2 has the longest branch in dictyostelids, which may account for it being drawn artefactually towards the base. As explained elsewhere (Cavalier-Smith, 2015), α-tubulin is not a good marker for single-gene trees as it has a huge diversity in evolutionary rates in numerous groups, just as does 18S rDNA in Amoebozoa. It is not surprising that combining data from two genes with two opposite long-branch artefacts (Schaap et al., 2006) gave three contradictory topologies (not just two as they implied) depending on algorithms, weighting, and choice of outgroups and nucleotides versus amino acids. Their analyses used only 1374 rDNA nucleotides; ours used 1470 and many more outgroup sequences and thus is expected to give much more accurate dictyostelid rooting. Though our Fig. S1 included fewer dictyostelids than theirs, as many have extremely similar sequences, our own ML trees including all dictyostelid sequences from Schaap et al. (2006) had exactly the same topology: the root is reliably between groups 1/2 and groups 3/4.

This conclusion is important because Romeralo et al. (2011) ignored the contradictory results, outgroup limitations, and caveats of Schaap et al. (2006), assuming their erroneous conclusion of early divergence of Group 1 to be correct. We conclude that the almost uniformly small spores of Group 1 is probably secondary reduction with Group 2 the deepest branch (no support shown, so <50%). If one roots that tree between Groups 1/2 and Groups 3/4, as our trees show to be correct, Group 2 has the longest branch in dictyostelids, which may account for it being drawn artefactually towards the base. As explained elsewhere (Cavalier-Smith, 2015), α-tubulin is not a good marker for single-gene trees as it has a huge diversity in evolutionary rates in numerous groups, just as does 18S rDNA in Amoebozoa. It is not surprising that combining data from two genes with two opposite long-branch artefacts (Schaap et al., 2006) gave three contradictory topologies (not just two as they implied) depending on algorithms, weighting, and choice of outgroups and nucleotides versus amino acids. Their analyses used only 1374 rDNA nucleotides; ours used 1470 and many more outgroup sequences and thus is expected to give much more accurate dictyostelid rooting. Though our Fig. S1 included fewer dictyostelids than theirs, as many have extremely similar sequences, our own ML trees including all dictyostelid sequences from Schaap et al. (2006) had exactly the same topology: the root is reliably between groups 1/2 and groups 3/4.

This conclusion is important because Romeralo et al. (2011) ignored the contradictory results, outgroup limitations, and caveats of Schaap et al. (2006), assuming their erroneous conclusion of early divergence of Group 1 to be correct. We conclude that the almost uniformly small spores of Group 1 is probably secondary reduction from medium-spored ancestors, probably not the ancestral dictyostelid state. As cell size usually correlates with genome size (Cavalier-Smith, 2005), this clade likely also underwent genome reduction compared with most Conosa, possibly stemming from selection for smaller spores for better aerial dispersion and/or ability to generate more spores from a given biomass. However, Romeralo et al. (2011) noted that two not directly related new Australian group 1 species (D. myxobasis and boomerasporum) have much larger spores than most dictyostelids, indicating secondary increases in spore size; one wonders if they have an unusual derived ecology. Romeralo et al. (2012) recognised that previous attempts to root dictyostelids were equivocal and the root position then still uncertain; our more firmly rerooting the tree does not alter the majority of their conclusions. Our CAT trees weakly group Dictostestium polycephalum with Group 3 containing D. caveatum, and somewhat more strongly reject its deeper branching than both groups 3 and 4 by ML (with only 58% support for this deeper position on ours) which could also be a long-branch artefact, so it may be better to include it in group 3 and not treat it as a separate clade (Schaap et al., 2006; Romeralo et al., 2011, 2012). CAT confirms that Polysphondylium violaceum is sister to group 4, and that Polysphondylium is polyphyletic (two independent origins of whorled branches) and Dictostestium paraphyletic (i.e. dictyostelids ancestrally lacked such branches and originated by evolving cellular stalks). That makes it highly likely that Actyostelium is polyphyletic (lost stalk cell differentiation twice), not paraphyletic as Romeralo et al. (2012) suggested (contradicting their reasonable conclusion that Dictostestium is paraphyletic not polyphyletic).

