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Molecular phylogeny of euglyphid testate amoebae (Cercozoa: Euglyphida) suggests transitions between marine supralittoral and freshwater/terrestrial environments are infrequent

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ABSTRACT

Marine and freshwater ecosystems are fundamentally different regarding many biotic and abiotic factors. The physiological adaptations required for an organism to pass the salinity barrier are considerable. Many eukaryotic lineages are restricted to either freshwater or marine environments. Molecular phylogenetic analyses generally demonstrate that freshwater species and marine species segregate into different sub-clades, indicating that transitions between these two environments occur only rarely in the course of evolution. It is, however, unclear if the transitions between freshwater and environments characterized by highly variable salinities, such as the marine supralittoral zone, are also infrequent. Here, we use testate amoebae within the Euglyphida to assess the phylogenetic interrelationships between marine supralittoral and freshwater taxa. Euglyphid testate amoebae are mainly present in freshwater habitats but also occur in marine supralittoral environments. Accordingly, we generated and analyzed partial SSU rRNA gene sequences from 49 new marine/supralittoral and freshwater Cyphoderiidae sequences, 20 sequences of the Paulinellidae, Trinematidae, Assulinidae, and Euglyphidae families as well as 21 GenBank sequences of unidentified taxa derived from environmental PCR surveys. Both the molecular and morphological data suggest that the diversity of Cyphoderiidae is strongly underestimated. The results of our phylogenetic analyses demonstrated that marine supralittoral and freshwater euglyphid testate amoeba species are segregated into distinct sub-clades, suggesting that transitions between these two habitats occurred only infrequently.

1. Introduction

The biotic and abiotic factors in marine and freshwater ecosystems differ considerably and impose physiological constraints on organisms that pass through this salinity barrier. As a consequence, the taxonomic compositions of the communities encountered in both environments are quite divergent. Some major eukaryotic lineages are restricted to either marine or freshwater environments. For example, radiolarians, echinoderms, most foraminiferans, most haptophytes, and pelagophytes are marine, whereas no representative of the Mycetozoa has ever been found in saltwater. In contrast, other eukaryote lineages occur in both marine and freshwater/terrestrial habitats. For instance, cryptophytes, diatoms and dinoflagellates are abundant in both environments. But even within these groups, phylogenetic studies have indicated a limited number of marine/freshwater transitions, suggesting that such events are rare in the evolutionary history of different lineages (von der Heyden and Cavalier-Smith, 2005; Alverson et al., 2007; Cavalier-Smith and von der Heyden, 2007; Logares et al., 2007; Shalchian-Tabrizi et al., 2008; Cavalier-Smith, 2009). Likewise, even though at the morphospecies level several microeukaryotic lineages appear to have wide salinity ranges, molecular phylogenies show that they are uncommon (Koch and Ekelund, 2005; Finlay et al., 2006; Scheckenbach et al., 2006; Bass et al., 2007).

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The order Euglyphida Copeland, 1956, is a group of testate amoebae with filamentous pseudopodia that build self-secreted silica tests. Euglyphids are currently divided into five families: the Assulinidae, Euglyphidae, Trinematidae, Paulinellidae and Cyphoderiidae (Meisterfeld, 2002; Adl et al., 2005; Lara et al., 2007). These organisms were considered as exclusive inhabitants of soil and freshwater habitats up to the second part of the 20th century. Early reports of Euglyphida from subsurface waters of the Pacific Ocean (Wailes, 1927) were interpreted as imports from continental freshwaters. Since then, Euglyphida were more intensively investigated in marine supralittoral environments and today, more than 50 species were described from the marine supralittoral of the Black Sea and other marine habitats of the World (Golemansky, 1974, 2007; Ogden and Couteaux, 1989; Chardez, 1991; Golemansky and Todorov, 1999). While the Assulinidae, Euglyphidae and Trinematidae have been found almost exclusively in terrestrial or freshwater habitats (for simplicity hereafter referred to as freshwater), the Cyphoderiidae and the Paulinellidae are found also in the marine supralittoral zone (Meisterfeld, 2002).

The marine supralittoral environment is characterized by variable salinity values, which can fluctuate relatively rapidly between typical seawater to less than 10‰ (Todorov and Golemansky, 2007; Todorov et al., 2009). Thus, organisms inhabiting such an environment must face huge selective pressure to adapt to these harsh conditions. The Cyphoderiidae are one of the few microeukaryotic groups that have successfully colonised both environments. Therefore, they represent an excellent model group to study the impact of salinity in eukaryotic cell evolution.

The current Euglyphida taxonomy is largely based on shell characters. Shells are composed of secreted plates which often differ in shape, size and arrangement among species (Meisterfeld, 2002). However, morphological data alone are often unreliable for testing hypotheses of colonization processes because such characters can be subject to convergent evolution during the marine to freshwater transition (or vice versa) (Lee and Bell, 1999).

In order to overcome these current limitations, a detailed phylogenetic study of freshwater and marine supralittoral Euglyphida, combining both molecular and morphological characters, is required. In this work, we inferred the molecular phylogenetic relationships between marine supralittoral and freshwater members of the Cyphoderiidae using SSU rRNA gene sequences and documented the morphology of isolated species with scanning electron microscopy. We hypothesised that only two separate marine and freshwater phylogenetic clades existed in the Cyphoderiidae.

2. Materials and methods

2.1. Sampling and species identifications

We sampled cyphoderiidae species from freshwater aquatic mosses and from subsurface waters of freshwater and marine sand beaches at five Bulgarian, two Canadian and three Swiss sites (Table 1). Following the most recent taxonomic revision (Chardez, 1991; Meisterfeld, 2002; Golemansky and Todorov, 2004, 2006; Todorov et al., 2009), we identified six *Cyphoderia*, one *Corythionella* and one *Pseudocorythion* morphotypes among a total of 15 populations (Table 1). The morphology of seven of these 15 populations was recently investigated by Todorov et al. (2009). This previous study revealed significant morphological differences among *Cyphoderia ampulla* populations from Moiry (CH), Rhodopes (BG) and Vitosha (BG), suggesting more than one taxon within the *C. ampulla* morphospecies. This morphological study however called for a complementary molecular study.

