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Evolutionary relationships among marine cercozoans as inferred from combined SSU and LSU rDNA sequences and polyubiquitin insertions

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ABSTRACT

An insertion of one or two amino acids at the monomer–monomer junctions of polyubiquitin is a distinct and highly conserved molecular character that is shared by two very diverse clades of microeukaryotes, the Cercozoa and the Foraminifera. It has been suggested that an insertion consisting of one amino acid, like that found in foraminiferans and some cercozoans, represents an ancestral state, and an insertion consisting of two amino acids represents a derived state. However, the limited number of cercozoan taxa examined so far limits inferences about the number and frequency of state changes associated with this character over deep evolutionary time. Cercozoa include a very diverse assemblage of mainly uncultivated amoeboflagellates, and their tenuous phylogenetic interrelationships have been based largely on small subunit (SSU) rDNA sequences. Because concatenated datasets consisting of both SSU and large subunit (LSU) rDNA sequences have been shown to more robustly recover the phylogenetic relationships of other major groups of eukaryotes, we employed a similar approach for the Cercozoa. In order to reconstruct the evolutionary history of this group, we amplified twelve LSU rDNAs, three SSU rDNAs, and seven polyubiquitin sequences from several different cercozoans, especially uncultured taxa isolated from marine benthic habitats. The distribution of single amino acid insertions and double amino acid insertions on the phylogenetic trees inferred from the concatenated dataset indicates that the gain and loss of amino acid residues between polyubiquitin monomers occurred several times independently. Nonetheless, all of the cercozoans we examined possessed at least one amino acid insertion between the polyubiquitin monomers, which reinforced the significance of this feature as a molecular signature for identifying members of the Cercozoa and the Foraminifera. Our study also showed that analyses combining both SSU and LSU rDNA sequences leads to improved phylogenetic resolution and statistical support for deeper branches within the Cercozoa.

1. Introduction

Cercozoans comprise a diverse clade of amoeboflagellates that was initially recognized and established using molecular phylogenetic analyses of SSU rDNA sequences (Cavalier-Smith, 1998a,b). An obvious morphological feature that unites the group has yet to be identified. Nonetheless, phylogenetic analyses of concatenated gene datasets have subsequently demonstrated that along with the Foraminifera and the Radiozoa (i.e., most of the traditional radiolarians), cercozoans are strongly nested within the supergroup Rhizaria (Adl et al., 2005; Burki et al., 2007, 2008; Keeling et al., 2005; Nikolaev et al., 2004; Polet et al., 2004). Cercozoans, along with euglenids and dinoflagellates, are among the most commonly encountered predatory flagellates in marine benthic environments, and environmental surveys of this diversity have demonstrated a huge number of cercozoan lineages that have yet to be adequately characterized (Bass and Cavalier-Smith, 2004; Chantangsi et al., 2008; Hoppenrath and Leander, 2006a,b). Marine benthic cercozoans, especially members of the Cryomonadida (e.g., *Cryothecomonas* and *Protaspis*) and their benthic and planktonic relatives (e.g., *Botuliforma*, *Clautriavia*, *Ebria*, *Thaumatomastix*, *Ventrifissura*, and *Verrucomonas*), have so far been relatively neglected by the protistological community (Chantangsi et al., 2008). Improved knowledge of these particular lineages is important for gaining a more comprehensive understanding of marine benthic ecosystems and shedding light on our view of the evolutionary history of marine benthic cercozoans.

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The Cercozoa and the Foraminifera share a significant molecular character involving the presence of novel amino acid insertion(s) at the junctions between monomers of polyubiquitin genes (Archibald et al., 2003). Ubiquitin is a small regulatory protein composed of 76 amino acids and functions to mark other proteins for destruction (Archibald et al., 2003; Bass et al., 2005). This protein is found only in eukaryotes and plays essential roles in many biological processes, such as cell cycle regulation, DNA repair, transcriptional regulation, signal transduction, endocytosis, embryogenesis, and apoptosis (Hershko and Ciechanover, 1998). Ubiquitin genes are also highly conserved, as indicated by only three residue differences between humans and yeast, and can be configured in three main ways: (1) individual genes with single open reading frames; (2) genes fused to ribosomal protein genes; and (3) genes organized as linear head-to-tail ubiquitin coding region repeats, called polyubiquitin genes (Bass et al., 2005). Although the amino acid sequences of polyubiquitin genes cannot be used directly to construct phylogenetic trees, the specific amino acids inserted between the monomers (e.g., serine or theonine) might provide molecular signatures for specific subclades within the Cercozoa and the Foraminifera (Archibald et al., 2003; Archibald and Keeling, 2004; Bass et al., 2005).

Phylogenetic analyses of multi-gene datasets have proven to be a powerful approach to better resolving relationships among eukaryotes (e.g., Baldauf et al., 2000; Bapteste et al., 2002; Burki et al., 2007, 2008; Burki and Pawlowski, 2006; Harper et al., 2005; Kim et al., 2006; Rokas et al., 2003). However, large-scale data (i.e., hundreds of genes or whole genomes) are only available for a very limited number of eukaryotes, and phylogenetic analyses of these datasets are computationally challenging, time consuming (Moreira et al., 2007), and currently impractical for studying uncultivated lineages. Phylogenetic analyses of combined SSU and LSU rDNA sequences, on the other hand, have been shown to be a pragmatic and effective way to significantly improve the resolution of distant relationships within the tree of eukaryotes (e.g., Moreira et al., 2007). These molecular markers are, therefore, expected to provide a promising avenue for evaluating the genealogical relationships within the Cercozoa, where most lineages need to be isolated from the ocean one cell at a time. Accordingly, we amplified and sequenced LSU rRNA, SSU rRNA, and polyubiquitin genes from several uncultivated cercozoans collected from marine benthic habitats in order to better understand the biodiversity and evolutionary history of this group.

