J. Eukaryot. Microbiol., 51(1), 2004 pp. 113–118

Actin and Ubiquitin Protein Sequences Support a Cercozoan/Foraminiferan Ancestry for the Plasmodiophorid Plant Pathogens

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ABSTRACT. The plasmodiophorids are a group of eukaryotic intracellular parasites that cause disease in a variety of economically significant crops. Plasmodiophorids have traditionally been considered fungi but have more recently been suggested to be members of the Cercozoa, a morphologically diverse group of amoeboid, flagellate, and amoeboflagellate protists. The recognition that Cercozoa constitute a monophyletic lineage has come from phylogenetic analyses of small subunit ribosomal RNA genes. Protein sequence data have suggested that the closest relatives of Cercozoa are the Foraminifera. To further test a cercozoan origin for the plasmodiophorids, we isolated actin genes from *Plasmodiophora brassicae*, *Sorosphaera veronicae*, and *Spongospora subterranea*, and polyubiquitin gene fragments from *P. brassicae* and *S. subterranea*. We also isolated actin genes from the chlorarachniophyte *Lotharella globosa*. In protein phylogenies of actin, the plasmodiophorid sequences consistently branch with Cercozoa and Foraminifera, and weakly branch as the sister group to the foraminiferans. The plasmodiophorid polyubiquitin sequences contain a single amino acid residue insertion at the functionally important processing point between ubiquitin monomers, the same place in which an otherwise unique insertion exists in the cercozoan and foraminiferan proteins. Taken together, these results indicate that plasmodiophorids are indeed related to Cercozoa and Foraminifera, although the relationships amongst these groups remain unresolved.

Key Words. Cercomonads, Cercozoa, chlorarachniophytes, euglyphids, Foraminifera, phylogeny, Plasmodiophora, Plasmodiophorida, Plasmodiophoromycota, polyubiquitin.

THE plasmodiophorids are an enigmatic group of obligate intracellular parasites, best known as pathogens of economically important plants and as vectors for disease-causing plant viruses. The order Plasmodiophorida (informally plasmodiophorids) contains 10 genera and 35 species, and includes *Spongospora subterranea*, the causative agent of powdery scab disease in potato, and *Plasmodiophora brassicae*, which causes club root disease in cabbage and other Brassicales (Braselton 2000). The most striking feature of plasmodiophorids is their possession of a peculiar form of closed mitosis known as cruciform nuclear division, in which a persistent nucleolus elongates perpendicular to the condensed metaphase chromosomes (Braselton, Miller, and Pechak 1975; Dylewski, Braselton, and Miller 1978). The group also has several other unusual cell biological characteristics, including the 'Rohr and Stachel', a cellular protrusion used by plasmodiophorid zoospores in the infection of host cells (Aist and Williams 1971), and the formation of multinucleate plasmodia inside their hosts (reviewed by Braselton 1995).

A great deal is known about the life cycles, infection strategies, and basic ultrastructure of plasmodiophorid parasites. However, their highly derived nature has made it difficult to place these organisms in the context of eukaryotic evolution. Historically, the plasmodiophorids have been allied with fungi (e.g. Waterhouse 1972), due to the fact that they produce spores and have been intensely studied by mycologists from the perspective of plant disease. However, they have also been considered protozoa, and the nomenclature for the group has varied considerably depending on their presumed phylogenetic affinities. The recent acquisition of molecular sequence data from plasmodiophorids has shed considerable light on their evolutionary origins. Phylogenetic analyses of small subunit ribosomal RNA (SSU rRNA) genes have revealed that these organisms are not true fungi (Castlebury and Domier 1998; Ward and Adams 1998) and have suggested that they belong to the protist phylum Cercozoa (formerly Rhizopoda, Bulman et al. 2001; Cavalier-Smith 2000; Cavalier-Smith and Chao 1997; Cavalier-Smith and Chao 2003a; Cavalier-Smith and Chao 2003b; Van de Peer et al. 2000). The Cercozoa are an extraordinarily diverse assemblage of flagellate, amoeboflagellate, and amoeboid protists that have only recently been recognized as constituting a monophyletic group through the consideration of molecular data. Included in the Cercozoa are the chlorarachniophytes, the cercomonad flagellates, the thaumatomonads, and the euglyphid amoebae (Cavalier-Smith and Chao 2003b).