We used the classification proposed in the Illustrated Guide to the Protozoa (Meisterfeld, 2002). Thus, *Corythionella* and *Pseudocorythion* species belong to the Cyphoderiidae family although they were initially described as members of the Psammonobiotidae family (Golemansky, 1970; Valkanov, 1970; Chardez, 1991). In this paper we use the terms "Euglyphid testate amoebae", or "euglyphids" to refer to the Euglyphida sensu stricto.

2.2. Testate amoebae isolation for DNA extractions and scanning electronic imaging

The testate amoebae were isolated by sieving and back sieving. With the exception of *Cyphoderia* cf. *compressa*, all samples from the marine sand beaches were incubated between 4 and 8 weeks in the laboratory, at about 20 °C prior to the isolation. For each DNA preparation, between 5 and 100 individuals were isolated individually under light microscope using fine diameter glass pipettes. Cells were washed by transferring them three times into distilled water. A guanidine thiocyanate protocol was used to extract DNA (Chomczynski and Sacchi, 1987). The shell ultrastructure of selected individuals from each populations, excepting *Cyphoderia ampulla* from Dragichevo, *C. ampulla* from Sofia and *C.* cf. *compressa* from Tsawassen, were investigated by scanning electron microscopy (SEM) by Todorov et al. (2009) or in the present study (Figs. 1 and 2). For SEM, testate amoeba shells were mounted on stubs and kept for 2 weeks in a desiccator. The shells were coated with gold in a vacuum coating unit and observed either with a JEOL JSM-5510 microscope at a tension of 10 kV or with a PHILIPS XL30 FEG microscope at a tension of 5 kV.

2.3. SSU rDNA amplification and sequencing

The 3′ terminal fragment (708–765 bp) of the SSU rRNA gene and a selected number of near full-length (1697–1795 bp) portions of this gene were amplified by nested polymerase chain reaction (PCR) with the universal eukaryotic primers in the first PCR (Table 2) and then using a specific Cyphoderiidae primer and a universal eukaryotic primer in the second PCR (Table 2). The PCR cycling profile was the same for all PCRs: 30 s initial denaturation step (95 °C), followed by 40 cycles of 95 °C for 30 s, 50 °C for 30 s, and 72 °C for 90 s and a final extension at 72 °C for 10 min. The PCR products were purified using the High Pure PCR Purification Kit (Roche Diagnostics) and cloned in TOPO TA cloning Kit (Invitrogen) or sequenced directly. Sequencing was carried out using a BigDye197 Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and analysed either with an ABI-3130xl or a 3730S 48-capillary DNA sequencer (Applied Biosystems). Sequences are deposited in GenBank with the Accession Numbers GU228850–GU228898.

2.4. Dataset constructions

Three data sets were used for phylogenetic analyses. The first included 50 short Cyphoderiidae SSU rDNA sequences (682 bp). The second comprises 43 near full-length SSU rDNA Euglyphida sequences (1461 bp) and the third included 43 Euglyphida as well as 21 environmental sequences (1461 bp). Publicly available SSU rDNA environmental sequences from the Euglyphida were downloaded from GenBank through the taxonomy web site at National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). The sequences were found by BLAST searches using query sequences from all main Euglyphida families (i.e., Assulinidae, Cyphoderiidae, Euglyphidae, Paulinellidae and Trinematidae). These searches were finalized on October 20th 2009. Euglyphid sequences were manually fitted to a general alignment of eukaryotic SSU rRNA gene sequences (Berney and Pawlowski, 2004) using the BIOEDIT 7.0.9 sequence alignment editor (Hall, 1999). This last alignment was based on a universal model of eukaryotic SSU rRNA secondary structure (Van de Peer et al., 2000). Ambiguously

Table 1
List of the Cyphoderiidae morphotaxa analysed and sampling locations.

Taxa	Habitat	Sampling location	Country	Sampling date	Co-ordinates		Altitude (m)	Number of SSU rDNA sequences (nb of extractions)		PCR products cloned (C) or sequenced directly (SD)
								Short fragment	Long fragment	
Corythionella minima	Marine supralittoral	Underground waters of marine sand beach, Galata, Black Sea	Bulgaria	July 2006	43°10′N	27°56′E	0	2 (1)	1 (1)	(C)
Cyphoderia amphoralis	Freshwater	*Sphagnum* mosses, Rila	Bulgaria	August 2005	42°12′N	23°22′E	1960	3 (3)	1 (1)	(SD)
Cyphoderia ampulla	Freshwater	Aquatic mosses, Moiry	Switzerland	July 2006	46°08′N	07°34′E	2310	3 (2)	2 (2)	(SD)
Cyphoderia ampulla	Freshwater	*Sphagnum* mosses, Dragichevo	Bulgaria	August 2006	42°36′N	23°09′E	960	3 (2)	1 (1)	(SD)
Cyphoderia ampulla	Freshwater	*Sphagnum* mosses, Rhodopes	Bulgaria	July 2005	41°59′N	24°10′E	1109	6 (2)	1 (1)	(SD)
Cyphoderia ampulla	Freshwater	*Sphagnum* mosses, Sofia, South Park	Bulgaria	August 2006	42°39′N	23°18′E	610	2 (2)	–	(SD)
Cyphoderia ampulla	Freshwater	*Sphagnum* mosses, Vitosha	Bulgaria	August 2006	42°36′N	23°17′E	1850	2 (2)	1 (1)	(SD)
Cyphoderia ampulla	Freshwater	Underground waters of freshwater sand beach, Lake Geneva, St-Sulpice	Switzerland	May 2008	46°30′N	06°32′E	375	3 (3)	3 (3)	(SD)
Cyphoderia cf. *compressa*	Marine supralittoral	Underground waters of marine supralittoral sand beach, Tsawassen, Pacific Ocean	Canada	October 2008	49°01′N	123°06′	0	1 (1)	1 (1)	(SD)
Cyphoderia compressa	Marine supralittoral	Underground waters of marine supralittoral sand beach, Galata, Black Sea	Bulgaria	July 2006	43°10′N	27°56′E	0	11 (3)	3 (3)	(C)
Cyphoderia littoralis	Marine supralittoral	Underground waters of marine supralittoral sand beach, Galata, Black Sea	Bulgaria	July 2006	43°10′N	27°56′E	0	3 (1)	1 (1)	(C)
Cyphoderia major	Freshwater	*Sphagnum* mosses, Rila	Bulgaria	August 2005	42°12′N	23°22′E	1960	2 (1)	1 (1)	(SD)
Cyphoderia ampulla	Freshwater	Aquatic mosses, Cape Breton, Nova Scotia	Canada	July 2008	46°48′N	60°49′W	236	2 (1)	1 (1)	(SD)
Cyphoderia trochus ssp. *palustris*	Freshwater	Wet mosses, Marchairuz	Switzerland	February 2007 and May 2008	46°33′N	06°14′E	1359	4 (4)	4 (4)	(SD)
Pseudocorythion acutum	Marine supralittoral	Underground waters of marine supralittoral sand beach, Galata, Black Sea	Bulgaria	May 2008	43°10′N	27°56′E	0	2 (2)	2 (2)	(SD)

