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SSU rRNA Reveals a Sequential Increase in Shell Complexity Among the Euglyphid Testate Amoebae (Rhizaria: Euglyphida)

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The existing data on the molecular phylogeny of filose testate amoebae from order Euglyphida has revealed contradictions between traditional morphological classification and SSU rRNA phylogeny and, moreover, the position of several important genera remained unknown. We therefore carried out a study aiming to fill several important gaps and better understand the relationships among the main euglyphid testate amoebae and the evolutionary steps that led to the present diversity at a higher level.
We obtained new SSU rRNA sequences from five genera and seven species. This new phylogeny obtained shows that (1) the clade formed by species of genera *Assulina* and *Placocista* branches unambiguously at the base of the subclade of Euglyphida comprising all members of the family Trinematidae and genus *Euglypha*, (2) family Trinematidae (*Trachelocorythion*, *Trinema,* and *Corythion*) branches as a sister group to genus *Euglypha*, (3) three newly sequenced *Euglypha* species (*E.* cf. *ciliata*, *E. penardi*, and *E. compressa*) form a new clade within the genus.
Since our results show that *Assulina* and *Placocista* do not belong to the Euglyphidae (unless the Trinematidae are also included in this family), we propose the creation of a new family named Assulinidae. Consequently, we give a family status to the genera *Euglypha* and (tentatively) *Scutiglypha*, which become the new family Euglyphidae.
The evolutionary pattern suggested by SSU rRNA phylogeny shows a clear tendency towards increasing morphological complexity of the shell characterised by changes in the symmetry (migration of the aperture to a ventral position and/or compression of the shell) and the appearance of specialised scales at the aperture (in families Trinematidae and Euglyphidae).

Key words: phylogeny; testate amoebae; protist; protozoa; evolution; morphology.

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Introduction

The eukaryotic super-group Rhizaria (Adl et al. 2005) is a large assemblage of morphologically and ecologically dissimilar organisms, comprising both flagellate and amoeboid forms. They can be phagotrophic, secondarily phototrophic (like the Chlorarachniophyta) or parasitic (such as the Haplosporidia and the Plasmodiophorida). The rhizarian amoeboid forms are characterised by filose pseudopodia, in contrast to the lobose pseudopodia generally found in "true amoebae" belonging to the supergroup Amoebozoa. In addition, the presence of a test is a recurring feature in rhizarians, such as the Foraminifera, Gromiida and the Euglyphida.

Extant members of the Euglyphida are typical inhabitants of soil and freshwater, being most abundant in permanently wet mosses such as *Sphagnum*. They are chiefly bacterivorous, and seem to play an important role in the food and energy turnover of terrestrial ecosystems (Schönborn 1992). Reproduction is achieved by binary fission; there is evidence that some species at least undergo meiosis and possibly gene exchange (Iudina and Sukhanova 2000; Schönborn and Peschke 1990). They are characterised by a self-secreted siliceous test, made of scales of different size and shape, which are bound together by organic cement. The morphological classification of the species and genera is based mainly on the shape and disposition of these scales.

The order Euglyphida Copeland, 1956 is divided into four families: Paulinellidae, Cyphoderiidae, Euglyphidae and Trinematidae (Meisterfeld 2002). The first two families are characterised by a rather simple scale pattern, while the latter two often show scale dimorphism, which leads to the apparition of specialised structures on the shell, such as spines and pseudostome-surrounding "teeth". Species belonging to the family Euglyphidae secrete tests with an acrostome opening, while species from family Trinematidae are characterised by a ventrally positioned pseudostome.