While SSU rRNA phylogenies have been extremely useful in recognizing the Cercozoa as a monophyletic protist lineage, they have been less successful at placing these organisms in the global picture of eukaryotic phylogeny (although see Cavalier-Smith and Chao 2003a). Significant advances in this area have come from consideration of protein sequence data. Phylogenetic analyses of the cytoskeletal protein actin have suggested that Cercozoa are closely related to the Foraminifera (Keeling 2001), an abundant group of marine and freshwater protists, characterized by the presence of organic or mineralized tests (shells) and granulose pseudopodia (Lee et al. 2002). Support for the idea of a specific cercozoan/foraminiferan relationship has been significantly bolstered with the recent discovery of an unusual polyubiquitin gene structure shared between Cercozoa and Foraminifera but absent in all other known eukaryotes (Archibald et al. 2003). Collectively, therefore, actin and polyubiquitin are presently two of the best markers with which to test a possible relationship with Cercozoa or Foraminifera. Here we present the first protein sequence data bearing on the question of the evolutionary position of the plasmodiophorids. We sequenced actin and polyubiquitin genes from plasmodiophorids in three different genera. The results support the hypothesis that they are related to Cercozoa and Foraminifera.

MATERIALS AND METHODS

DNA samples and protist cultures. A sample of *Plasmodiophora brassicae* genomic DNA (gDNA) was kindly provided by Shin-ichi Ito (Yamaguchi University, Japan) and gDNA samples of *Spongospora subterranea* and *Sorosphaera veronicae* were provided by Simon R. Bulman (New Zealand Institute for Crop and Food Research, New Zealand) and James P. Braselton (Ohio University, USA). A culture of the chlorarachniophyte *Lotharella globosa* (strain CCMP1729) was provided by Ken-ichiro Ishida (Kanazawa University, Japan) and maintained in f2-Si medium at 20 °C using a 16-h light/ 8-h dark cycle. Genomic DNA was extracted from a 100-ml culture of

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L. globosa cells using the DNeasy Plant Mini Kit (Qiagen, Santa Clarita, CA).

Amplification, cloning, and sequencing of actin and ubiquitin genes [2]. Actin coding regions were amplified from the gDNAs of *P. brassicae*, *S. subterranea*, *S. veronicae*, and *Lotharella globosa* using the following PCR primers: ActF2: 5′-GAGAAGATGACNCARATHATGTTYGA-3′; ActR1: 5′-GGCCTGGAARCAYTTNCGRTGNAC-3′. Polyubiquitin gene fragments were amplified with the following primer pair: UBIQ1: 5′-GGCCATGCARATHTTYGTNAARAC-3′; IUB2: 5′-GATGCCYTCYTTRTCYTGDATYTT-3′. PCR reactions were performed as follows: after an initial 3-min denaturation at 94 °C, 45 cycles of 45 sec at 92 °C, 1 min at 50 °C, and 1 or 1.5 min at 72 °C were performed. All reactions were finished with a final 5-min extension at 72 °C. The UBIQ1/IUB2 primer set generates a ladder of ubiquitin fragments ranging from a half-monomer to increasing numbers of the polyubiquitin tract. Amplification products corresponding to 1.5 or 2.5 polyubiquitin repeat units were purified using the UltraClean™ 15 DNA purification kit (MOL BIO Laboratories, Solana Beach, CA), cloned into pCR2.1 using the Topo TA cloning kit (Invitrogen, Carlsbad, CA) and sequenced. Actin PCR products of the expected size were purified and cloned using the same procedure. For each gene, three or more independent clones were sequenced from each species.

Phylogenetic analyses. Conceptual amino acid translations for the actin genes of the plasmodiophorids and *L. globosa* were added manually to a comprehensive alignment of actin protein sequences. Phylogenetic analyses were performed on three different data sets. The first data set contained 76 sequences from organisms representing the full spectrum of eukaryotic diversity and the second was a smaller alignment of 49 sequences in which particularly divergent and/or highly similar sequences were removed. The third data set was the 49-sequence alignment with the foraminiferan sequences excluded. Alignments are available from the authors upon request.