aligned regions, gaps, highly divergent sequences (e.g., *Tracheleuglypha dentata* and three environmental sequences) and two environmental sequences (AY620296 and AY620297) with unclear affiliation within the Euglyphida were excluded from the analyses. Additionally, only one environmental sequence was selected when several identical or nearly identical environmental sequences were available in GenBank. *Corythionella minima* and *Pseudocorythion acutum*, which branch as a sister group to the *Cyphoderia* genus (Fig. 3), were used as outgroups in the first tree. The second and the third trees were rooted with six thaumatomonad sequences, the inferred sister group of the Euglyphida (Adl et al., 2005).

2.5. Phylogenetic analyses

Phylogenetic analyses were performed with maximum likelihood using RAxML (Stamatakis et al., 2008) and Bayesian inference methods using MrBayes v. 3.1.2 (Huelsenbeck and Ronquist, 2001). The MrAIC program (Nylander, 2004) identified the general-time-reversible model with invariable sites and gamma distribution (GTR + G) as the most appropriate model of sequence evolution for the first and third data sets and the general-time-reversible model with invariable sites and gamma distribution (GTR + I + G) as the most appropriate model for the second data set. For the three data sets, maximum likelihood analyses were run for 1000 replicates and the most likely tree chosen from those runs. Bootstrap proportions (BS) were estimated under the same conditions for 1000 pseudoreplicates. Bayesian analyses were performed for the second and third data sets only. Three simultaneous Markov chains were run up to 10 million generations from a random starting tree. Trees were sampled every 10 generations. The first 250,000 trees were discarded as the burn in after checking that the chains had converged. The resultant trees were used to calculate the posterior probabilities (PP) for each node. The convergence of the Markov chains were graphically estimated by plotting the sample values versus the iteration values as well as by using diagnostics criteria produced by the "sump" command in MrBayes (PSRF = 1.00). Bayesian analyses were run through the Bioportal web-based service platform for phylogenomic analysis at the University of Oslo (www.bioportal.uio.no).

3. Results

3.1. Phylogenetic trees based on Euglyphida SSU rRNA sequences

We first performed a phylogenetic analysis based on a short SSU rDNA alignment including 50 sequences (682 bp) from marine supralittoral and freshwater Cyphoderiidae populations (Table 1). Extractions from the same population always gave almost identical sequences (between 99.5% and 100% identity), and revealed 14 distinct clades. However, the relationships among clades were not well

