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Sphenoderiidae (fam. nov.), a New Clade of Euglyphid Testate Amoebae Characterized by Small, Round Scales Surrounding the Aperture

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Euglyphid testate amoebae are a highly conspicuous group of Cercozoa whose systematics is based mainly on the shape and ultrastructure of the shell. However, only a couple of species have been studied with molecular methods. As a consequence, there are still some genera whose classification remains uncertain. Amongst those are *Sphenoderia* and *Trachelocorythion*, two genera with diverging ecological requirements that share a collar composed of small scales around the aperture. We demonstrate here with a molecular and morphological approach that they are closely related, and propose a new family, Sphenoderiidae fam. nov. to group these species. Some species share almost similar morphology in spite of being genetically distantly related (*Sphenoderia minuta* and *S. pseudominuta* sp. nov.), underlining the importance of combining ultrastructural and morphological data when describing new species of protists. In addition, we describe here *Sphenoderia valdiviana* sp. nov., a new species isolated from Southern Chile temperate rainforests.

Key words: Euglyphida; Rhizaria; Cercozoa; phylogeny; cryptic diversity; ultrastructure.

Introduction

Euglyphid filose testate amoebae are a group that is most common in soil litter and mosses, but that can also be encountered in various freshwater and marine habitats (Meisterfeld 2002). They have also conquered relatively extreme environments, such as the thin water layer covering the ice on certain Chilean glaciers (Santibanez et al. 2011). Although their fossil record is relatively poor in compari-son to other protists with mineral shells, there is unambiguous evidence that the main extant gen-era were already present since the beginning of the Neogene (Boeuf and Gilbert 1997; Foissner and Schiller 2001; Frenquelli 1933); earlier fossils have been dated back to the Permian (Kumar et al. 2011), although the exact affiliation of these early forms remains dubious. Some Precambrian micro-fossils also show resemblances to extant forms

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(Porter and Knoll 2000; Porter et al. 2003). Meiotic processes and nuclear fusion have been documented in many forms, and it is assumed that some are obligate sexual organisms (Lahr et al. 2011). Most species typically harbour an ornamented shell composed of self-secreted siliceous plates that are held together by organic cement. Together with the Arcellinida (i.e. lobose testate amoebae), Euglyphida are often used for bioindication of environmental conditions such as pH and water-table depth in peat bogs (Charman and Warner 1992; Tolonen 1986; Warner 1987), and also to measure human impact on the environment (Nguyen-Viet et al. 2007). As the shells of some species (such as *Assulina muscorum* and *A. seminulum*) are preserved in peat, they have been used successfully as bioindicators in palaeoecology, and contributed to the reconstruction of ancient climates (Mitchell et al. 2008). Their ability for fast colonisation of new habitats and their large distribution areas (Lara et al. 2011) further validate their use in bioindication. Recently, they have been shown to play a major environmental role, as it has been demonstrated that they are responsible for the biomineralisation of half of total silica present in soils, roughly as much as all vascular plants together (Aoki et al. 2007; Wilkinson 2008).

The systematics of euglyphid testate amoebae was traditionally based on overall shell morphology (Meisterfeld 2002). The introduction of molecu-lar approaches and more precisely sequencing of the gene coding for the RNA present in the small subunit of the ribosome (SSU rRNA) placed these organisms within the Cercozoa, together with many amoeboflagellates, as well as the photo-synthetic chlorarachniophytes (Bhattacharya et al. 1995) and the equally silicified thaumatomonads (Cavalier-Smith and Chao 1997) and phaeodare-ans (Polet et al. 2004); their morphological similarity with lobose testate amoebae appeared to be the result of convergent evolution. Their mono-phyly has been demonstrated also with SSU rRNA gene sequences (Wylezich et al. 2002). Later, the relationships within euglyphid taxa were also determined using this molecular marker and led to the definition of five families, namely Cyphoderiidae, Paulinellidae, Assulinidae, Trinematidae and Euglyphidae. It appeared that the scaling pattern of the shells was the morphological criterion that allowed a classification that suited best the phylogenetic tree of the euglyphids as obtained with molecular methods; it was suggested that scal-ing patterns tended to gain complexity as the organisms adapted to life in relatively dryer conditions (Lara et al. 2007b). Assulinidae, with only one type of scales, were suggested to be the basal-most euglyphid family that conquered terrestrial environments, Euglyphidae and Trinematidae being more derived (Lara et al. 2007b). Scale size, shape and disposition patterns revealed to be equally important for species-level discrimination, as shown within family Cyphoderiidae (Heger et al. 2010; Todorov et al. 2009).