2. Materials and methods

2.1. Source of samples and light microscopy (LM)

Fourteen cercozoan taxa were examined in this study (Table 1 and Fig. 1); only three of these have been cultivated: *Cryothecomonas* sp. (strain APCC MC5-1Cryo), *Gymnochlora stellata* (strain CCMP 2057), and *Lotharella vacuolata* (strain CCMP 240). *Placocista* sp. was isolated from freshwater aquatic moss, and individual cells representing the remaining ten taxa were isolated from marine sand samples collected near Vancouver, British Columbia (details in Table 1). Cercozoan cells were extracted from the sand samples through a 48 μm mesh using a melted seawater–ice method described by Uhlig (1964). Briefly, 2–3 spoons of sand samples were placed into an extraction column wrapped with the mesh, and two to three seawater ice cubes were then put on top of the sand samples and left to melt over several hours. The cells of interest passed through the mesh and were concentrated in a seawater-filled Petri dish that was placed underneath the extraction column. The Petri dish containing the cells was then observed using a Leica DMIL inverted microscope. Cells were individually isolated and placed on a slide for imaging and identification using phase contrast and differential interference contrast (DIC) microscopy with a Zeiss Axioplan 2 imaging microscope (Fig. 1).

2.2. DNA extraction and PCR amplification

Cells were individually isolated and washed three times in either autoclaved distilled water or autoclaved filtered seawater, depending on the species. DNA was extracted using the protocol provided in the Total Nucleic Acid Purification kit by EPICENTRE (Madison, WI, USA). Semi-nested polymerase chain reaction (PCR) with a final reaction volume of 25 μl was performed in a thermal cycler using puReTaq Ready-To-Go PCR beads (GE Healthcare Bio-Sciences, Inc., Québec, Canada). The first PCR amplification was conducted using the outermost forward and reverse primers which were NPF1 and R4 for SSU rDNA, 28S-1F and 28S-4R for LSU rDNA, and UBIQ1 and IUB2 for ubiquitin gene (Table 2). The first PCR product was used as a template for a second round of PCR involving the internal primers provided in Table 2. For the amplification of SSU rDNA, the primers NPF1 and either 1242RD or CercR were used to obtain a 5′ fragment of the gene; the overlapping 3′ fragment was amplified with either 525F or Eugl_b

Table 1
Cercozoan protists whose genes were amplified and sequenced in this study. GenBank accession numbers for gene sequences, which were newly sequenced in this study, are underlined.

Taxa	Source of organisms	GenBank accession number		
		SSU	LSU	UBI
Botuliforma benthica	Pachena Beach, Vancouver Island, BC, Canada	FJ824126	GQ144685	–
Clautriavia biflagellata	American Type Culture Collection (ATCC) PRA-311; Brady Beach, Vancouver Island, BC, Canada	FJ919772	GQ144682	GQ144694
Cryothecomonas sp.	Antarctic Protist Culture Collection (APCC) MC5-1Cryo	GQ144679	GQ144683	GQ144695
Ebria tripartita	Bamfield Inlet, Vancouver Island, BC, Canada	DQ303922/DQ303923	GQ144684	GQ144696
Gymnochlora stellata	Center for Culture of Marine Phytoplankton (CCMP) 2057	AF076171	GQ144686	–
Lotharella vacuolata	Center for Culture of Marine Phytoplankton (CCMP) 240	AF076168	GQ144687	–
Placocista sp.	Grouse Mountain, Vancouver, BC, Canada	GQ144680	GQ144688	–
Protaspis grandis	Boundary Bay, Vancouver, BC, Canada	DQ303924	GQ144689	GQ144697
Protaspis oviformis	Spanish Banks, Vancouver, BC, Canada	FJ824125	–	GQ144698
Thaumatomastix sp.	American Type Culture Collection (ATCC) PRA-312; Boundary Bay, Vancouver, BC, Canada	GQ144681	GQ144693	–
Ventrifissura foliiformis	Boundary Bay, Vancouver, BC, Canada	FJ824128	GQ144690	–
Ventrifissura sp.	Boundary Bay, Vancouver, BC, Canada	–	–	GQ144699
Verrucomonas bifida	Boundary Bay, Vancouver, BC, Canada	FJ824129	GQ144691	GQ144700
Verrucomonas longifila	Spanish Banks, Vancouver, BC, Canada	FJ824130	GQ144692	–