Phylogenetic trees were inferred from all three data sets using maximum likelihood (ML) and ML-distance methods of tree reconstruction. ML trees were constructed with PROML in PHYLIP 3.6 (http://evolution.genetics.washington.edu/phylip.html) using the Dayhoff substitution matrix, one randomized sequence-input order, the global rearrangements option, and an among-site rate variation model using a six rate category discrete approximation to the Γ distribution plus an additional invariable sites category. TREE-PUZZLE 5.0 (Strimmer and von Haeseler 1996) was used to estimate the relative rates for each category. ML-distance trees were inferred from Γ-corrected distance matrices (calculated using TREE-PUZZLE 5.0, as above) using weighted neighbor-joining (WEIGHBOR; Bruno, Socci, and Halpern 2000), BIONJ (Gascuel 1997), and Fitch-Margoliash (FITCH in PHYLIP 3.6). For Fitch-Margoliash analyses, the global rearrangements option was used and the sequence input order was randomized once. Statistical support for ML and ML-distance trees was obtained by bootstrapping 100 data sets from the original data with SEQBOOT (PHYLIP 3.6). For PROML trees, 100 data sets were bootstrapped under a uniform-rates model with the global rearrangements option and a single randomization of the sequence input order. For the bootstrapping of ML-distance trees, Γ-corrected distance matrices were inferred from the re-sampled datasets using PUZZLEBOOT (A. Roger and M. Holder; www.tree-puzzle.de), with the parameters described above.

[2] New sequences were deposited in GenBank under the following accession numbers: AY452179-AY452196.

RESULTS AND DISCUSSION

Plasmodiophorid and chlorarachniophyte actin genes. For *P. brassicae* and *L. globosa*, three distinct copies of actin were isolated and two were sequenced from *S. veronicae*, while a single actin gene was amplified from *S. subterranea*. Within a given organism, the different actin gene copies were typically highly similar to one another (sharing > 90% identity), and in the case of *P. brassicae*, the actin-1 and actin-2 genes differed only at synonymous (silent) sites. No introns were found in any of the plasmodiophorid or *L. globosa* actin genes determined in this study.

Actin phylogeny. The newly obtained *P. brassicae*, *S. subterranea*, and *S. veronicae* actin sequences form a strongly supported monophyletic group in phylogenetic analysis, and the three *L. globosa* genes branch with the other chlorarachniophyte genes characterized in an earlier study (Fig. 1) (Keeling 2001). Together, the plasmodiophorid sequences weakly branch near the Cercozoa, consistent with the results of SSU rRNA analyses (e.g. Bulman et al. 2001; Cavalier-Smith and Chao 1997; Cavalier-Smith and Chao 2003b). More specifically, the plasmodiophorids form a poorly supported but consistently observed clade with the Foraminifera. This result is potentially significant, given that the present analysis represents the first instance in which molecular data from both groups can be reliably compared to one another. While SSU rRNA sequences are available from plasmodiophorids and foraminiferans, the foraminiferan genes are extraordinarily divergent (Pawlowski et al. 1996; Pawlowski et al. 1997) and SSU rRNA phylogenies do not recover the cercozoan/foraminiferan clade obtained in actin trees (Keeling 2001). In general, actin phylogeny recovers most of the well-established large-scale eukaryotic groups, such as the Euglenozoa, Fungi, and plants and green algae, but the support for these groups is typically low and the relationships amongst them unresolved. The phylogeny is also characterized by a number of divergent sequences, such as those of ciliates, red algae, diplomonads, and parabasalids (Fig. 1).

In an attempt to more accurately infer the evolutionary position of plasmodiophorids, a smaller data set was assembled in which select sequences were excluded from the alignment. This involved removing most of the long-branch sequences at the ''base'' of the tree shown in Fig. 1, such as the actins of *Giardia lambia* and *Trichomonas vaginalis*, as well as those from ciliates and euglenozoans. The two paralogs from the foraminiferan *Ammonia* sp. were also removed, as one of these was somewhat divergent. Finally, most of the highly similar lineage-specific actin paralogs present in various chlorarachniophyte, plasmodiophorid, and cercomonad species were deleted in order to reduce the total number of sequences. The resulting data set was analyzed using ML-distance and full ML methods.

As was observed in phylogenies inferred from the larger alignment, the plasmodiophorids branch with the cercozoan/foraminiferan sequences. The statistical support for this placement increased, although was still marginal (61%, 63%, and 54% using PROML, Fitch-Margoliash and BIONJ, respectively). A specific connection between the plasmodiophorids and foraminiferans was also observed with all phylogenetic methods, albeit with low support (41% to 50%). Within foraminiferans, two distinct actin gene families have been described, and the members of each family cluster together in phylogenetic analyses irrespective of species (Pawlowski et al. 1999a; Pawlowski et al. 1999b). Curiously, in PROML analyses (Fig. 2A) the plasmodiophorid sequences branch specifically with the foraminiferan actin-1 paralog. At face value, this would suggest that the duplication of the foraminiferan actin paralogs predated the plasmodiophorid/foraminiferan divergence. However, this to-