However, several genera still remain unclassified within euglyphids. *Trachelocorythion pulchellum*, initially described as *Corythion pulchellum* (Trine-matidae; Penard 1890) was subsequently removed from that family and placed in a new, monospecific genus due to its small scales around the shell aper-ture, which is slightly subterminal (Bonnet 1979). Molecular data did not confirm its placement within Trinematidae, nor did it contradict it, because the support for the group formed with the other Trine-matidae was weak (Lara et al. 2007b). However, as Bonnet created a new genus to accommodate *C. pulchellum*, he noticed the resemblance between this species and genus *Sphenoderia*, which dif-fered only by its round section and its asymmetrical pseudostome. Following this intuition, we gathered specimens from genus *Sphenoderia*, sequenced their SSU rRNA gene and documented them with light and electron microscopy. In the light of these results, we propose a new hierarchical classifica-tion of the Euglyphida based on these results.

Results

We obtained sequences of the SSU rRNA gene between 1600 and 1800 base pairs long; we did not detect large sized (i.e. over 10 base pairs) transcribed insertions or introns. All sequences obtained from the five picked clones per PCR product were exactly identical after careful examination of the pherograms. We therefore did not detect any hidden diversity within a sample.

In the first analysis, each Euglyphida fam-ily (Trinematidae, Assulinidae, Cyphoderiidae and Paulinellidae) as defined in (Lara et al. 2007a) was robustly supported, with the exception of family Euglyphidae whose monophyly was not supported. Our six sequences from genus *Sphen-oderia* branched together with *Trachelocorythion pulchellum* (AJ418789) with a 98% bootstrap value and 1.00 posterior probability; we called this new clade family Sphenoderiidae. The new family branched together with Trinematidae, Euglyphidae and Assulinidae in a robust clade, 88% bootstrap value and 0.95 posterior probability (Fig. 3). A basal position of Assulinidae was supported with

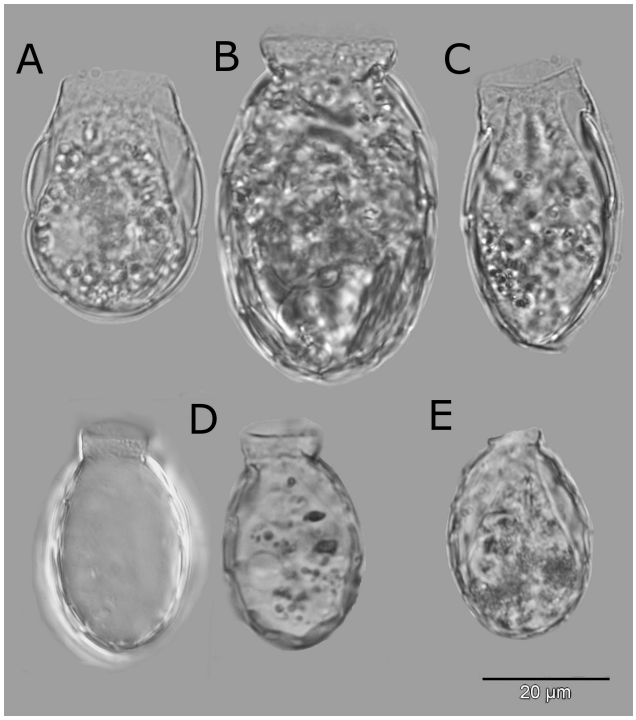

Figure 1. Light micrographs illustrating the five species studied in this manuscript: **A**. *Sphenoderia macrolepis* **B**. *S. lenta* **C**. *S. valdiviana* **D**. *S. pseudominuta* **E**. *S. minuta*. All micrographs are at the same scale.

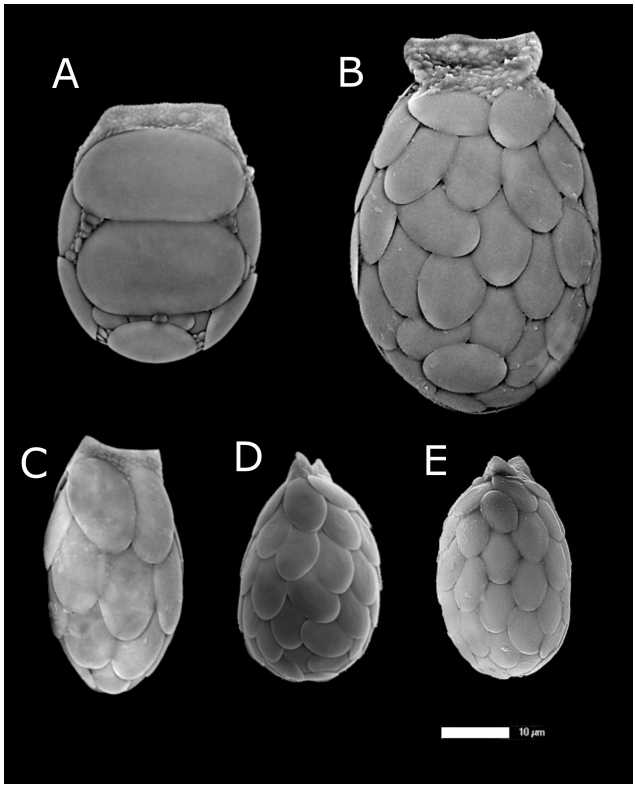

Figure 2. Scanning electron micrographs of the species studied in this manuscript: **A**. *Sphenoderia macrolepis* **B**. *S. lenta* **C**. *S. valdiviana* **D**. *S. pseudominuta* and **E**. *S. minuta*. All micrographs are at the same scale.