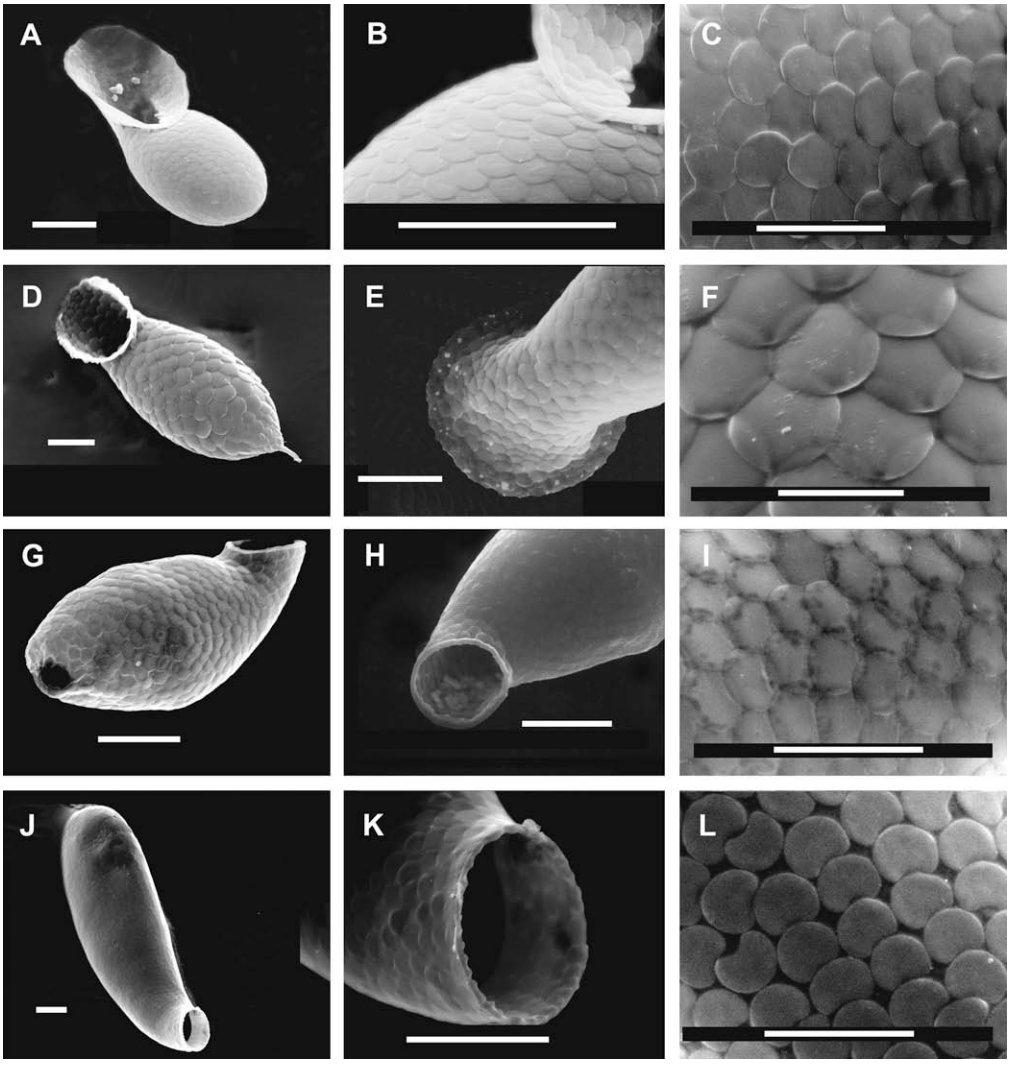

Fig. 1. Scanning electron micrographs of marine supralittoral Cyphoderiidae morphotaxa. The illustrated individuals correspond to the sequenced populations. The detailed pictures at the center and on the right show respectively the pseudostome and the arrangement of the scales. A–C, *Corythionella minima* from Galata (BG); D–F, *Pseudocorythion acutum* from Galata (BG); G–I, *Cyphoderia littoralis* from Galata (BG); J–L, *Cyphoderia compressa* from Galata, Black Sea (BG). Scale bars represent 10 μm in all pictures excepted for the detailed pictures of arrangement of the scales (C, F, I and L) scale bars represent 5 μm (G, H and I from Todorov et al., 2009).

resolved, showing no consistent groupings across Bayesian and maximum likelihood analyses (Supplementary material Fig. 1). In order to increase the phylogenetic signal and to clarify the relationships between these clades, we analysed a longer SSU rDNA data set (1461 bp) including 24 Cyphoderiidae sequences and 19 sequences of the Paulinellidae, Trinematidae, Assulinidae, and Euglyphidae families (Fig. 3). At least one sequence of each of 14 Cyphoderiidae clades mentioned above was represented in this analysis.

Phylogenetic trees inferred from Bayesian and maximum likelihood approaches showed quite similar topologies (Fig. 3). Marine supralittoral and freshwater Euglyphida species were segregated into three major clades (Fig. 3). The first clade included freshwater testate amoebae of the Trinematidae, Assulinidae and the Euglyphidae families, the second well-supported clade was constituted of marine and non-marine Cyphoderiidae phylotypes while the two freshwater *Paulinella chromatophora* sequences formed a third well-supported clade. The Paulinellidae clade branched as a sister group to the Cyphoderiidae clade with moderate statistical support (97% BS and 0.84 PP). Within the Cyphoderiidae clade, the marine supralittoral *Pseudocorythion acutum*, *Corythionella minima* and *Cyphoderia littoralis* phylotypes had an early diverging position relative to the other sequences in the analysis (Fig. 1A–I and Fig 3). The phylogenetic relationships among the marine supralittoral species *C. compressa* (Fig. 1J–L) and *C.* cf. *compressa* and the two separated freshwater sub-clades were moderately supported (Fig. 3). The highly supported freshwater sub-clades 1 (97% BS and 1.00 PP) comprised *Cyphoderia amphoralis*, *C. trochus* ssp. *palustris* and *C. ampulla* from Cape Breton, Rhodopes, Vitosha and Dragichevo – this clade was composed of isolates having a shell built of overlapping or slightly overlapping scales (Fig. 2A–O); and the highly supported subclade 2 (100% BS and 1.00 PP) comprised freshwater lineages *C. major* and *C. ampulla* from Lake Geneva (Switzerland), Aachen (Germany), and Moiry (Switzerland) – this clade was composed of isolates having a shell built of non-overlapping scales (Fig. 2P–X). The morphospecies *C. ampulla* was thus a polyphyletic entity including five distinct phylotypes (sequence divergence >1% among the five *C. ampulla* clusters), (Supplementary material Fig. 1, Fig. 2D–L and P–U, Table 1).

3.2. Phylogenetic trees inferred from euglyphid and environmental SSU rRNA sequences

In order to evaluate the phylogenetic relationships between marine supralittoral and freshwater Euglyphida species more