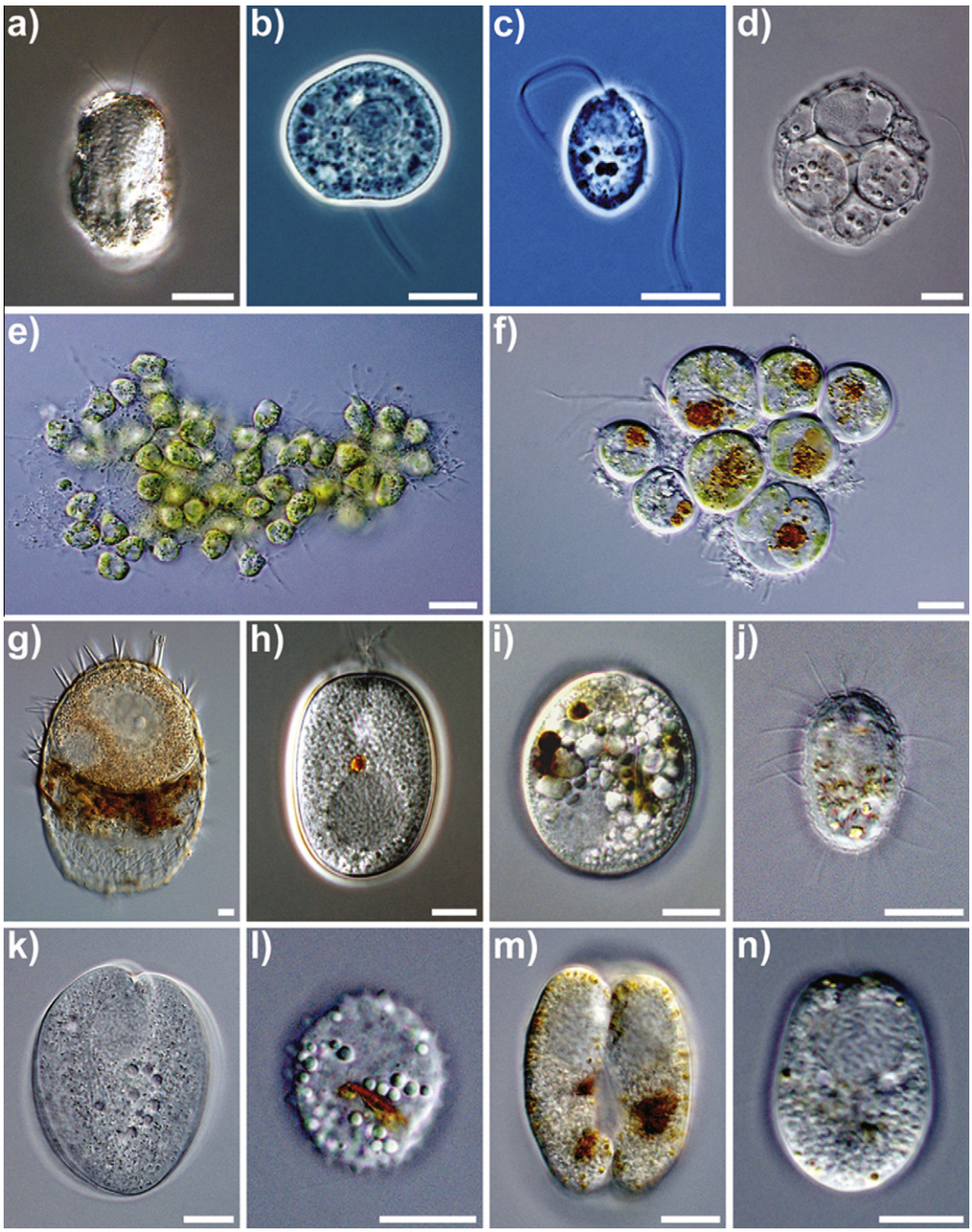

Fig. 1. Light micrographs (LM) of the cercozoans examined in this study. (a) *Botuliforma benthica*. (b) *Clautriavia biflagellata*. (c) *Cryothecomonas* sp. (strain APCC MC5-1Cryo). (d) *Ebria tripartita*. (e) *Gymnochlora stellata* (strain CCMP 2057). (f) *Lotharella vacuolata* (strain CCMP 240). (g) *Placocista* sp. (CC-Grouse Mountain). (h) *Protaspis grandis*. (i) *Protaspis oviformis*. (j) *Thaumatomastix* sp. (CC002-Boundary Bay). (k) *Ventrifissura foliiformis*. (l) *Ventrifissura* sp. (CC005-Boundary Bay). (m) *Verrucomonas bifida*. (n) *Verrucomonas longifila*. (Bars = 10 μm).

and R4. For the amplification of LSU rDNA, the 5′ fragment of the gene was obtained using 28S-1F and either 28S-2R, 28S-1638R, 28S-1611R, or 28S-3R; the 3′ fragment of the LSU rDNA was obtained using 28S-1426F and either 28S-3093R, 28S-3116R, or 28S-4R. For some taxa, specific primers were designed in order to amplify the LSU rDNA: 28S-1499F for *Ebria tripartita*; 28S-1903R for *Lotharella vacuolata*; and 28S-PlacoR, 28S-PlacoR2, and 28S-PlacoR3 for *Placocista* sp. Moreover, the specific sequencing primers used are also listed in Table 2. In all cases, the primers UBIQ1 and IUB2 were used to amplify sections of the polyubiquitin tract. This primer pair generated a ladder of ubiquitin gene products ranging from a small fragment representing one half of a monomer (~120 bp) to increasing numbers of tandem repeats of the polyubiquitin tract that spanned the monomer–monomer junctions (e.g., ~340 bp representing 1.5 monomers and 570 bp representing 2.5 monomers).

The thermal cycler was programmed as follows: hold at 94 °C for 4 min; 5 cycles of denaturation at 94 °C for 30 s, annealing at 45 °C for 1 min, and extension at 72 °C for 105 s; 35 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 1 min, and extension at 72 °C for 105 s; and hold at 72 °C for 10 min. The annealing temperatures of each primer for the last 35 cycles of

Table 2
Oligonucleotide primers used for amplification and sequencing in this study.

Primers	Direction	Sequence 5′–3′	Annealing region[a]	Annealing temp. (°C)[b]
SSU rDNA				
NPF1	Forward	5′-TGCGCTACCTGGTTGATCC-3′	1–19	55
525F	Forward	5′-AAGTCTGGTGCCAGCAGCC-3′	568–586	55
Eugl_b	Forward	5′-ACGACTCCATTGGCA-3′	1099–1085	50
1242RD	Reverse	5′-GTCYGGACCTGGTAAGTTTTC-3′	1243–1223	55
CercR	Reverse	5′-TCGAGGTCTCGTTCGTTAACGG-3′	1359–1338	50
R4	Reverse	5′-GATCCTTCTGCAGGTTCACCTAC-3′	1826–1804	55
LSU rDNA				
28S-1F	Forward	5′-ACCCGCTGAATTTAAGCAT-3′	1–19	50
28S-568F	Forward	5′-TTGAAACACGGACCAAGGAG-3′	762–781	55
28S-713F[c]	Forward	5′-CTAACATATRTGCGAGTATTTG-3′	783–804	50
28S-1426F[c]	Forward	5′-AAYTAGCCCTGAAAATGGATGG-3′	1417–1438	50
28S-1499F[c]	Forward	5′-ATGAGTASGHGGGCGTG-3′	1490–1506	50
28S-2F	Forward	5′-GCAGATCTTGGTGGTAG-3′	1557–1573	50
28S-2R[c]	Reverse	5′-CTMCCACCAAGATCYGC-3′	1573–1557	50
28S-1527F[c]	Forward	5′-CAAATGAGAACTTTGAAGACT-3′	1585–1605	50
28S-1638R[c]	Reverse	5′-CACRHGRAACCTTTCTCCACTTCA-3′	1628–1605	50
28S-PlacoR2[c]	Reverse	5′-YTTCCCTATCTCTTAGGAY-3′	1719–1701	50
28S-1903R[c]	Reverse	5′-ACGTGARGTGCTTTACCAGC-3′	1759–1740	50
28S-PlacoR[c]	Reverse	5′-TCATCCGAAGACAACCTGC-3′	1760–1742	50
28S-1759F[c]	Forward	5′-GCCYGRGAAGAGTTA-3′	1795–1809	50
28S-1611R	Reverse	5′-CTTGGASACCTGMTGCGG-3′	1986–1969	50
28S-3R	Reverse	5′-CACCTTGGAGACCTGCT-3′	1989–1973	50
28S-PlacoR3[c]	Reverse	5′-CATTGCGTCAACATCYTTTC-3′	2425–2406	50
28S-3093R[c]	Reverse	5′-CAATCCKACACTTGGCCYC-3′	3071–3053	50
28S-3116R[c]	Reverse	5′-CGTTCCCTGTTGGWGGA-3′	3092–3076	50
28S-4R	Reverse	5′-TTCTGACTTAGAGGCGTTCAG-3′	3081–3061	50
Ubiquitin				
UBIQ1	Forward	5′-GGCCATGCARATHTTYGTNAARAC-3′	−4–20	55
IUB2	Reverse	5′-GATGCCYTCYTTRTCYTGDATYTT-3′	108–85	55