Bayesian analysis (posterior probability 0.94), but only weakly with maximum likelihood.

In the second analysis with a smaller dataset (Fig. 4), the monophyly of Euglyphidae is robustly recovered (bootstrap value 72%; posterior probability 0.99). Within family Sphenoderiidae, two well supported groups appeared, comprising for the first *S. macrolepis*, *S. valdiviana* and the two isolates of *S. minuta* from Belgium (bootstrap value 70%, posterior probability: 0.96) and *S. lenta* and *S. min-uta* from Spain for the second (bootstrap value 98%, posterior probability: 1.00). The exact position of *Trachelocorythion pulchellum* within the family remained uncertain.

Light and electron micrographs revealed a large variability in scaling pattern, with some forms showing extreme scale polymorphism (such as *S. macrolepis* and *S. valdiviana*) and others with regular scaling patterns such as *S. minuta* and *S. lenta* (Fig. 2). Regular-scaled and strongly divergently scaled forms were intermixed in the Sphenoderi-idae. In contrast, sequences from cells identified morphologically as *S. minuta* were genetically dis-tantly related. A closer examination of *S. minuta* from Spain and Belgium revealed differences in the shape of the scales of the two forms, the isolates from Belgium having slightly but significantly more elongated shell scales (see Table 2). In addition, the Belgian form had visible protruding collar scales, which were not visible (maybe absent) from the Spanish isolates.. As the rounded shell scales of the Spanish isolate corresponded better to the original drawing of Deflandre (Deflandre 1931) we decided to keep the original name for this form and to call the Belgian isolates *S. pseudominuta* as a reference to the very slight morphological differ-ences that discriminate this form and the nominal *S. minuta*.

Discussion

Because the different *Sphenoderia* species and *Trachelocorythion pulchellum* branched together robustly, and also because of the presence of typical round and very small scales around the pseudostome in both taxa (as pointed out by

Table 1. Description of the samples from which the studied species derived, with geographical localisation and type of sample.

Species	Sampling site	Country	Coordinates	Sample type
Sphenoderia minuta	Sierra do Xistral	Spain	N 042° 52′ 22″; W 006° 49′ 37″	*Sphagnum fallax*
Sphenoderia pseudominuta	Venn auf Hochscheit	Belgium	N 050° 36′ 60″; E 006° 13′ 23″	*Sphagnum* sp. (fen)
Sphenoderia lenta	Ebano Verde National Park	Dominican Republic	N 019° 02′ 22″; W 070° 31′ 04″	Mixed broadleaf litter
Sphenoderia macrolepis	Sierra do Xistral	Spain	N 042° 52′ 22″; W 006° 49′ 37″	*Sphagnum pylaesi*
Sphenoderia valdiviana	Monumento Nacional Alerce Costero	Chile	S 040° 12′ 47″; W 073° 23′ 12″	Mixed broadleaf litter

Meisterfeld 2002), we decided to unite them in a new family, the Sphenoderiidae.

As expected, the family branched within the strongly supported clade that comprised fami-lies Assulinidae, Trinematidae and Euglyphidae. This group is most common in habitats such as forest litter and bryophytes, and although some species are strictly aquatic, like *Euglypha acan-thophora* (Bobrov et al. 2010), most species have adapted to terrestrial habitats. It has been hypothe-sized that this group made the transition towards drier environments by increasing the complexity of the scaling pattern (Lara et al. 2007b). These authors hypothesized that *Trachelocorythion pul-chellum* might be a transition form between the mostly monomorphically scaled Assulinidae (with the exception of spines in genus *Placocista*) and the Trinematidae with their specialised denticu-lated pseudostome scales, because of its slightly lateral opening surrounded by smaller and differ-ently shaped scales that prefigure more specialised structures. In our new analysis, the Sphenoderi-idae, with their varying scale shape and size, appear also derived with respect to Assulinidae, although this position is not robustly supported (Fig. 3). In contrast, the position of the aperture varies in Sphenoderiidae from terminal (i.e. genus *Sphenoderia*) to slightly subterminal (*Trachelocory-thion*). If we admit that the newly described species *Deharvengia japonica* belongs indeed to family Assulinidae as suggested in Bobrov et al. (2012), this would be a second case where aperture posi-tion can vary within a family (other genera, i.e. *Placocista* and *Assulina* have terminal apertures). Therefore, this criterion would be of lower hierarchi-cal level than the complexity of scaling pattern.