3.13. Significance of Cutosa and low mobility Variosea for the origin of Conosa and Amoebozoa

Most Variosea except Flammellidae (Flamella, Telaepolella) are much less mobile than Glycopoda. Filamoebidae, non-ciliate members of Protostelida and Ramamoebida, and all trophic Phalansteriida except non-amoeboi Phalanterium itself are essentially non-mobile cells with highly branched tree-like pseudopodia (Smirnov et al., 2011; Dyková et al., 2005, 2010; Berney et al., 2015). Though some Filamoeba species have a motile form (Page, 1967) a branched immobile form appears to predominate (Page, 1967; Dyková et al., 2005). Flammellidae however, alone in Conosa, have fan-shaped active locomotion (Kudryavtsev et al., 2009) indistinguishable from that of Vannellidae. This locomotory morphotype therefore apparently evolved twice independently in Amoebozoa, and is thus a rare derived condition within Variosea. Because the many uniciliate forms in Phalansteriida (Cavalier-Smith, 2013 and Table 2) do not show significant amoeboid movement, their relationship to Amoebozoa was only realised after we sequenced Phalanterium rDNA (Cavalier-Smith et al., 2004). Thus as Cavalier-Smith (2013) noted, Conosa were probably ancestrally largely immobile, like Sulcozoa using pseudopods mainly for feeding not locomotion as in most Glycopoda. The more mobile Flamma, Myctetozoa, and certain Archamoebae (e.g. Entamoeba and Tricholimax, with eruptive pseudopodia that evolved independently of each other and of Percolozoa) probably independently evolved greater pseudopodia-based locomotion.

That Cutosa are the most divergent Lobosa suggests that ancestral Lobosa also were low mobility protists, effective rapid pseudopodial locomotion of Glycopoda evolving secondarily significantly after loss of posterior ciliary gliding, the sulcozoan groove, and dorsal pellicle during the origin of Amoebozoa (Cavalier-Smith, 2013). Our site-heterogeneous rDNA trees are consistent with this; all disagree with a site-homogeneous two-gene tree weakly suggesting that Cutosa may be sister to Vestilifera (Lahr et al., 2015); Fig. 1 clearly excludes them from holophyletic Dactylopodina (Fig. 1, posterior probability 0.81). Fig. 1 weakly placed them as sister to all other Amoebozoa, like our much stronger multigene trees showing they are not Discosea. However, unconverged Fig. S1 including very long branch Amoebozoa did not recover holophyletic Dactylopodina and weakly placed Cutosea within Discosea as sister to Coelopodium similarly to a Mr Bayes tree (Kudryavtsev et al., 2014, who excluded Parvamoeba which wrongly attracted Ovalopodium away from Coelopodium in Fig. S1, and wrongly grouped with Sappinia in Lahr et al., 2015). As neither rDNA tree consistently resolves the deepest branchings within Amoebozoa these contradictory positions are all meaningless – there are too many extremely closely spaced and too variable length basal amoebozoan branches for single-gene trees to be reliable. Even a six-gene tree that also showed a Coelopodium grouping was basally totally unresolved (Lahr et al., 2015), making further trees with many scores of proteins and many more taxa than here essential for testing and extending our conclusions.
4. Major conclusions

1. Trichosida are not Discosea as recently supposed, but belong in Tubulinea and are here grouped with orders Euamoebida and Arcellinida as subclass Neolobosa. The transcriptome-sequenced strain is probably not *Trichosphaerium*, so we described it as a new non-spiculate trichosid species *Atrichosa algivora*.

2. *Nolandella* and *Copromyxa* branch so closely together that we include both in Euamoebida in new suborders Nolandina and Amoeboina.

3. Better taxon sampling of Discosea including all orders but one largely establishes the branching order of the main subgroups congruently with their diverse pseudopodial forms and cell coat ultrastructure. Five multiprotein trees strongly favour holophyly of Discosea, but one suggested paraphyly.

4. Vexilliferidae and Paramoebidae are robustly sisters within holophytic Dactylopodina. The *Perkinsela*-like ichthyobodon endosymbionts of both *Paramoeba* (parasomes) together form a robust prokinetoplastid clade that is firmly sister to metakinetoplastids within Euglenozoa.