Molecular phylogenetic investigation on the position of euglyphid testate amoebae date back to 1995, when Bhattacharya, Helmchen and Melkonian (Bhattacharya et al. 1995) demonstrated the position of *Euglypha rotunda* and *Paulinella chromatophora* next to the Chlorachniophyta; Cavalier-Smith (1996/1997) added to this group the sarcomonad flagellates; this assemblage of morphologically diverse organisms was later emended Cercozoa (Cavalier-Smith 1998). However, although the position of the euglyphid testate amoebae in the tree of life was clarified since then, the phylogenetic relationships within this taxon remained unresolved. Wylezich et al. (2002) first studied the relationships within this group. Their study confirmed a close relationship between all sequenced euglyphid testate amoebae, the monophyly of which is also well supported by the morphology. They also showed that the different morphospecies that could be identified within genus *Euglypha* using available descriptions did not correspond to monophyletic units. This confirmed the uncertain status of many forms, as pointed out previously by Coûteaux et al. (1979) on the basis of morphology. However, the position of the other genera of euglyphids, and particularly of the family Trinematidae, still remained unresolved. The low number of sequences from genera other than *Euglypha* led to conclusions, which were in contradiction with the morphological data, such as the grouping of the divergent *Assulina* and *Trinema*. Finally, the single sequence representing family Trinematidae (*Trinema enchelys*) branched inside the Euglyphidae (Wylezich et al. 2002), which did not agree with its morphological characteristics.

To shed more light on the evolution of the euglyphid testate amoebae and the phylogenetic relationships among the different taxa, we carried out a comparison of SSU rRNA-based phylogeny with the morphological classification based on light-and scanning electron microscopy. We have analysed the most common representatives of euglyphids, which are encountered in mosses and *Sphagnum* peat bogs (Fig. 2), and we have added these new sequences to the existing database.

Results

The obtained sequences were aligned, and a phylogenetic tree was inferred using both ML and Bayesian methods; the robustness of the nodes was inferred by bootstrapping and posterior probabilities. The methods gave congruent phylogenetic trees, thus suggesting that the evolutionary relationships among investigated taxa are robust.

The two sequences from genera *Paulinella* and *Cyphoderia* branch at the base of the Euglyphida. The rest of the species appear closely related, and the addition of seven new taxa revealed the existence of three major clades; the genus *Euglypha*, the Trinematidae and a group formed

by genera *Placocista* and *Assulina* (Fig. 1). The analyses presented here suggest that the order Euglyphida is monophyletic. The group formed by *Placocista* and *Assulina* appears basal to genus *Euglypha* and to the Trinematidae, the last two appearing as sister taxa.

The species *Assulina muscorum*, *A. seminulum* and the phototrophic symbiont-bearing *Placocista spinosa* form a clade, which obtains maximal statistical support in both Bayesian and ML analyses. The two *Assulina* species branch together and *Placocista* branches at the base. Genus *Euglypha* and the Trinematidae form together a clade strongly supported by Bayesian posterior probabilities, but moderately supported by ML bootstrap values. The monophyly of genus