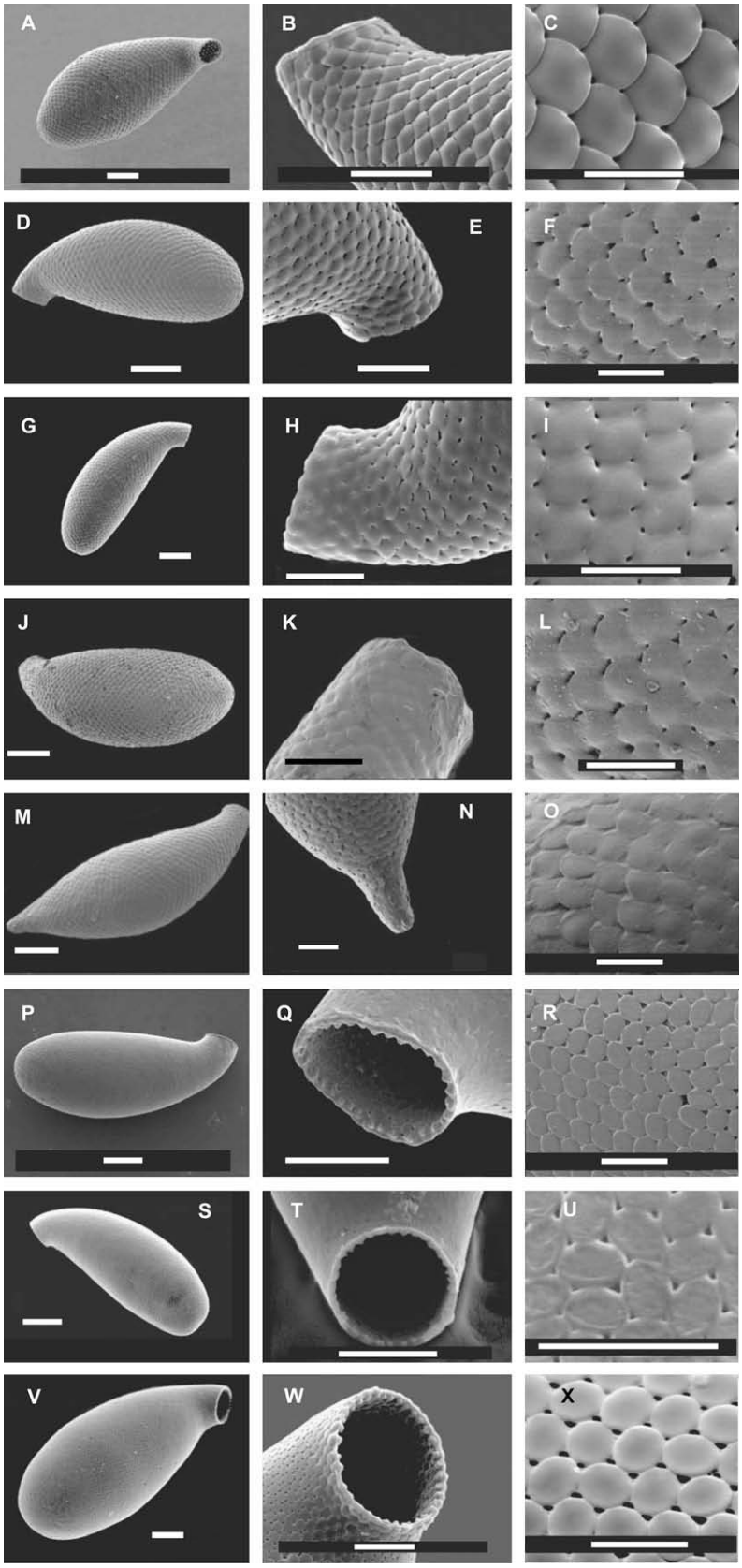

Fig. 2. Scanning electron micrographs of freshwater Cyphoderiidae morphotaxa. The illustrated individuals correspond to the sequenced populations. The detailed pictures show the arrangement of the scales, the pseudostome or the extremity of the shell. (A–O) Represent individuals of the subclade 1 characterized by species having overlapping or slightly overlapping scales and. (P–X) Represent individuals of the subclade 2 characterized by species having non-overlapping scales. (A–C) *Cyphoderia amphoralis* from Rila (BG). (D–F) *Cyphoderia ampulla* from Rhodopes (BG). (G–I) *Cyphoderia ampulla* from Vitosha (BG). (J–L) *Cyphoderia ampulla* from Cape Breton (CAN). (M–O) *Cyphoderia trochus* ssp. *palustris* from Marchairuz (CH). (P–R) *Cyphoderia ampulla* from Moiry (CH). (S–U) *Cyphoderia ampulla* from Geneva Lake (CH). (V–X) *Cyphoderia major* from Rila (BG). Scale bars on the left, at the center and on the right correspond respectively to 20, 10 and 5 μm (pictures A, B, P–R and V–X from Todorov et al., 2009).

Table 2
Sequences of SSU and COI primers used in this study.

Name	Specificity	Sequence (5′–3′)	Direction	Location (on *E. rotunda* X77692)
A10S1	Most eukaryote	CTCAAAGATTAAGCCATGC	Forward	35
CercoR	Most cercozoa	GGTCGAGGTCTCGTTCGTTAACGG	Reverse	1331
Cyphrevb[a]	Most Cyphoderiidae	CACATAATCTGCCAATGGAGTCG	Reverse	1078
Eugl b[a]	Most Cyphoderiidae	CGACTCCATTGGCA	Forward	1078
s12.2	Universal eukaryotic primer	GATCAGATACCGTCGTAGTC	Forward	1013
sB	Universal eukaryotic primer	TGATCCTTCTGCAGGTTCACCTAC	Reverse	1781
SSUcypho[a]	Most Cyphoderiidae	CTATACCGACTATCGATCAGTG	Forward	1044

[a] Primers newly designed in this study.