5. Dactylopodids, *Stygamoeba*, and vannellids are a robust discosean clade here treated as revised order Glycostylida (ancestrally with cell coat of glycostyles) with these three groups ranked as suborders of contrasting locomotory shapes. The *Stygamoeba* culture used for transcriptome sequencing was heavily contaminated by *Mayorella* sp. (actually *Cunea*) sequences but we identified genes for each component of the mixed culture unambiguously using 187 single-gene trees. *Mayorella* sp. is not a *Mayorella* but a third, undescribed species of the paramoebid genus *Cunea* and robustly sister to *Paramoeba*.

6. Thecamoebida represented by *Stenamoeba* are sister to Glycostyldia.

7. *Streptomyxa* (misidentified; should be new genus) and *Acanthamoeba* are robust sisters.

8. *Sapocribrum* and PRA-29 are robust sisters, forming new order Squamocutida and class Cutosea characterised by a pellicle with embedded scale-like oval structures and unusual very slow moving pseudopodia. PRA-29 was probably misidentified as *Pessonella* sp. and should be a new genus. Cutosea include *Squamamoebida* and are sisters of Discosea plus Tubulinea and thus the most divergent Lobosa.

9. Recognition of Cutosea together with the overall robustness of the better sampled amoebozoan multigene tree enabled substantially novel interpretations of lobosan evolution, making ultrastructure, pseudopodial form, and phylogeny more congruent, and simplifying discosean taxonomy.

10. Our trees raise the possibility that ancestral Lobosa were enveloped and naked groups (gymnamoebae) multiply derived from them by losing the envelope.

11. Our site-heterogeneous rDNA trees confirm polyphyly of Protostelida and Protostelida, so Protostelida is abandoned and Protostelida restricted to a robust clade transferred to Variosea. Other variosean protosteloids group with five morphologically and genetically related non-fruiting recently described variosean genera not previously assigned to orders; some are transferred to Phalansterida with related non-fruiting amoebae; Cavosteliidae is grouped with others as new reticulose order Ramamoebida. A protosteloid clade closely related to Ceratiomycida is made a new order of Mycetozoa: Protosporangida within expanded subclass Exosporeae of revised class Stelamoebida.

12. Our rDNA trees show that the position previously assumed for the root of Dictyostelida was incorrect because of an overlooked long-branch artefact. Group 1 dictyostelids are not the most divergent clade but are extra-long-branch sisters of Group 2; the root lies between Groups 1/2/Dictyostelium polycarpum and Groups 3/4.

5. Note added in proof

During proof correction another multigene amoebozoan prepublication appeared online: Tekle et al. (2016).

It convincingly groups *Parvamoeba* with Himatisenidena as Smirnov et al. (2011) argued, even within Cochliopodiidae; *Goe西亚 fonbrunei* with Centramoebida not Himatisenidena (confirming their independent origin of a dorsal tectum); and *Vermistella* with Thecamoebida not Glycostyldia; and shows monophyly of Vannelina and the robust Glycostyldia/Thecamoebida clade we called Flabellinia. Unfortunately it omitted *Stygamoeba* and *Copromyxa*; did not realise that *Mayorella* is actually *Cunea* so mistakenly concluded that Dactylopodida is not a clade and therefore wrongly moved *Mayorella* from Dermamoebida to Dactylopodida; used no site-heterogeneous method; and ran no Amoebozoa-only trees. Resolution on their LG ML tree (not the better LGF) was far too weak to resolve amoebozoan deep phylogeny (neither revealing the deep branching of Cutosea nor even recovering Conosa or Lobosa clades) or to justify their conclusion of non-monophyly of Discosea; apparent topological differences from our better resolved trees (notably exclusion of Himatisenidena from Discosea; 41% ‘support’) are statistically insignificant. ‘Trichosphaerium’ was with Tubulinea as in our trees, but less strongly. Consistently with our Table 2 not listing *Unda* separately, *U. shaefferi* Sawyer, 1975, Trans. Am. Micros. Soc. 94, p. 319 unambiguously nests within *Vannella*; it is just another *Vannella* (*V. schaefferi* Sawyer) Cavalier-Smith comb. n., as morphology hinted (Page, 1983).

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2016.03.023.

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