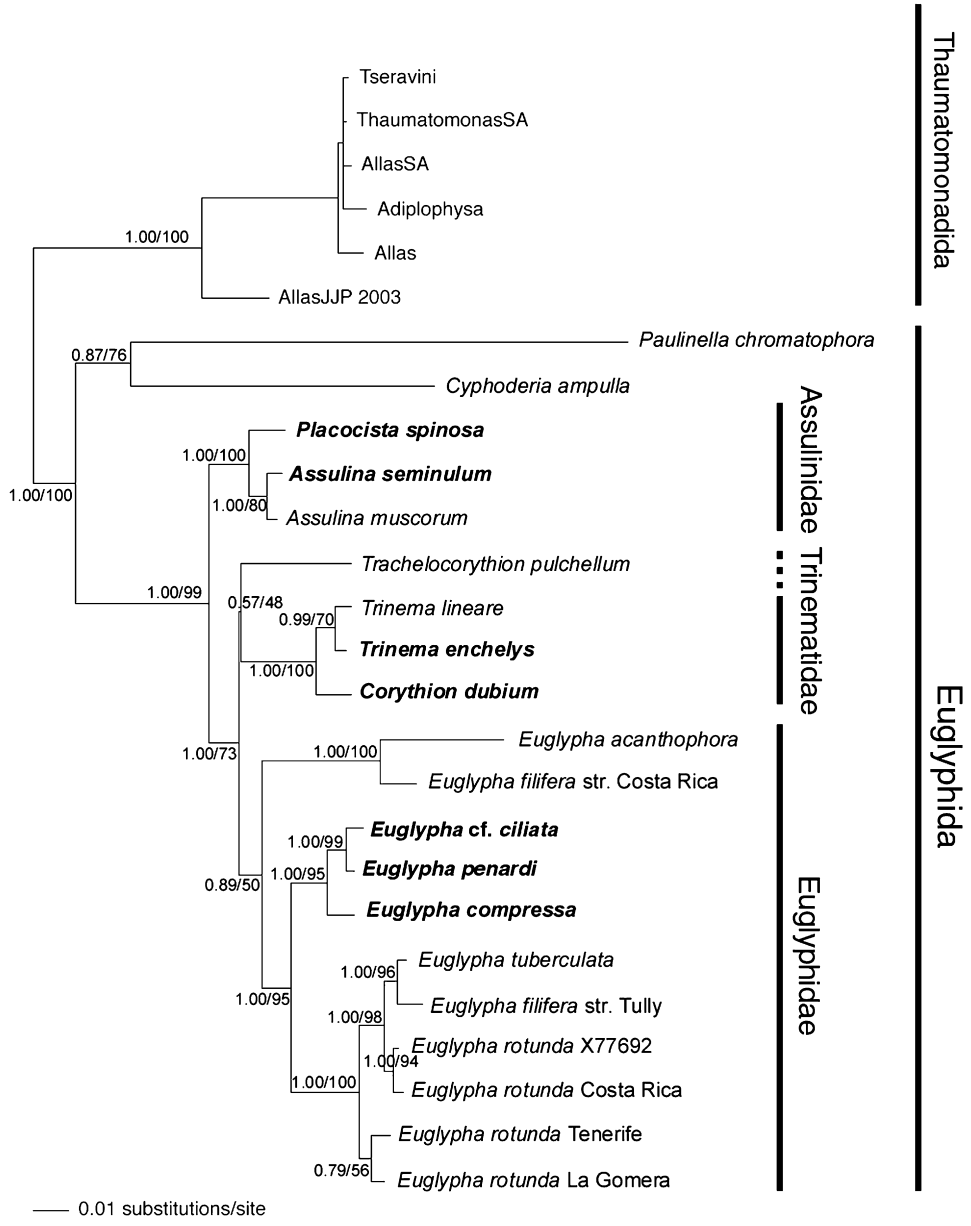

Figure 1. Maximum-likelihood tree of euglyphid testate amoebae, including all SSU rRNA gene sequences from Euglyphida present in GenBank except *Tracheleuglypha dentata*. The tree was rooted with six thaumatomonads. The numbers above the branches on each node indicate the posterior probabilities as calculated with Bayesian analyses and the bootstrap values obtained with ML. Bayesian values under 0.5 are omitted.

Euglypha is well supported by both ML and Bayesian analyses, with the exception of the taxa *E. acanthophora* and *E. "filifera"* from Costa Rica. These two long.branch sequences are linked to genus *Euglypha* taxa only topologically, with very low/no support in both Bayesian and ML analyses. The large, spiny species *E. compressa, E.*cf. *ciliata* and *E. penardi* form a strongly supported clade in all analyses, *E.* cf. *ciliata* and *E. penardi* being most closely related to each other. The small, spineless species *E. rotunda*, plus *E. tuberculata* and *E. filifera* (Australia) also form a well-supported group. The Trinematidae *Trinema lineare*, *T. enchelys* and *Corythion dubium* form a well-supported clade, with the two *Trinema* species branching together; the assignment of *Trachelocorythion pulchellum* to this group is much more dubious, since the statistical support of this phylogenetic position is much lower, in both Bayesian and ML methods (0.57/48).