Within genus *Sphenoderia*, however, the shape and size of the scales can be highly variable. For instance, *S. macrolepis*, a species with very large scales branches closely to both isolates of *S. pseudominuta*, with their regular body scales. As regularly and strongly dimorphically scaled forms appear intermixed in the Sphenoderiidae tree (*Tra-chelocorythion pulchellum* has a regular scaling pattern), we can postulate that it is a fast-evolving trait in the family. Conversely, *S. minuta* and *S. pseudominuta* are two species that are genetically very distinct but whose scaling patterns have under-gone a strong evolutionary convergence. It is a clear case of pseudocryptic diversity (i.e. when two genetically distinct species can be only discrim-inated by examining their ultrastructure). Similar cases are well known in other scale bearing pro-tists such as prymnesiophytes (Medlin and Zingone 2007), diatoms (Kooistra et al. 2010; Poulickova

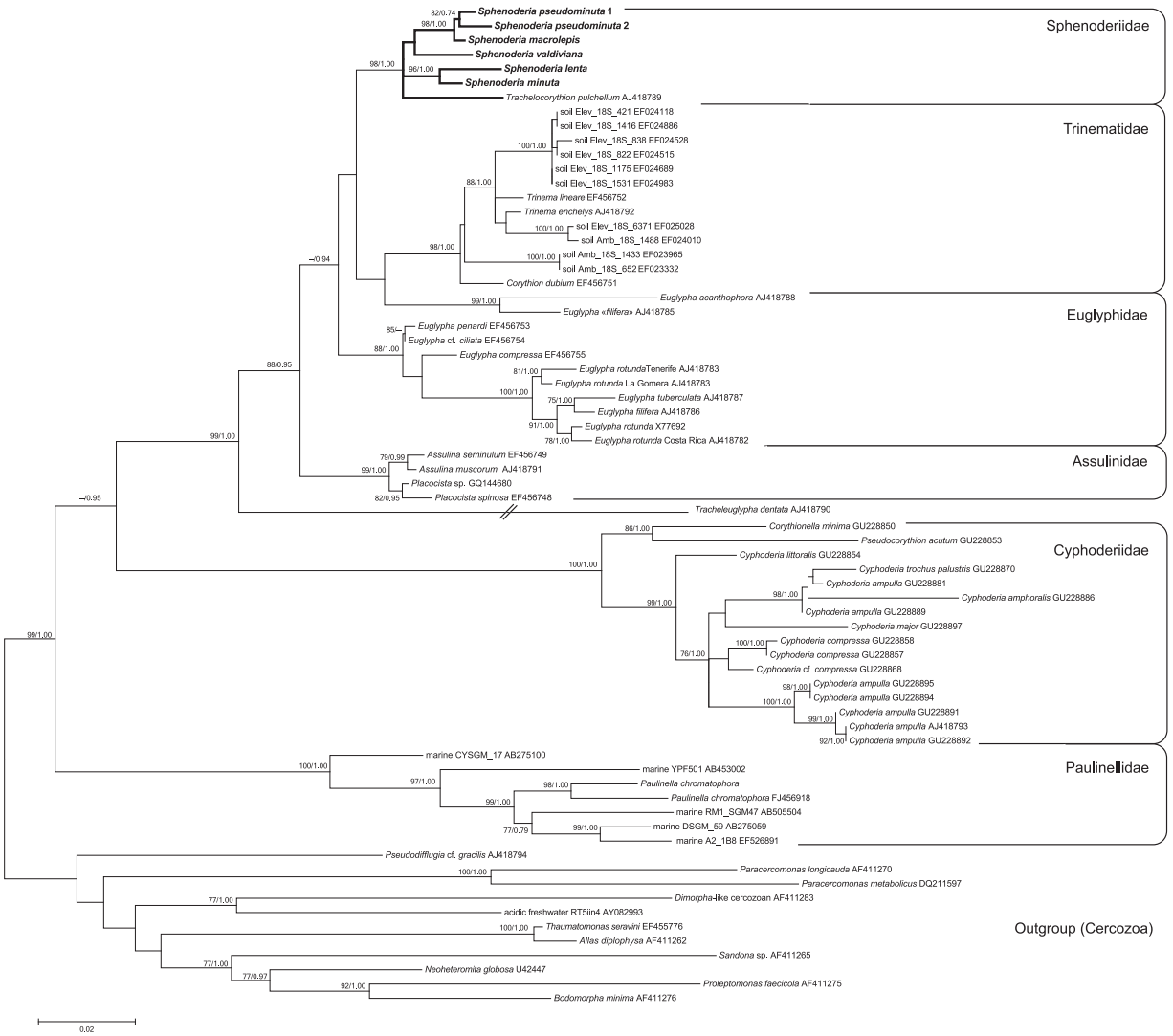

Figure 3. Maximum likelihood tree representing the phylogenetic position of family Sphenoderiidae with respect to other euglyphid families. Numbers at the nodes indicate respectively maximum likelihood bootstrap supports and posterior probabilities as calculated using a Bayesian approach. Values below, respectively, 70 and 0.7 were not indicated. A selection of non-euglyphid cercozoa was used to root the tree. A double bar symbol at the base of the branch leading to *Tracheleuglypha dentata* indicates that branch length has been reduced by half for clarity reasons.

et al. 2010), and, also, other euglyphids (Heger et al. 2010). Therefore, a careful examination of the ultrastructure of euglyphid testate amoebae shells is necessary to identify individuals to the species level.