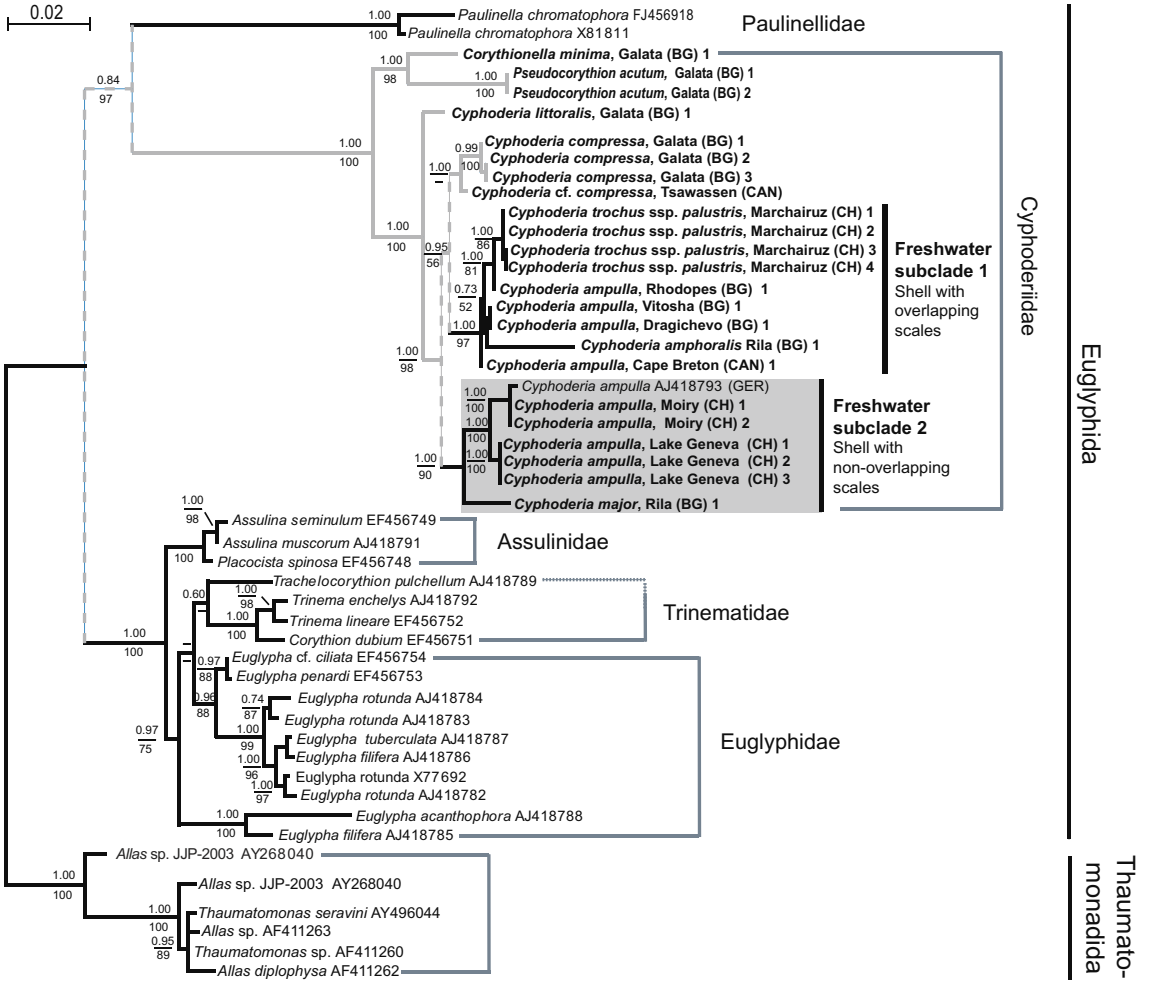

Fig. 3. Phylogenetic tree of 43 SSU rDNA Euglyphida sequences based on 1461 nucleotide positions. Distances of the phylogenetic tree are derived from a RAxML analysis. Numbers represent values of posterior probabilities as calculated with Bayesian analyses and the bootstraps obtained by the maximum likelihood method. A dash indicates that the topology shown is not supported with Bayesian analyses. Marine supralittoral and freshwater Euglyphida lineages are marked with grey and black lines respectively. Data obtained in this study are denoted in bold. The tree was rooted with six thaumatomonads.

broadly, we analyzed an additional dataset (1461 bp) including 21 short environmental sequences assigned to the Euglyphida (Fig. 4). The validity of the two major groups inferred in the former analysis was confirmed (100% BS and 1.00 PP). Ten environmental sequences derived from soil samples branched within the clade formed by the families Trinematidae, Assulinidae and the Euglyphidae (more specifically inside Trinematidae). Five marine environmental sequences and the freshwater *Paulinella chromatophora* were closely related and formed a third robust monophyletic group (Paulinellidae clade, Fig. 4). Two environmental sequences from the marine supralittoral environment (AY620307 and AY620315) branched at the base of all other Cyphoderiidae sequences. Two additional marine supralittoral environmental sequences (AY620326 and AY620325) branched among the marine supralittoral Cyphoderiidae sequences and the soil sequence AY620259 branched within the freshwater Cyphoderiidae subclade 1. Supralittoral environmental sequence AY620293 and *C. compressa* (sensu lato) formed a sister group to the freshwater subclade 1.

4. Discussion

4.1. Marine supralittoral–freshwater transitions

Several studies have suggested that the physiochemical differences between marine and freshwaters environments represent a strong barrier that cannot be crossed by most eukaryotic species