[a] Annealing region was provided with reference to SSU rDNA sequence of *Thaumatomastix* sp. CC002 [GenBank Accession No. GQ144681]; LSU rDNA sequence of *Thaumatomastix* sp. CC002 [GenBank Accession No. GQ144693]; and ubiquitin gene sequence of *Cryothecomonas* sp. APCCMC5-1Cryo [GenBank Accession No. GQ144695]. Annealing region of 28S-PlacoR, 28S-PlacoR2, and 28S-PlacoR3 was provided with reference to LSU rDNA sequence of *Placocista* sp. CC [GenBank Accession No. GQ144688]; that of 28S-1903R to LSU rDNA sequence of *Lotharella vacuolata* [GenBank Accession No. GQ144687]; and that of 28S-4R to LSU rDNA sequence of *Gymnochlora stellata* [GenBank Accession No. GQ144686].
[b] Annealing temperatures of primers that were programmed in the last 35 cycles of a 40-cycle PCR reaction.
[c] Primers that were newly designed in this study.

PCR reaction are provided in Table 2. PCR products corresponding to the expected size were separated by agarose gel electrophoresis and cleaned using the UltraClean™ 15 DNA Purification Kit (MO BIO Laboratories, Inc., CA, USA). The cleaned DNA was cloned into pCR2.1 vector using the TOPO TA Cloning® kits (Invitrogen Corporation, CA, USA). Plasmids with the correct insert size were sequenced using BigDye 3.1 and the vector primers, and appropriate internal primers, with an Applied Biosystems 3730S 48-capillary sequencer (Table 2).

2.3. Sequence alignment

Sequences were assembled and edited using Sequencher™ (version 4.5, Gene Codes Corporation, Ann Arbor, Michigan, USA). Acquired sequences were initially identified by Basic Local Alignment and Search Tool (BLAST) analysis. New sequences and other additional sequences retrieved from GenBank database were aligned using online MAFFT version 6 (Katoh et al., 2002). The aligned sequences were then imported to the MEGA (Molecular Evolutionary Genetics Analysis) program version 4 (Tamura et al., 2007) and further refined by eye. Three multiple sequence alignments were created for phylogenetic analyses: (1) an 85-taxon cercozoan SSU rDNA alignment covering representatives from different cercozoan subgroups (1443 sites); (2) a 32-taxon SSU rDNA alignment (1469 sites); and (3) a 32-taxon combined SSU and LSU rDNA alignment (4131 sites: 1469 SSU and 2662 LSU). The last two alignments were each composed of the same composition of taxa. SSU rDNA sequences from foraminiferans were excluded from the analyses because of their extremely high nucleotide substitution rates. The alignment files are available upon request.

2.4. Phylogenetic analyses

MrBayes version 3.1.2 was used to perform Bayesian analyses on three datasets (Ronquist and Huelsenbeck, 2003). Four Markov Chain Monte Carlo (MCMC) chains — 1 cold chain and 3 heated chains — were run for 5,000,000 generations, sampling every 50th generation (tree). The first 5000 trees were discarded as burn-in (trees sampled before the likelihood plots reached a plateau). The remaining trees were used to compute the 50% majority-rule consensus tree. Branch lengths of the trees were saved.

Maximum likelihood analyses were performed on the three datasets using PhyML (Guindon and Gascuel, 2003). The General Time Reversible (GTR) model of nucleotide substitution and optimization of equilibrium frequencies were chosen. The proportion of invariable sites, transition/transversion ratio, and gamma shape parameter were estimated from the original dataset. Eight categories of substitution rates were selected. A BIONJ distance-based tree was used as a starting tree. Nearest neighbor interchange (NNI) and subtree pruning and regrafting (SPR) were chosen as approaches of tree topology improvement with optimization of tree topology and branch length in effect. PhyML bootstrap trees with 1000 replicates for the 85-taxon, 32-taxon SSU rDNA, and 32-taxon combined SSU and LSU rDNA datasets were constructed using the same parameters as the individual ML trees.

2.5. Sequence availability

The nucleotide sequences generated in this study were submitted to the GenBank database and listed in Table 1.

3. Results and discussion

3.1. Preliminary phylogeny of cercozoans as inferred from SSU rDNA sequences

Phylogenetic analyses of the 85-taxon dataset (SSU rDNA) containing representatives of all major cercozoan lineages recovered three main clades: (1) a well-supported clade containing parasitic cercozoans (i.e., Ascetosporea, Phagomyxida, and Plasmodiophorida) and free-living gromiids; (2) a clade consisting of chlorarachneans and *Metromonas simplex*, a free-living gliding flagellate; and (3) a large clade consisting of all other cercozoans in the analysis (Fig. 2). ML analyses grouped the large cercozoan clade and the chlorarachnean + *Metromonas* clade together with very strong statistical support (bootstrap value = 96% data not shown), to the exclusion of the clade consisting of the parasitic cercozoans and gromiids. The SSU rDNA phylogenies also showed several well-supported subclades, namely the Ascetosporea, Auranticordida, Chlorarachnea, Cryomonadida, Euglyphida, Glissomonadida, Gromiidea, Pansomonadida, Phaeodarea, Phagomyxida, Plasmodiophorida, Thaumatomonadida, Ventrifissuridae, Verrucomonadidae, some lineages of Cercomonadida, and a clade consisting of the Botuliformidae and the Ebriida (Fig. 2). These results are consistent with several previous studies (Bass et al., 2005, 2009; Bass and Cavalier-Smith, 2004; Burki et al., 2002; Cavalier-Smith and Chao, 2003; Chantangsi et al., 2008; Hoppenrath and Leander, 2006a,b; Karpov et al., 2006; Polet et al., 2004; Vickerman et al., 2005; Wylezich et al., 2002, 2007). However, several of the earliest cercozoan branches were only weakly or modestly supported by the data, and in some cases, the statistical values for these branches were lower than those recovered in previous analyses (Bass et al., 2005, 2009; Bass and Cavalier-Smith, 2004; Cavalier-Smith and Chao, 2003). Our primary aim in this study was to assess and bolster the robustness of these relationships with the inclusion of additional taxa (i.e., several uncultivated marine and freshwater lineages shown in Table 1 and Fig. 1) and additional comparative molecular data for these taxa, namely LSU rDNA sequences and polyubiquitin insertions.