Discussion

Our analysis clearly identified three groups of euglyphid testate amoebae. The first group comprises the genera *Assulina* and *Placocista*. All analyses show this group branching at the base of genus *Euglypha*/Trinematidae. This very robust clade is also supported by morphology: the strongly flattened shape of the shell characterises these two genera (Fig. 2A, B). Also, the shape of the scales is similar, strongly resembling the scales found in genus *Euglypha*. The scales are disposed in a regular, alternated pattern. No specialised apertural scales are found in members of this clade but some species in genus *Placocista* (*P. spinosa*, but also *P. jurassica*, *P. ventricosa* and *P. lapponum*, not included in this study) possess spines. Hence, we propose family status for this clade, which we name Assulinidae.

The next two clades (genus *Euglypha* and Trinematidae) are characterised by the presence of two or more different types of scales (Fig. 2D—H). The genus *Euglypha* is characterised by specialised denticulate scales surrounding the pseudostome (Fig. 2H), which are not found in any other Euglyphida (Meisterfeld 2002). While this remarkable synapomorphy supports the monophyly of the genus, the molecular phylogenetic analyses do not support the monophyly of the genus. This is likely due to the sequences of *Euglypha acanthophora* and "*Euglypha filifera*" (Costa Rica). The monophyly of the rest of the *Euglypha* sequences was very well supported in all analyses. These sequences showed also to be divergent in the analysis of Wylezich et al. (2002), their assignation to the genus based only on SSU rDNA sequences being unclear. However, we favour the hypothesis of a monophyletic genus *Euglypha*, given the consistency of the morphological criteria. In this sense, and to be consistent with the new status of the Assulinidae, we propose to give the family status to this genus, which becomes then the family Euglyphidae. Until the relationships within genus *Euglypha* are better resolved and valuable criteria for species definition in agreement with genetic data are found, the family remains monogeneric. It is, however, highly likely that the Euglyphidae will include at least a second genus, *Scutiglypha*, which is characterised by the scutiform shape of the body plates (Foissner and Schiller 2001). Further morphological and molecular work is needed to clarify this.

The three newly sequenced species, *Euglypha* cf. *ciliata*, *E. compressa* and *E. penardi* (Fig. 3), form a well-supported clade. This is also consistent with their morphology and ecology: these are three large spiny species which are often found in wet forest mosses, the larger *E. compressa* being especially abundant in wet *Sphagnum* habitats (Booth 2001; Charman and Warner 1992; Lamentowicz and Mitchell 2005; Mitchell et al. 1999; Payne et al. 2006). *Euglypha* cf. *ciliata* and *E. penardi*, branch together. This is in agreement with their morphology: Both have an oval cross section, and their test is covered with slender spines, whereas *E. compressa* has a more flattened test, angular in cross section, and has spines only on the angular edge of the shell (Fig. 2). It is interesting to note the evolutionary convergence of this species with *P. spinosa*; the spines are disposed the same way, and the compressed shape of the shell is quite similar.

On morphological grounds the family Trinematidae has been considered as separate from the Euglyphidae (Meisterfeld 2002). This is justified by the ventral, often invaginated, opening of their pseudostome (Fig. 2). The shell of the studied species is built of either two types of scales (large, round and small, elliptic) in the case of *Trinema*, or of only one type of scale (the small and elliptic type) in the case of *Corythion*. These scales are disposed in an irregular pattern. In the previous work on the phylogeny of filose testate amoebae by Wylezich et al. (2002), *Trinema* was placed inside the rest of the Euglyphidae. In the present work, the addition of two more sequences confirms that the Trinematidae are indeed closely related to the Euglyphidae, but they now are