As most testate amoebae (and not only euglyphids but also arcellinids (see Kosakyan et al. 2012) have been described solely by light microscopy, it is very likely that many (pseudo)cryptic species have been overlooked in earlier research; therefore, our estimations of their diversity will certainly have to be upscaled. For instance, the early description of *Sphenoderia fissirostris* by Penard (Penard 1890) depicted a figure where four scales start at the same point on the test, and a long collar. Later, a scanning electron microg-raphy by Ogden (Ogden 1984) revealed a shell with only two scales in the first row and two others start-ing slightly shifted backwards, and a short collar. These cells, collected in Great Britain, correspond exactly to our *Sphenoderia valdiviana* (Fig. 2), and because some traits such as the long collar appear unmistakable, we posit that they are clearly different from the ones described in Penard's early work.

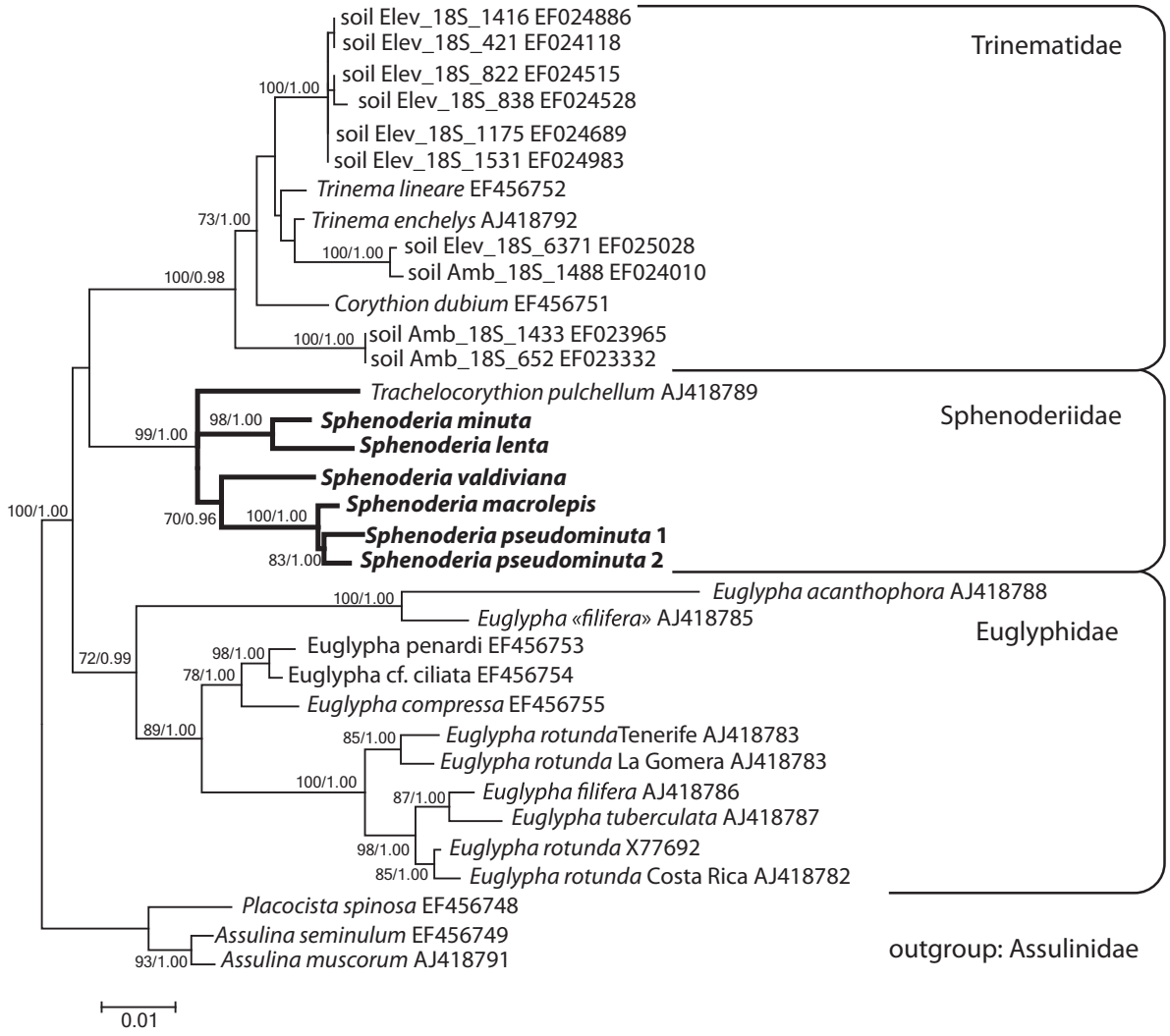

Figure 4. Maximum likelihood tree representing the families Assulinidae, Euglyphidae, Trinematidae and Sphenoderiidae. Numbers at the nodes indicate respectively maximum likelihood bootstrap supports and posterior probabilities as calculated using a Bayesian approach. Values below, respectively, 70 and 0.7 were not indicated. The tree was rooted with Assulinidae.