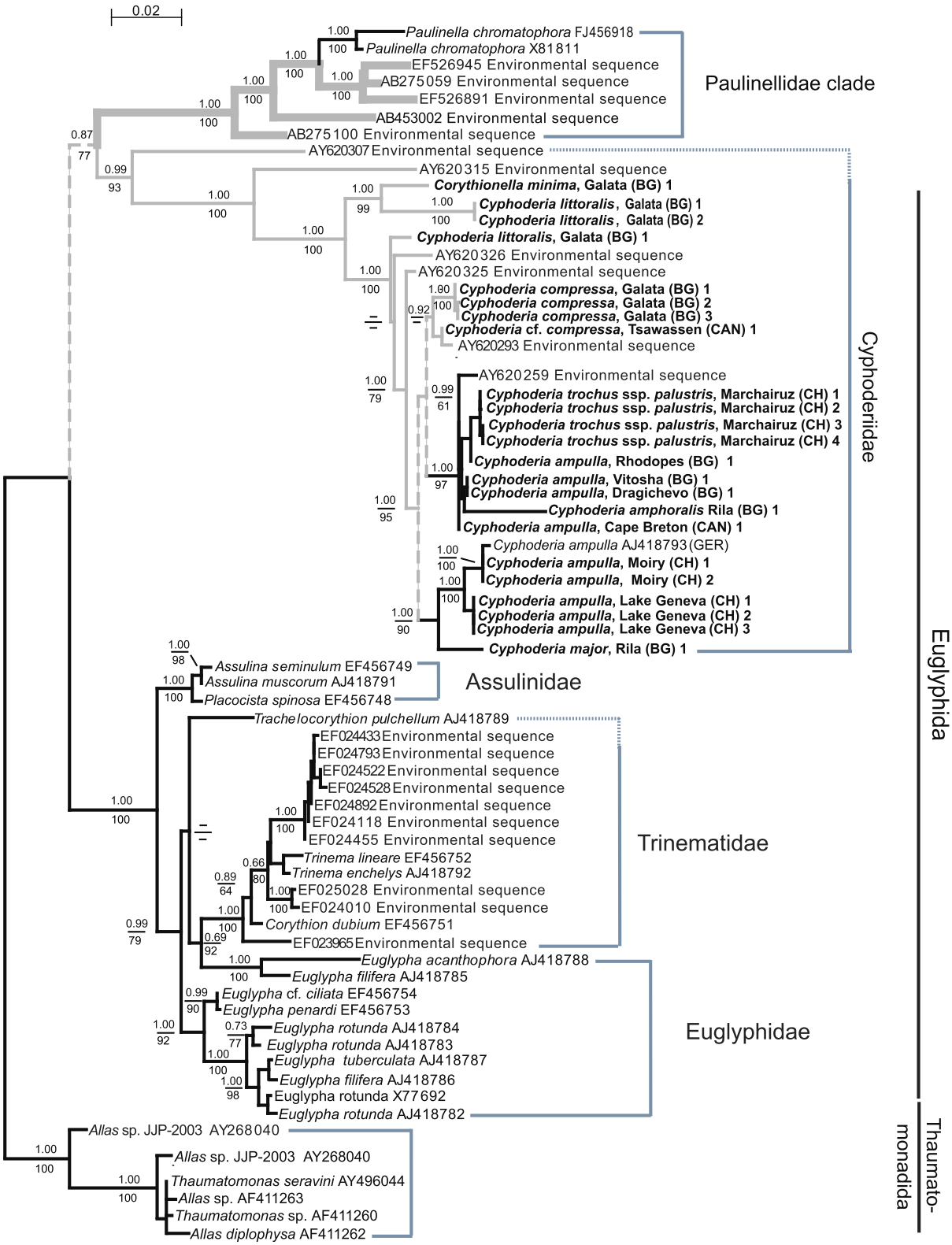

Fig. 4. Phylogenetic tree of 43 Euglyphida and 21 environmental SSU rDNA sequences based on 1461 nucleotide positions. The best-fit model selected in MrAIC (Nylander) was the general-time-reversible model with gamma distribution (GTR + I + G). Numbers represent values of posterior probabilities as calculated with Bayesian analyses and the bootstraps obtained by the maximum likelihood method. A dash indicates that the bootstraps values are lower than 50. Marine and freshwater Euglyphida lineages are marked with grey and black lines, respectively. Marine, marine supralittoral and freshwater Euglyphida lineages are marked with large grey, grey and black lines, respectively. Lines with unclear marine or freshwater origin are indicated with dashed lines. Data obtained in this study are denoted in bold. The tree was rooted with six thaumatomonads.

(Alverson et al., 2007; Logares et al., 2007, 2009; Shalchian-Tabrizi et al., 2008; Cavalier-Smith, 2009). As an extension of this research, our aim was to study the molecular phylogenetic relationships between marine supralittoral and freshwater cyphoderiid testate amoebae as a model system for inferring the frequency of habitat transitions in microbial eukaryotes.

The phylogenetic analyses of isolated euglyphids and related environmental sequences from GenBank demonstrated the existence of three highly divergent clades. The first clade comprised one *Paulinella chromatophora* sequence isolated from freshwater and five marine environmental sequences of unknown morphology (Fig. 4). Previous species descriptions based on morphology also suggest that the only truly marine species within the Euglyphida belong to the genus *Paulinella* (Wulff, 1916; Vors, 1993; Hannah et al., 1996; Nicholls, 2009). *Paulinella chromatophora* was reported from both freshwater habitats and brackish waters (Pankow, 1982; Yoon et al., 2009). It is unclear whether the *P. chromatophora* morphospecies corresponds to a single euryhaline species or to different phylotypes having potentially distinct salinity requirements. This genus alone, therefore, represents another interesting subject for studying marine–freshwater transitions.

The second clade comprises freshwater and marine supralittoral sequences within the Cyphoderiidae. The third clade comprises the freshwater Trinematidae, Assulinidae and Euglyphidae. Neither studies based on comparative morphology nor molecular phylogenetic data have yet convincingly demonstrated the existence of any marine or marine supralittoral species within these three families, which includes more than 80% of the total number of described Euglyphida taxa (Meisterfeld, 2002). Indeed, the only specimens within these three families reported in marine environments were mostly observed in the Baltic Sea where the salinity is very low (less than 4.5‰) and in most cases it was unclear if these reports concerned living individuals or empty shells.

The concordance between the morphology-based taxonomy and molecular phylogenetic data at the genus/major morphotype level suggests that a more extensive phylogeny will demonstrate only few additional transitions between marine supralittoral and freshwater environments within the Euglyphida.

The phylogenetic clustering of the Cyphoderiidae in our analyses suggests that transitions between marine supralittoral and freshwater habitats are infrequent and occurred only twice in this clade. Freshwater *Cyphoderia* species emerge as two monophyletic sub-clades (Figs. 3 and 4). However, the phylogenetic relationships among the two freshwater sub-clades and *C. compressa* (sensu lato) are not strongly supported. We therefore cannot exclude that all freshwater phylotypes constitute a single clade. Such a scenario would suggest only one transition between marine supralittoral and freshwater habitats. This hypothesis would be consistent with the morphological characteristics of the two freshwater sub-clades. Freshwater morphotaxa are characterized by circular cross-sections while *C. compressa* (sensu lato) are characterized by laterally compressed shells. Clarifying the phylogenetic relationships among the two freshwater sub-clades and the *C. compressa* (sensu lato) would require sequencing additional genes and/or including potential missing organisms in our phylogenies.