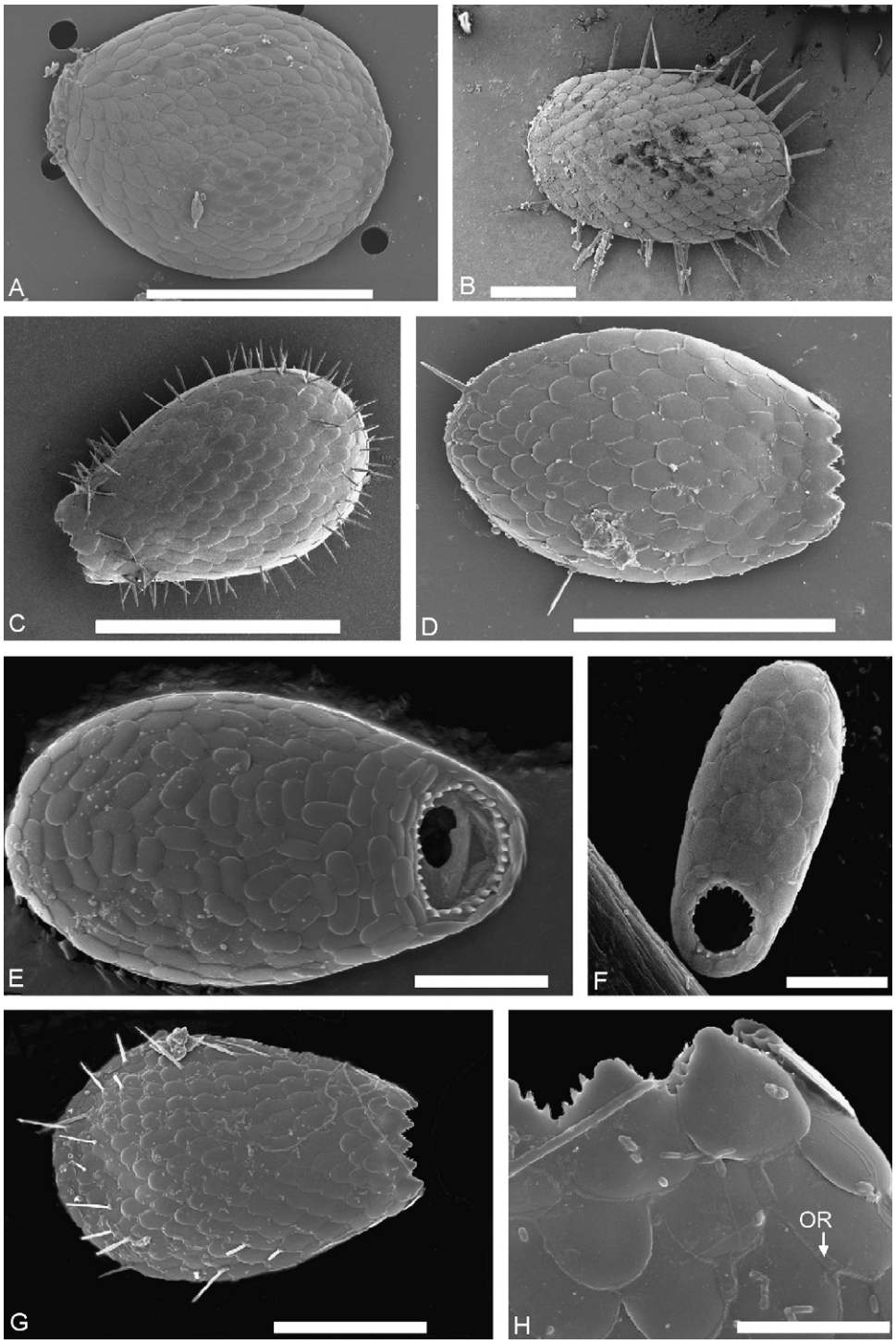

Figure 2. Scanning electron micrographs of the tests of the species studied in this manuscript. **A**: *Assulina seminulum*, **B**: *Placocista spinosa*, **C**: *Euglypha* cf. *ciliata*, **D**: *Euglypha compressa*, **E**: *Corythion dubium*, **F**: *Trinema lineare*, **G**: *Euglypha penardi*, **H**: *E. penardi*, detail of the pseudostome, showing the organic rin between the scales, characteristic of that species (OR). Scale bars represent 50 μm in all pictures excepted pictures E, F and H (10 μm).