As a consequence, family Sphenoderiidae hosts now two genera, *Sphenoderia* and *Trachelocorythion*. Our phylogenetic analyses did not allow determining whether *Trachelocorythion pulchellum* was basal to the rest of genus *Sphenoderia*, or if it should be included within; until more data are made available, we favour the separation of genera, as the ecological niches of *Sphenoderia* and *Trachelocorythion* are quite distinct. Indeed, the first genus is typical for wet habitats, such as *Sphag-num* bogs and fens were they can be found in microhabitats with a low water table, and with pH ranging from very acidic to neutral (Bonnet 1991; Charman and Warner 1997; Mitchell 1999; Mitchell et al. 2000; Payne and Mitchell 2007), and also in strictly aquatic systems (Schönborn 1966). Our specimen of *Sphenoderia lenta* from the Dominican Republic was encountered in forest litter, but the very high humidity conditions (tropical cloud forest) probably made its settlement possible. This species has been also found under similar conditions in the Ecuadorian Andes, where the forest litter testate amoeba communities were associated to semi-aquatic habitats (Krashevska et al. 2007). In contrast, *Trachelocorythion pulchellum* can be found in much drier habitats, such as skeletal soil, alpine and subalpine meadows (Bonnet 1960) and drier forest litter (Bonnet 1991), but tolerates also wet conditions such as *Sphagnum* mosses (Penard 1902). The shell presents characteristics

that have been interpreted as adaptations to drier environments, such as a lateral location of the pseudostome (Bonnet 1964) and a compressed shell. Therefore, it can be hypothesised that genus *Trachelocorythion* separated from *Sphenoderia* based on a diverging ecological niche.

Taxonomic Rearrangements

Because euglyphids were historically classified as Rhizopoda and therefore ruled by the International Code for Zoological Nomenclature (ICZN), we followed this principle here, as recommended in Lahr et al. (2012).

Sphenoderiidae fam. nov.

Testate amoeboid organisms with a test covered with self-secreted circular to elliptical silica scales that can be of different sizes and shapes, but without indentations. The pseudostome is surrounded with small round or oval scales. Because usually one side ("ventral") of the aperture is shorter the opening lies subterminal. Family Sphenoderiidae includes two genera: *Sphenoderia* and *Trachelocorythion*. Type genus: *Sphenoderia*.

- *Sphenoderia* Schlumberger, 1845
  Scales of one or more type s on the core of the shell, pseudostome slit-like, mostly surrounded by a collar that comprises small scales that can be sometimes invaginated (*S. sphaerica*). Round or oval cross section (e.g. *S. compressa, S. labiata*). Occurs from aquatic (*S. compressa, S. lenta* var. *longicollis*) to litter and moss environments. Type species: *Sphenoderia lenta*. Other species: *S. splendida, S. foveosa, S. australis, S. longicollis, S. minuta*, *S. labiata, S. macrolepis, S.ovoidea, S. compressa*, *S. sphaerica, S. fissirostris, S. valdiviana* (spec. nov.) and *S. pseudominuta* (spec. nov.).
- *Trachelocorythion* Bonnet 1979
  Scales of regular size and shape on the core of the shell, upper lip of the pseudostome larger than the lower resulting in a slightly subterminal opening, no collar. Flattened cross section. Occurs from dry soils to forest litter and *Sphagnum* mosses. Type species: *Trachelocorythion pulchellum*. Other species: only type species described

Description of two new species, *Sphenoderia valdiviana* spec. nov. and *Sphenoderia pseudominuta* spec. nov.

New species description:

Sphenoderia valdiviana Chatelain, Meisterfeld, Roussel-Delif and Lara

Diagnosis:

- Description: Test acrostome, colourless, fusiform, including different-sized ovoid plates. The largest scales measure about 40% of the total shell length. Scales around the collar terminated in two different levels, four scales protruding giving the appearance of a notch.
- The two species *S. fissirostris* and *S. ovoidea* have a similar morphology. However, both can be differentiated by the scales on the first row placed at the same level (in *S. valdiviana*, scales are inserted at two different levels, see Figure 2). Moreover, *S. fissirotris* has a longer collar and *S. ovoidea* has a more rounded test and smaller scales.
- Shell dimensions (based on 17 individuals): length: average 42.4 μm, SD = 2.3, width: average 23.2 μm, SD = 1.0, length of the collar: average 4.8 μm, SD = 0.6, width of the collar (inner): average 14.7 μm, SD = 1.3, width of the collar (outer): 13.7 μm, SD = 1.1.