3.2. Phylogeny of mostly marine cercozoans as inferred from SSU+LSU rDNA sequences

Phylogenetic analyses of the two 32-taxon datasets — one of only SSU rDNA sequences and one of combined SSU and LSU rDNA sequences — demonstrated generally similar topologies; different branching patterns for some lineages reflected very weak statistical support (Fig. 3). For example, *Lotharella globosa* and *L. vacuolata* were positioned differently within the chlorarachnean clades in both SSU rDNA and SSU + LSU rDNA trees (Fig. 3). Similarly, placement of *Limnofila* sp. and *Mesofila limnetica* remained unresolved in the two trees (Fig. 3). The placement of *Clautriavia biflagellata* was uncertain; however, it clustered with euglypids in the SSU rDNA phylogeny with a posterior probability of 0.93 (Fig. 3). Likewise, *Ventrifissura foliiformis* branched as the sister lineage to verrucomonads in the SSU + LSU rDNA tree but branched with a clade containing *Clautriavia*, euglyphid, and thaumatomonad lineages in the SSU rDNA tree (Fig. 3). Although the precise phylogenetic positions of *C. biflagellata*, *Limnofila* sp., *M. limnetica* and *V. foliiformis* remained unresolved, our analyses of both alignments did recover several well-supported subclades within the non-chlorarachnean cercozoans, including the Cryomonadida, Glissomonadida, Thaumatomonadida, Verrucomonadidae, and the clade consisting of the Botuliformidae and Ebriida.

Moreover, some relatively deep branches that received only low bootstrap support in the SSU rDNA tree received stronger support in the analyses of the SSU + LSU rDNA alignment. For instance, the large clade consisting of non-chlorarachnean cercozoans was recovered with a posterior probability (PP) of 1.00 and a bootstrap percentage (BP) of 55.2% in the SSU rDNA tree, but received PP = 1.00 and BP = 98.5% in the analyses of SSU + LSU rDNA (Fig. 3). The close relationship between *Paulinella chromatophora* and *Placocista* sp. also gained greater bootstrap support (BP of 66.0% in the SSU tree but 98.2% in the combined tree; Fig. 3). This relationship is consistent with previous molecular phylogenetic studies and morphological features of the two species (i.e., both are testate and filose amoebae; Lara et al., 2007). In addition, the number of nodes obtained full statistical support values (PP of 1.00 and BP of 100%) was increased considerably in the SSU + LSU rDNA tree (13 nodes) in comparison to those in the SSU rDNA tree (7 nodes) (Fig. 3). We were also able to determine a more reliable phylogenetic position for some lineages in the combined analyses (SSU + LSU rDNA). *Partenskyella glossopodia*, for example, was positioned as a sister taxon of *Chlorarachnion reptans* with very low support values (PP of 0.20; data not shown) in the SSU rDNA tree; however, in the combined analyses, *P. glossopodia* was placed next to *C. reptans* with strong support (PP of 1.00 and BP of 80.3%) (Fig. 3).

These results help demonstrate the utility of combined SSU and LSU rDNA sequence alignments for inferring the evolutionary relationship within the Cercozoa. It is widely appreciated that increased taxonomic sampling improves phylogenetic resolution (Graybeal, 1998; Hillis, 1996), and the relatively small number of ingroup taxa included in our combined analyses is a direct reflection of the limited number of LSU rDNA sequences currently available for cercozoans. Accordingly, our study has almost doubled the taxonomic sampling of LSU rDNA sequences from non-chlorarachnean cercozoans, by providing 12 new sequences from mostly poorly understood lineages collected from marine habitats. The high copy number of SSU and LSU rRNA genes in nuclear genomes facilitates the acquisition of these sequences via PCR from a very small amount of starting material. The SSU and LSU rDNA sequences reported here were derived mostly from uncultivated, albeit identified, marine microeukaryotes. Fewer than five uncultured cells were isolated per DNA extraction, and in some cases, we acquired SSU and LSU rDNA sequences from only one uncultured cell that was manually isolated from heterogenous sand samples (Table 1 and Fig. 1). This overall approach offers a pragmatic and effective way to evaluate the cellular and molecular biodiversity of cercozoans within a robust phylogenetic context.

3.3. Patterns of polyubiquitin insertions within the Cercozoa

An insertion of one or two amino acid residues at the intermonomeric junction of polyubiquitin molecules has been found in both the Cercozoa and the Foraminifera, and this feature is, so far, absent in all other eukaryotes (Archibald et al., 2003; Bass et al., 2005) (Fig. 4). Because ubiquitin genes are so highly conserved across eukaryotes, the novel insertion is potentially a robust molecular marker for identifying members of the Cercozoa and specific subclades within the group. This is significant, because cercozoans currently lack unifying features at the morphological or behavioral levels, although recently, Cavalier-Smith et al. (2008) proposed the proximal hub-lattices and distal nonagonal fibers in the ciliary transition zone as ultrastructural synapomorphies of flagellated cercozoans.