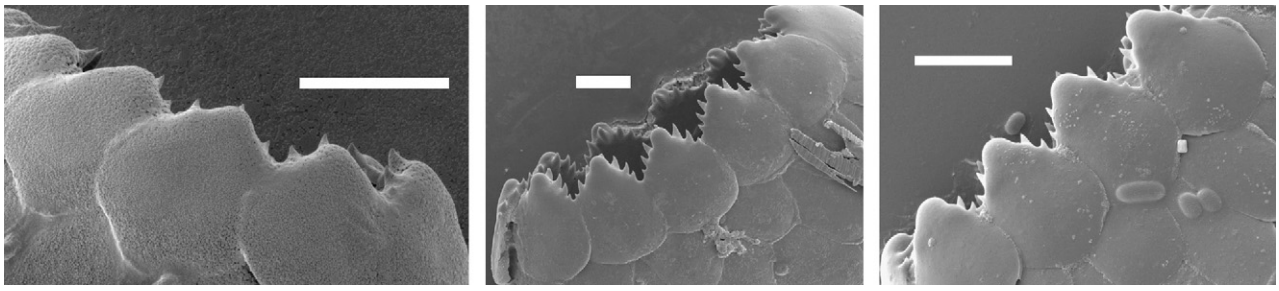

Figure 3. Scanning electron micrographs showing the denticulate plates around the pseudostome of the *Euglypha* species studied in this manuscript. **A**: *Euglypha* cf. *ciliata*, **B**: *E. penardi*, **C**: *E. compressa*. Scale bars represent 5 μm.

placed as a sister taxon to the family Euglyphidae. The three sequences of Trinematidae form a monophyletic, robust clade. In contrast, the affiliation of *Trachelocorythion pulchellum* to this group is still uncertain. From a morphological point of view, the slightly ventral position of a pseudostome surrounded by smaller scales, which may be interpreted as a transition towards the specialised "teeth" in *Corythion* and *Trinema*, could justify the assignation of this species to the family Trinematidae. However, the scales are placed in a regular alternate pattern as found in the Assulinidae and the Euglyphidae; in that sense, the morphological data place *T. pulchellum* as a transitional form prefiguring the family Trinematidae. In its first description, Penard (1890) included it in the genus *Corythion*. Later, Bonnet (1979) transferred it to a new monospecific genus, *Trachelocorythion*, based on the type of scales that resemble those of *Euglypha* spp., and the *Assulina-Placocista* clade, and the absence of denticulate mouth plates. Meisterfeld (2002) placed *T. pulchellum* in Euglyphidae (sensu lato), separately from Trinematidae. In this paper, the phylogenetic analysis joins *T. pulchellum* with the Trinematidae, although with a weak support. Hopefully, addition of new sequences will clarify its affiliation and confirm this view.

Besides the mentioned species, the fast-evolving *Tracheleuglypha dentata* was excluded from our analysis because it perturbed the analyses, resulting in a long branch and nodes with a low robustness. In the analyses in which *T. dentata* was included, its position was at the base of the clade formed by Trinematidae and Euglyphidae. This derived position would be in contradiction with the simple structure of its test, which only has one type of scales, a circular cross-section, and a terminal aperture. We interpret this position as a long-branch attraction artefact. Its exact position on the tree will probably be further clarified when sequences from morphologically similar species are added, as for instance *Pareuglypha reticulata,* which has only one type of scales and a circular cross section.