Scale dimension: 16.3 μm, SD = 0.8; 10.2 μm, SD = 0.4 (N = 12)

Type material: Organisms were collected in *Sphagnum magellanicum* hummock found growing in valdivian temperate rainforest in the Monumento Nacional Alerce Costero, dominated by the endangered coniferous species *Fitzroya cupressoides* (40° 12′ 47″ S, 73° 23′ 12″ W). Dry moss samples containing this species are deposited in the sample collection of the Laboratory of Soil Biology, University of Neuchâtel, Switzerland (code: EM 1450). SSU rRNA gene sequence was deposited in Genbank with accession number KF539411. One SEM stub with several specimens has been deposited at the Oberösterreichisches Landesmuseum in Linz.

Etymology: the epithet refers both to the city of Valdivia (Chile, Región de los Lagos) where the type specimens from this species were described and to the particular biome where it originated, the Valdivian temperate rainforests.

Sphenoderia pseudominuta

Diagnosis:

- Description: Test acrostome, colourless, rounded, with similar sized ovoid plates. Collar

scales well-visible under scanning electron microscopy and high power LM (Fig. 1). The nucleus is vesicular.
- Very similar to *Sphenoderia minuta*, excepted that the scales are slightly smaller and less elongated (see Table 2 and Fig. 2)
- Shell dimensions (based on 32 individuals): length: average 33.6 μm, SD = 1.8, width: average 21.1 μm, SD = 1.4, length of the collar: average 4.2 μm, SD = 0.6, width of the collar (inner): average 10.1 μm, SD = 0.8, width of the collar (outer): 9.8 μm, SD = 0.9.

Scale dimensions: 9.3 μm length (SD = 0.5) and 6.2 μm width SD = 0.6), as measured over 16 scales from 9 individuals.

Type material: Specimens were isolated from *Sphagnum* collected at the bank of a drainage ditch close to Fringshaus on the Belgian side of the Venn auf Hochscheit 50°36′59.94″N 6°13′23.08″E. SSU rRNA gene sequence was deposited in Genbank with accession number KF539407-8. A permanent mount has been deposited at the Oberösterreichisches Landesmuseum in Linz.

Etymology: "pseudo (Gk. false); minuta (Lt. small) in reference to a strong and misleading similarity to *S. minuta*, despite being genetically distinct".

Methods

Microscopic observations: The following species were docu-mented: *Sphenoderia lenta, S. minuta* (three different isolates, one from Northern Spain and two from Belgium)*, S. macrolepis* and a new, unidentified species that we described as *Sphen-oderia valdiviana* (see below). Cells were collected with a stirred Pasteur pipette under an inverted microscope and set aside for both electron and light microscopy. The origin of the sampled species and their respective habitats are summarised in Table 1. Between 15 and 32 cells were observed for morphometrical analyses under light microscopy (LM) using an Olympus IX81 (see Table 2), a Zeiss Axioskop II and a Leitz Diavert; LM pic-tures of the species are in Figure 1.

The tests were then rinsed with demineralised water, 70% ethanol and finally with 95% ethanol. Tests were then kept 1 week in a desiccator. They were coated with gold in a Bio Rad Polaron division SEM Coating system A5400 or a Hum-mer sputter coater. Samples were observed alternatively in a PHILIPS ESEM XL40 microscope at a tension of 10 kV and in a Philips SEM 525 at 15 kV; micrographs are illustrated in Figure 2.

Sampling, DNA extraction and specific amplification: Samples of soil litter, mosses and *Sphagnum* were kept at 12 °C before cells were extracted; references concerning their characteristics are given in Table 1. Living and active cells were collected and DNA was extracted as described in (Lara et al. 2007b); between 1 and 20 cells were collected for each extraction. PCR amplifications were conducted as follows: a first PCR including SSU and partial LSU rRNA genes were

Table 2. Measurements taken of different parameters of the shells of the described species. N = number of shells measured, SD = standard deviation.