The Cyphoderiidae contains five genera: *Corythionella, Cyphoderia, Messemvriella*, *Pseudocorythion* and *Schaudinnula*; Nicholls (2003b) transferred *Campascus* into the Psammonobiotidae. The genera *Pseudocorythion* and *Corythionella*, which are each represented in our phylogeny by only one taxon, comprise four and nine species, respectively. While *Pseudocorythion* comprises only marine supralittoral species (Meisterfeld, 2002), two among 10 *Corythionella* species occur in freshwater (Nicholls, 2003a, 2005, 2007, 2009). This suggests one more transition between marine supralittoral and freshwater environments among the *Corythionella* genus. Based on morphology, we expect the two marine supralittoral *Messemvriella* species to be closely related to *Pseudocorythion* and *Corythionella* because they share several distinct morphological features. However, *Messemvriella* species differ from *Pseudocorythion* by the lack of a caudal horn and from *Corythionella* by the circular transverse section and the arrangement of the scales (Golemansky, 1973; Meisterfeld, 2002). Additionally, it would be very interesting to determine the phylogenetic position of the only species of *Schaudinnula*. By contrast to all other Cyphoderiidae species, the shell of this very rare and poorly documented freshwater species is composed of irregularly overlapping scales (Schönborn, 1965; Meisterfeld, 2002).

4.2. Other potential factors

The marine supralittoral is a specific environment characterized by fluctuating salinity. Several macroorganisms such as some littorinid snails (Judge et al., 2009) or microorganisms such as some Cyphoderiidae species are restricted to this environment (Golemansky, 2007). However, given the fact that data on marine microeukaryotic diversity remain quite limited with of the exception of the pelagic euphotic zone, we can not completely exclude the presence of Cyphoderiidae species in benthic or pelagic marine environments (Cuvelier et al., 2008; Epstein and Lopez-Garcia, 2008). Detecting Cyphoderiidae species in truly marine environments would require a taxon-specific primer approach as successfully used by Bass and Cavalier-Smith (2004) Bass et al. (2007) or Lara et al. (2009) for revealing poorly explored protist lineages in environmental DNA surveys.

Besides salinity, numerous other factors (e.g., pH, oxygen content, organic versus mineral substrate) may influence protist communities and restrict some species to specific habitats (Fallu and Pienitz, 1999; Booth et al., 2008). In this study, substrates generally differ between marine and freshwater Cyphoderiidae samples (Table 1). However, substrates differences are unlikely to account for the infrequent transition between marine supralittoral and freshwater habitats. The freshwater *Cyphoderia ampulla* isolated from sandy habitat branches among other freshwater *Cyphoderia* species isolated from aquatic mosses and not within marine supralittoral *Cyphoderia* species isolated from a sandy habitat. Several freshwater and marine supralittoral species of unrelated taxonomical groups, such as *Pseudocorythion acutum* or *Corythionella minima*, are characterized by large apertural collars. This morphological feature is considered an adaptation to the substrate (i.e., to life on sand grains rather than to freshwater or marine environments per se (Meisterfeld, 2002).

4.3. Cryptic cyphoderiid diversity

The existence of cryptic or pseudo-cryptic species may have high relevance in explaining disjunctive geographic distribution patterns or functional niches. Among microeukaryotes, such as within the Foraminifera or within Bacillariophyceae, cryptic species are relatively abundant (de Vargas et al., 1999; Pawlowski and Holzmann, 2002; Beszteri et al., 2005; Darling and Wade, 2008). Within the Euglyphida, cryptic species were so far reported only from two *Euglypha* morphospecies (Wylezich et al., 2002).

Our molecular phylogenetic data revealed the existence of cryptic species within the Cyphoderiidae. The environmental sequence data suggests the existence of two marine supralittoral species from Canada (AY620325 and AY620326) closely related to *C. littoralis* and therefore of the existence of undescribed diversity within this taxon. The morphospecies *Cyphoderia ampulla* is represented by five different phylotypes distributed throughout two distinct freshwater sub-clades in our trees and is therefore a polyphyletic taxon. The phylotypes of these two sub-clades are characterized by distinct arrangements of the scales as revealed by SEM analyses.

The first one includes *C. amphoralis*, *C. trochus* ssp. *palustris* and *C. ampulla* from Cape Breton, Rhodopes, Vitosha and Dragichevo and is composed of isolates having a shell built of overlapping scales. *C. ampulla* sequences from Vitosha and Dragichevo are almost identical to each other (sequence divergence <1%) but differ from *C. ampulla* from Cape Breton and Rhodopes. The second freshwater subclade (Fig. 2), represented by *C. major* and *C. ampulla* from Geneva Lake (Switzerland), Aachen (Germany), and Moiry (Swiss Alps), is characterized by specimens having a shell composed of non-overlapping scales. The *Cyphoderia ampulla* sequence from Moiry is almost identical to the *C. ampulla* from Germany which was the only Cyphoderiidae sequence previously available in GenBank.

These results are consistent with a previous biometrical study that suggested the existence of at least two different taxa within *C. ampulla* morphotype (Todorov et al., 2009). In our study, different *C. ampulla* phylotypes were isolated from distinct ecological habitats such as underground water of a sandy beach on Lake Geneva, aquatic mosses in an Alpine stream or *Sphagnum* mosses of an oligotrophic peatbog (Table 1). Because *C. ampulla* morphotypes includes cryptic species having probably different ecological requirements, the *Cyphoderia ampulla* morphotype should be used with extreme caution for biogeographical, paleoecological or ecological studies. The taxonomic status of some Cyphoderiidae species should be revised. Therefore, a DNA barcoding approach coupled with traditional taxonomic tools would be very useful for clarifying the cyphoderiid taxonomy. The cryptic and pseudo-cryptic diversity revealed by this study and the one of Todorov et al. (2009) suggest that the total diversity of genus *Cyphoderia*, and therefore most likely the Euglyphida as a whole is much higher than currently recognised.

5. Conclusions

The results of this study provide the first insights into phylogenetic relationships between freshwater and marine supralittoral species. In our phylogenies, transitions between marine supralittoral and freshwater habitats occur only once or twice within the *Cyphoderia* genus.

Although our phylogenies do not include all described species, morphological-based taxonomy suggests only a small number of additional transitions within the Cyphoderiidae but none within the exclusively freshwater Trinematidae, Assulinidae and Euglyphidae clades, which comprise the majority of the known euglyphid species.

This reinforces the hypothesis that transitions of microeukaryotes are infrequent between truly marine (s. str. or supralittoral) and freshwater environments (or vice versa) and shows that the Euglyphida offer a valuable system for studying marine–freshwater transitions.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2009.11.023.

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