These new results based on 20 SSU rRNA gene sequences from 16 morpho-species lead us to propose an evolutionary scenario for the euglyphid testate amoebae. The ellipsoid scales disposed on a regular alternate pattern of the Assulinidae seem to be the most ancient character, the presence of polymorphic scales being a derived character. From this ancestral state appeared the specialised denticulated pseudostome plates (Euglyphidae) and the polymorphic plates of the Trinematidae. According to this interpretation, the evolution of the Euglyphida is characterised by an increasing shell complexity. The ancestral euglyphid had most likely a terminal pseudostome. In these analyses, it is clear that the ventral opening evolved from symmetric ancestors, as family Trinematidae diverged from inside the Euglyphidae. As in the case of the lobose testate amoebae Arcellinida, the ventral aperture represents an adaptation to dryer conditions which allowed the colonisation of terrestrial environments (Bonnet 1964), as these species are typically found in forest mosses and other less predictable environments (Nikolaev et al. 2005).

Based on this scenario, we can hypothesize the position of some taxa from which sequences are not available at the moment. For instance, *Playfairina* presents the particularities of a typical Trinematidae, while *Dehvarengia* and *Sphenoderia* share some characteristics with *Trachelocorythion* (one type of body scales in alternated position, smaller specialised scales around the pseudostome); sequences from these last two species could help clarify the position of *T. pulchellum*. The position of *Pileolus* is less clear, because of the

shape of its scales is quite atypical, and its affiliation to Trinematidae remains to be demonstrated.

Taxonomic summary

Eukaryota
Rhizaria Cavalier-Smith, 2002
Cercozoa Cavalier-Smith, 1998 emend. Adl, 2005
Silicofilosea Adl, 2005
Euglyphida Copeland, 1956 emend. Cavalier-Smith, 1997

Assulinidae fam. nov.

Definition: testate amoebae with an acrostome test that is composed of elliptic or round plates which are disposed in a regular, alternate pattern. The test is strongly compressed, and the pseudostome is surrounded by a thin organic rim. No specialised type of scales around the pseudostome.

Genera: *Assulina*, *Placocista*
Euglyphidae Wallich, 1864 emend. Lara et al., 2006

Definition: testate amoebae with an acrostome test composed of elliptic, sub-rectangular, scutiform, or almost round body plates which are disposed in a regular, alternate pattern. The pseudostome is surrounded by denticulate plates.

Genera: *Euglypha*, *Scutiglypha?*

Methods

Cell sorting and DNA extraction: For each species, between five and 20 individuals were extracted from moss samples and picked individually under the dissecting microscope using small diameter pipettes. The amoebae were washed and filtered in clean tap water. DNA was extracted using a guanidine thiocyanate protocol (Chomczynski and Sacchi 1987). The sequence of *Trinema lineare* was obtained from a culture (RM; the organism is available upon request). The complete list of taxa studied and sampling locations is given in Table 1.

PCR protocol and sequencing: SSU sequences were obtained in two steps, using for the first half of the SSU gene two group specific primers (euglyph1F and cercoR), and for the second half a combination of a group specific primer (euglyph 2F) and a domain specific

Table 1. Taxa studied, origin of the samples and shell measurements.

Taxon	Sampling location	Coordinates		Altitude (m a.s.l.)	Shell dimensions ±SD (µm)		number of shells measured
Euglypha cf. *ciliata*	*Sphagnum*, La Chaux d'Abel Peatland (CH)	47°10′ N	06°56′ E	1006	54±2	31±2	12
Euglypha penardi	*Sphagnum* Ryggmossen Peatland (SW)	60°00′ N	17°15′ E	58	120±5	84±4	15
Euglypha compressa	*Sphagnum*, La Chaux d'Abel Peatland (CH)	47°10′ N	06°56′ E	1006	78±3	48±2	13
Trinema lineare	Moss on a tree, Sieben Quellen, Aachen (D)	50°46′ N	06°01′ E	207	29.7±0.7	12.8±0.7	32
Corythion dubium	*Sphagnum*, Ponts-de-Martel (CH)	47°00′ N	06°44′ E	1035	41±5	26±2	9
Assulina seminulum	*Sphagnum*, Hummock, Holmegaard Mose (DK)	55°16′ N	10°37′ E	87	77±4	64±4	10
Placocista spinosa	*Sphagnum*, Pacific Rim National Park (CA)	48°38′ N	124°46′ W	2	129±11	89±9	10

Table 2. List of the primers used in this study.