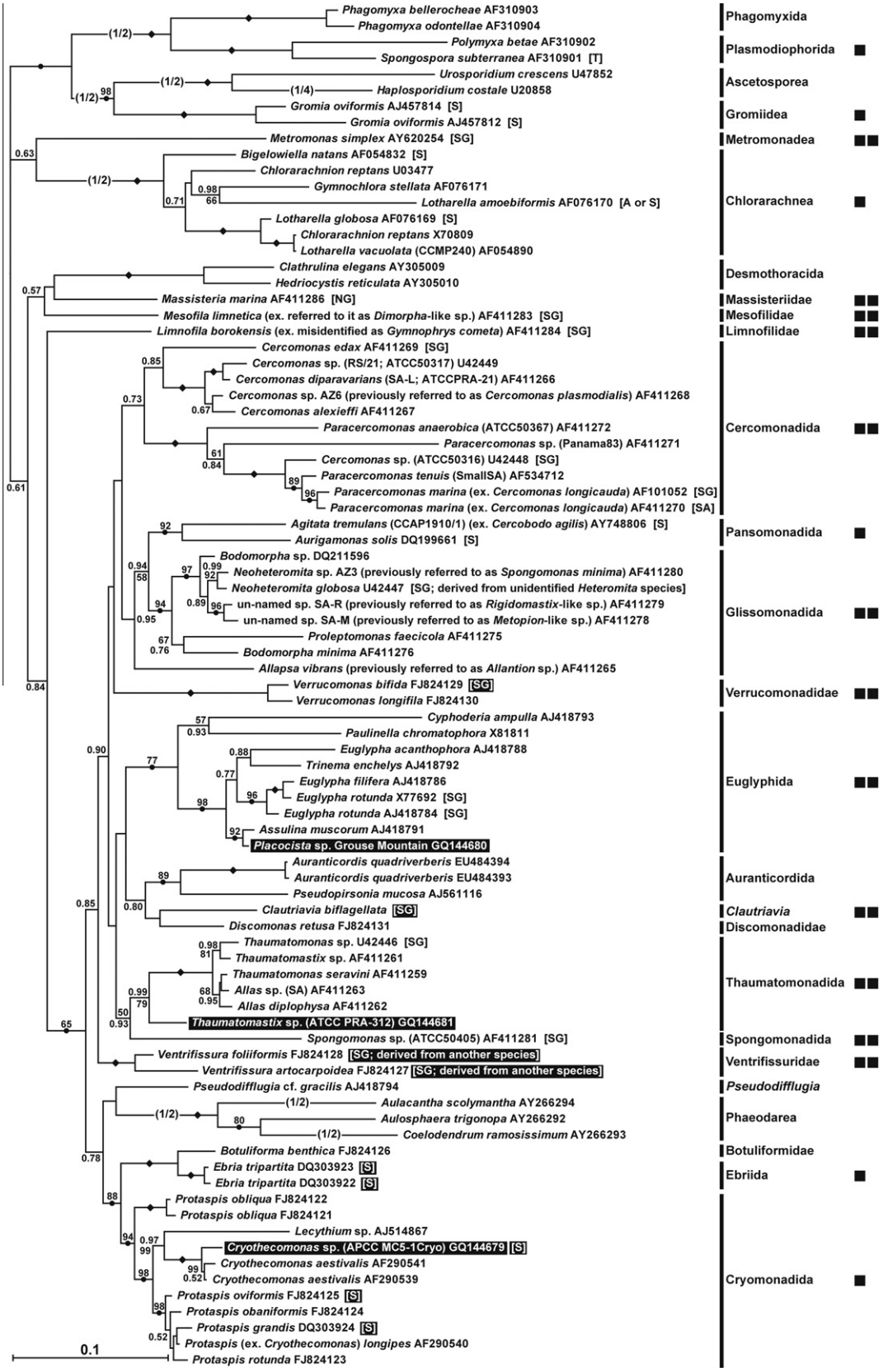

Fig. 2. Phylogenetic tree inferred from Bayesian analysis of 1443 bp of SSU rDNA sequences from 85 cercozoan taxa; the phylogenetic positions of the cercozoan sequences derived from this study are highlighted in black boxes. The tree (mean ln $L = -20{,}900.61$) is a consensus of 95,001 trees with the GTR + I + G using 8 rate categories implemented. Numbers of 0.50 or higher at the notes indicate Bayesian posterior probabilities and PhyML bootstrap percentages higher than 50%. Black circles represent Bayesian posterior probability of 1.00. Black diamonds represent Bayesian posterior probability of 1.00 and phyML bootstrap value of 100%. Several long branches were shortened to one half (labeled 1/2) or one fourth (labeled 1/4) of their actual length. The scale bar corresponds to 0.1 substitutions per site. Capital letters in square brackets indicate amino acid insertions of polyubiquitin molecules. Polyubiquitin-amino-acid insertions generated from this study are highlighted in black boxes. Black squares to the right of the tree represent the number of amino acid residues in the polyubiquitin insertion in the representative member(s) of the taxa indicated.