Species	Shell measurments					Collar measurments					Scale measurments				
		Length		Width		Length		Width (inner)		Width (outer)		Length		Width	
	n	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	n	Mean	SD	Mean	SD
Sphenoderia minuta	15	31.8	1.5	20.4	0.9	3.5	0.5	9.9	0.6	10.0	0.7	10	7.8	0.6	5.5	0.5
Sphenoderia pseudominuta	32	33.6	1.8	21.1	0.6	4.2	0.6	10.1	0.8	9.8	0.9	16	9.3	0.5	6.2	0.6
Sphenoderia lenta	18	56.4	2.2	35.1	1.7	7.1	1.3	18.3	1.1	19.5	1.3	12	13.2	1.5	8.2	0.6
Sphenoderia macrolepis	16	39.1	0.9	28.5	0.9	5.4	0.5	18.8	0.5	15.4	0.4	15	12.9	1.0	20.7	1.3
Sphenoderia valdiviana	17	42.4	2.3	23.2	1.0	4.8	0.6	14.7	1.3	13.7	1.1	12	16.3	0.8	10.2	0.4

conducted using the following primers EK 1F (CTG GTT GAT CCT GCC AG) and EuglyLSU1R(GT TTG GCA CCT TAA CTC GCG), the latter being specifically designed for eug-lyphids based on the sequences of Wylezich et al. (2007). Cycling profile included an initial touchdown step 62 °C and 54 °C (1 °C/cycle), and a total of 30 cycles. PCR products were stored and used subsequently for a second round of (nested) PCR. This was performed with the euglyphid-specific primers EuglySSU1F (GCG TAC AGC TCA TTA TAT CAG CA) and EuglySSU2R (GCA CCA CCA CCC ATA GAA TCW AGA AAG ATC), with an annealing step at 59 °C and 30 cycles; a fragment comprising the first ca. 1200 bp of the SSU rRNA gene was amplified. In order to amplify the last part of the gene (i.e. the last 1400 bp of the SSU rRNA), a specific protocol developed using the primers spheno1F (GAY TCG CTT TGT GGT GAC TC) and the same cycling profile as for the first fragment of the gene. *For S. pseudominuta* nine cells were isolated under an inverted microscope and washed in sterile distilled water. DNA was extracted using the InstaGene Matrix BIORAD method (10 µl, 56 °C, 20 min).The SSU rRNA gene was amplified by a nested PCR using universal eukaryote specific primers as described in (Medlin et al. 1988), but except for the annealing temperature (46 °C) and the number of temperature cycles (35),The amplification products were used for a reamplification with nested primers designed for SSU rRNA of Testaceafilosia (18Si for: 5′GATCCTGCCAGTAACATATGC 3′, 18Si rev: 5′ACCTACGGAAACCTTGTTACG 3′). The PCR-product was then cloned with a StrataClone PCR Cloning Kit (Agilent Technologies, Santa Clara, CA, USA). Two clones were custom sequenced at the Fraunhofer Institute for Molecular Biology and Applied Ecology (Aachen).

All other amplicons were cloned into pCR2.1 Topo TA cloning vector (Invitrogen) and transformed into *E. coli* TOP10' One Shot cells (Invitrogen) according to the manufacturer's instructions. Up to five clones per PCR product were sequenced. Clone inserts were amplified with vector T7 and M13R primers, and inserts of the expected size were sequenced directly using a BigDye197 Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Carlsbad, CA, USA) and analysed with an ABI-3130xl DNA Sequencer (Applied Biosystems).

Sequence analysis and phylogenetic trees: SSUrRNA sequences were deposited in GenBank with the accession numbers KF539406 - KF539411. Sequences were visual-ized using the software BioEdit 7.0.9.0 (Hall 1999) aligned using the software MUSCLE v. 3.6 (Edgar 2004). We built two phylogenetic trees, the first comprising sequences from all Euglyphida families plus environmental clones and rooted with other Cercozoa sequences, and the second containing sequences from closely related families Assulinidae, Euglyphidae, Trinematidae (i.e. terrestrial euglyphids), to which we added related sequences from environmental clones searched through BLAST (Altschul et al. 1997) and the new species. While the first tree included sequences from all euglyphids (analysis performed on 1596 positions), the second one did not include long-branch sequences (i.e. *Tracheleuglypha dentata*), thus allowing the use of more positions in the variable regions of the SSU rRNA gene (1705 characters). We restricted also the analysis to members of the families Assulinidae, Trinematidae, Sphenoderiidae and Euglyphidae, and rooted the tree with the Assulinidae as they appeared basal to Trinematidae and Euglyphidae in previous work (Lara et al. 2007a).

Trees were built based on a Maximum likelihood algorithm as provided by the software MEGA v5.10 (Tamura et al. 2011). The model used was General Time Reversible with a Gamma distribution of rates among sites (4 categories). All other parameters were estimated over the duration of the search. Gaps were treated as non-existing characters. In addition, a Bayesian analysis was performed in each of the datasets (respectively, the tree with all euglyphids and the tree with only closely related families); using the software MrBayes v. 3.1.2 (Huelsenbeck and Ronquist 2001). Node robustness was evaluated by bootstrapping (1000 bootstraps). We performed two simultaneous MCMC chains, and 500,000 generations. The generations were added until standard deviation of split frequencies fell below 0.01, according to the instruction in the manual. For every 1,000th generation, the tree with the best likelihood score was saved, resulting in 10,000 trees. The burn in value was set to 25%.Topology of the consensus trees was compared with the obtained maximum likelihood trees.

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