Name	Sequence 5′—3′	Specificity	Location (on *E. rotunda* X77692)
euglyph1F	ACATATGCTTGTCTCAAAGACTAAG	Euglyphidae+Trinematidae	23
euglyph2F	TATACCGACTMAGGATCAGTG	Euglyphidae+Trinematidae, except *Tracheleuglypha dentata*	1045
cercoR	GGTCGAGGTCTCGTTCGTTAACGG	Most cercozoa	1331
Euk3b'	ATCCTTCYGCAGGTTCAC	Most eukaryotes	1793

eukaryotic primer (Euk3b'). The sequences of these primers as well as their specificity are given in Table 2. The reason for this indirect protocol is that it is virtually impossible to remove eukaryotic contaminants (mainly fungi) by washing the cells, owing to undigested preys, epibionts and also the presence of symbionts in *Placocista spinosa*. Both first and second parts of the SSU gene were amplified using the following PCR protocol: a first denaturation at 95 °C (5 min), followed by 35 cycles of 94 °C for 45 s, 58 °C for 60 s and 72 °C for 90 s; final extension at 72 °C for 10 min. The PCR products where purified with the Nucleo-Fast®96 PCR Clean Up kit from Macherey-Nagel (Düren, Germany) and sequenced at MWG Biotech (Martinsried, Germany) and Fraunhofer IME (Aachen, Germany). The sequencing primers were the same as the ones used for PCR.

Phylogenetic analysis: The SSU rRNA gene sequences obtained in this study were aligned manually using the BioEdit software (Hall 1999). Other sequences belonging to the Silicofilosea were introduced into the analysis; the Thaumatomonadida being used as outgroups in the phylogenetic analyses. This order is considered as the sister-group of the Euglyphida (Adl et al. 2005). The highly divergent sequence *Tracheleuglypha dentata* was not taken into account in the analysis because the branch it formed with rest of the Euglyphida was significantly longer than the other branches and perturbed the analyses; the topology of the tree was identical, but nodes were less supported; *T. dentata* appeared at the base of the group formed by Trinematidae and *Euglypha* (data not shown). A total of 1613 positions were kept for phylogenetic analyses. The alignment is available from the authors upon request. A maximum likelihood tree was built using the program PAUP* 4.0 b10 (Swofford 1998). The best likelihood model was inferred using the software Modeltest v3.06 (Posada and Crandall 1998). The model chosen was the TIM+G+I. A total of 1000 bootstrap replicates were run under this model.

In addition, a Bayesian analysis was achieved using the software MBayes v. 3.1.2 (Huelsenbeck and Ronquist 2001). Four simultaneous chains were run for 1,000,000 generations, and 10,000 trees were sampled, 50 of which were discarded as the burn-in. The model chosen here was the GTR model of substitution, with five rate categories (Lanave et al. 1984; Rodriguez et al. 1990), the number of invariable sites being estimated, and a gamma-shaped distribution of variable sites. Posterior probabilities at all nodes were estimated from the 9950 remaining trees. The tree obtained with maximum likelihood and the one obtained with Bayesian analysis had the same topology (see Fig. 1)

SEM microscopy observations: Tests were fixed in 6% glutaraldehyde before dehydration. The tests were then rinsed with demineralised water, 70% ethanol and finally with 95% ethanol. Tests were then kept 1 week in a desiccator. The samples were coated with gold in a Bal-Tec SCD005 sputter, and also in a Bio Rad Polaron division SEM Coating system A5400. Samples were observed alternatively in a PHILIPS ESEM XL40 microscope at a tension of 10 kV and in a XL30 FEG at a voltage of 5 kV.

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