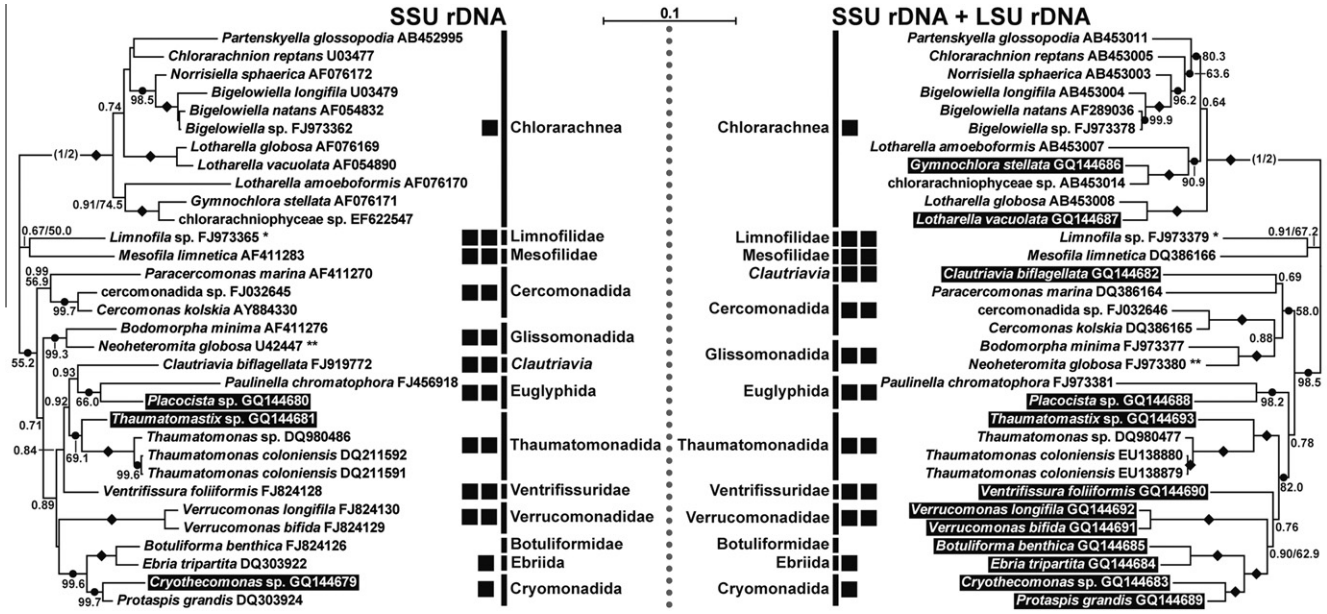

Fig. 3. Comparison of phylogenetic trees inferred from Bayesian analysis of a 32-taxon alignment; the left tree was inferred from 1469-bp of SSU rDNA sequences (mean ln $L = -9431.83$), and the right tree was inferred from 4131-bp of SSU + LSU rDNA sequences (mean ln $L = -28{,}380.10$). The phylogenetic positions of the cercozoan sequences derived from this study are highlighted in black boxes. Each tree is a consensus of 95,001 trees with the GTR + I + G using 8 rate categories implemented. Numbers at the nodes indicate Bayesian posterior probabilities and PhyML bootstrap percentages. Diamonds represent Bayesian posterior probability of 1.00 and PhyML bootstrap value of 100%. Two long branches leading to chlorarachnean clade were shortened to one half (labeled 1/2) of their actual length. The scale bar corresponds to 0.1 substitutions per site. Black squares represent the number of amino acid residues in the polyubiquitin insertion in the representative member(s) of the taxa indicated. *The sequences FJ973365 and FJ973379 were actually derived from an isolate of *Limnofila* sp. These two GenBank entries carry over a misidentification (i.e., *Gymnophrys* sp.) from the ATCC catalogue. **_Neoheteromita globosa_ was previously known as _Heteromita globosa_.

In our phylogenetic analyses, cercozoans clustered into three major clades and these clades reflect different patterns of polyubiquitin insertions. For instance, members of the clade consisting of parasitic cercozoans and free-living gromiids possessed one amino acid insertion (i.e., Serine = S or Threonine = T); chlorarachneans also possessed a single amino acid insertion between the intermonomeric junctions of polyubiquitin molecules, with an exception of *Metromonas*. *Metromonas*, a putative sister taxon of chlorarachneans, possessed an insertion of two amino acids (i.e., serine plus glycine = SG); however the phylogenetic position of *Metromonas* is tenuous because of low statistical support (e.g., posterior probability of only 0.63 uniting *Metromonas* with chlorarachneans). Members of the clade consisting of the remaining cercozoans possessed an insertion of either one amino acid (if one, all with serine = S) or two amino acids (most with SG) (Fig. 2). *Paracercomonas marina* and *Massisteria marina* possessed two uncommon amino acid insertions, namely SA (or SG) in the former and asparagine plus glycine = NG in the latter (Fig. 2).

It has been suggested that the ancestral state for cercozoans possesses one amino acid insertion, like that in Foraminifera, and more derived cercozoans possess two residues between monomers of ubiquitin (Bass et al., 2005). Archibald et al. (2003) and Bass et al. (2005) demonstrated polyubiquitin sequences for three foraminiferan genera — *Bathysiphon*, *Haynesina*, and *Reticulomyxa* — and showed that all of them possess only one amino acid insertion between the monomers, namely either A or T. Subsequent investigations have demonstrated that the following cercozoans also possess one amino acid insertion between the monomers of the polyubiquitin tract: *Agitata* (formerly referred to as *Cercobodo*), *Aurigamonas*, *Bigelowiella*, *Gromia*, *Helkesimastix*, *Lotharella*, *Metopion*, *Plasmodiophora*, and *Spongospora* (Archibald et al., 2003; Archibald and Keeling, 2004; Bass et al., 2005; Vickerman et al., 2005). Our study expanded this dataset by demonstrating seven additional polyubiquitin sequences from mainly uncultivated marine cercozoans: *Cryothecomonas* sp. APCC, *Ebria tripartita*, *Protaspis oviformis*, and *Protaspis grandis* have an insertion consisting of serine; *Clautriavia biflagellata*, *Ventrifissura* sp., and *Verrucomonas bifida* have an insertion consisting of serine plus glycine (Fig. 4). The distribution of different states for the polyubiquitin insertions on our phylogenetic tree inferred from SSU and SSU + LSU rDNA sequences (Figs. 2 and 3) shows that some lineages have reverted to one amino acid insertion. For example, the pansomonadida clade, consisting of *Agitata* and *Aurigamonas*; the cryomonadida clade, comprising *Cryothecomonas* and *Protaspis*; and the ebriida clade, composed of *Ebria*, possessed a single amino acid residue (insertion = S) (Figs. 2 and 4). Moreover, members of the clade consisting of the Ebriida and the Cryomonadida were united by a consistent pattern of polyubiquitin insertions (i.e., one [S]). Although members of the Ebriida and the Cryomonadida occupy different ecological niches (planktonic and benthic/planktonic habitats, respectively), these clades are sister taxa (Fig. 3). *Botuliforma benthica* is the nearest benthic relative of *Ebria*, and it is expected that this taxon will possess a single amino acid insertion (possibly S) as observed in *Cryothecomonas*.

Our data indicate that the possession of one amino acid insertion between the monomers of the polyubiquitin tract is an ancestral state for some lineages (e.g., chlorarachneans and foraminiferans) and a derived state (a reversal) for other lineages (e.g. ebriids and cryomonads) that had ancestors with a two-residue insertion. In other words, the gain and loss of amino acids within the polyubiquitin insertion is inferred to have happened multiple times independently within the Cercozoa. If so, then the degree of evolutionary conservation of the polyubiquitin gene, specifically the insertions, might be lower than previously appreciated. The phylogenetic framework inferred from the SSU + LSU rDNA sequences (Fig. 5) enabled us to more confidently show reversals in polyubiquitin-amino-acid insertions. For instance, the ancestral state of one amino acid insertion found in non-filosan cercozoans (i.e., Endomyxa [e.g., Ascetosporea, Gromiidea, Phagomyxida, and Plasmodiophorida]), chlorarachneans, and foraminif-

Fig. 4. Illustration showing the junction between two ubiquitin monomers (UBIQ) within the polyubiquitin tract of rhizarians (i.e., cercozoans, foraminiferans, and radiozoans) and an array of other eukaryotes. Insertions of one or two amino acids between the ubiquitin monomers are marked in bold. The seven cercozoan sequences generated by this study are highlighted in black boxes.

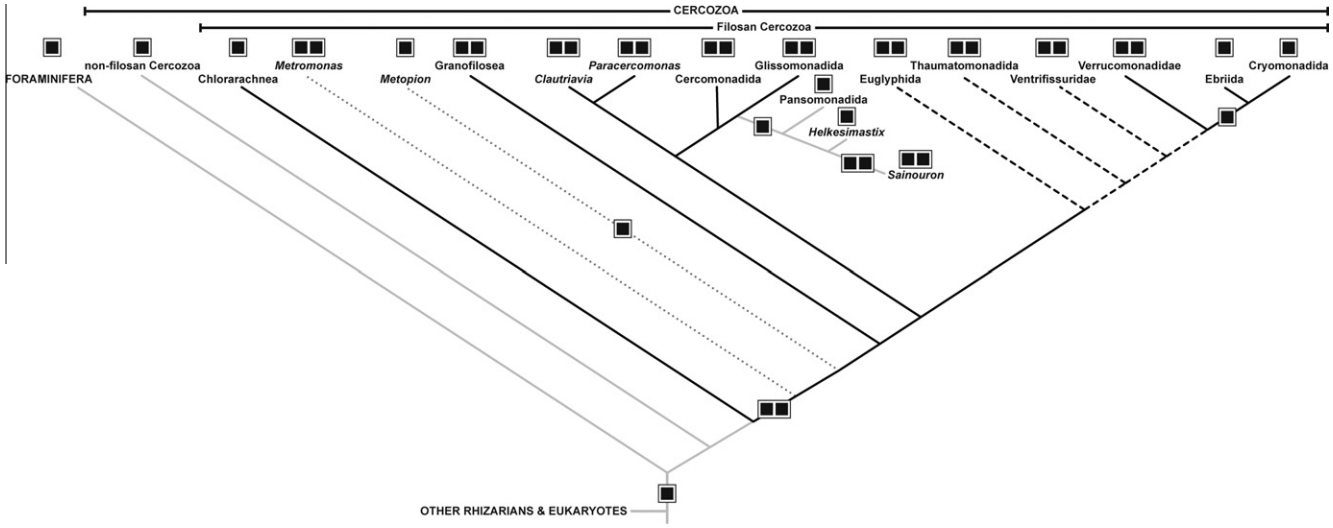

Fig. 5. Schematic tree inferred from the combined SSU and LSU rDNA sequences of the taxa studied (black branches both solid and dashed) showing inferred reversions in the number of polyubiquitin-amino-acid insertions in the evolutionary history of the Cercozoa. The distribution of presence/absence and the number of the polyubiquitin insertion are indicated by filled black boxes. Gray branches, both solid and dotted, indicate positions of other taxa, which were not available to include in our combined SSU and LSU rDNA analyses. Dashed branches indicate the uncertainty of branching order of Euglyphida, Thaumatomonadida, and Ventrifissuridae. Dotted branches indicate hypothetical phylogenetic positions of *Metopion* and *Metromonas* as suggested by several previous studies (Bass et al., 2005, 2009; Bass and Cavalier-Smith, 2004; Cavalier-Smith and Chao, 2003).

erans is inferred to have given rise to the derived state of two amino acid insertions observed in non-chlorarachnean filosan cercozoans. Moreover, the state of two amino acid insertions in these taxa is inferred to have given rise to one amino acid insertion observed in the Cryomonadida and the Ebriida. It should also be mentioned that several other taxa also possess only one amino acid insertion [e.g., *Agitata tremulans* (formerly referred to as *Cercobodo agilis*), *Aurigamonas solis*, *Helkesimastix* sp., and *Metopion fluens*] (Bass et al., 2005, 2009; Vickerman et al., 2005). *Helkesimastix*, in particular, is a sister taxon of *Sainouron acronematica*, which has two amino acid insertions, and pansomonads, which has one amino acid insertion (Cavalier-Smith et al., 2009). Altogether, current data suggests that reversion from two polyubiquitin-amino-acid insertions to one insertion occurred several times independently (Fig. 5).

Although the overall heterogeneity in the size and states of the polyubiquitin insertions are becoming clearer with more data, these data also help reinforce our understanding of the universality of insertions within the Cercozoa; the complete absence of insertions has never been definitively observed in any cercozoan examined so far. This suggests that the presence of the insertion is more biologically/functionally significant, and therefore more evolutionarily conserved, than the specific number and identity of the amino acids inserted (Archibald and Keeling, 2004). However, current data demonstrate that serine is a particularly common polyubiquitin insertion within the Cercozoa. Additional polyubiquitin sequences from both foraminiferans and cercozoans will continue to help establish the universality of the insertions and the utility of this molecular character for recognizing members of this extremely diverse assemblage of microeukaryotic diversity.

3.4. Patterns of polyubiquitin insertions within the Rhizaria

The Rhizarian supergroup is comprised of not only the Cercozoa and the Foraminifera but also the Radiozoa (i.e., acanthareans and polycystines). A putative subclade consisting of the latter two groups is referred to as the Retaria and is based primarily on phylogenetic analyses of combined SSU and LSU rDNA datasets (Cavalier-Smith, 1999, 2002; Moreira et al., 2007). However, the sister relationships between the Foraminifera and the Radiozoa can be a result of long branch attraction artefact. In addition, radiozoans appear to lack the novel polyubiquitin insertions found in cercozoans and foraminiferans (Archibald and Keeling, 2004; Bass et al., 2005). Because polyubiquitin gene sequences are extremely conserved across the full breadth of eukaryotic diversity, the presence of polyubiquitin insertions has been used as a robust molecular synapomorphy that, instead, unites the Cercozoa with the Foraminifera to the exclusion of the Radiozoa (Archibald et al., 2003; Archibald and Keeling, 2004; Bass et al., 2005). The Retaria hypothesis and the hypothesis that the Cercozoa and the Foraminifera are sister lineages are, thus, mutually exclusive. Moreira et al. (2007) reconciled these conflicting data by proposing that the most recent ancestor of the Radiozoa subsequently lost the polyubiquitin insertions after their origin in the most recent ancestor of all rhizarians; in other words, the presence of polyubiquitin insertions in cercozoans and foraminiferans is inferred to be symplesiomorphic in both groups. Although Moreira et al. (2007) provided good support for the Retaria hypothesis, the sister relationship between the Foraminifera and the Cercozoa cannot be ruled out at this time, especially in light of the high degree of evolutionary conservation known for polyubiquitin gene sequences. Therefore, more comprehensive phylogenetic analyses involving more radiozoan taxa and more gene sequences will be required to more definitively establish the deepest relationships within the Rhizaria and the intriguing evolutionary history of the polyubiquitin gene.

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