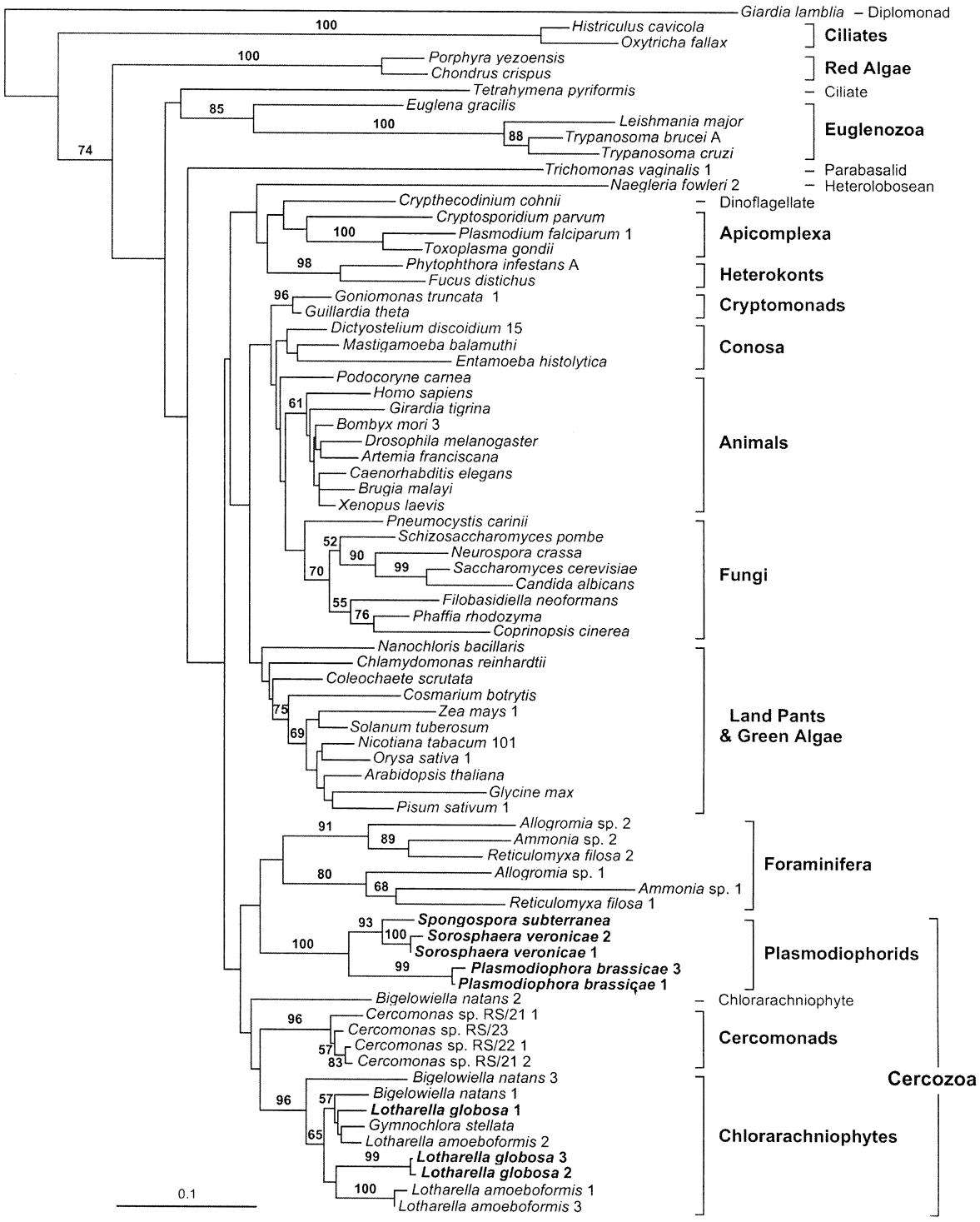

Fig. **1.** Phylogenetic position of plasmodiophorids based on actin protein sequences. The newly obtained plasmodiophorid and chlorarachniophyte actin sequences were added to a comprehensive alignment of actin proteins and phylogenetic trees were inferred. The phylogeny shown is a maximum likelihood (ML)-distance tree inferred from an alignment of 76 actin sequences covering the full breadth of eukaryotic diversity, and is arbitrarily rooted with the actin sequence from the diplomonad *Giardia lamblia*. Bootstrap values (100 replicates) are shown where > 50%. The major eukaryotic groups are highlighted and the plasmodiophorid and chlorarachniophyte sequences determined in this study are in bold. The scale bar indicates the estimated number of amino acid substitutions per site.

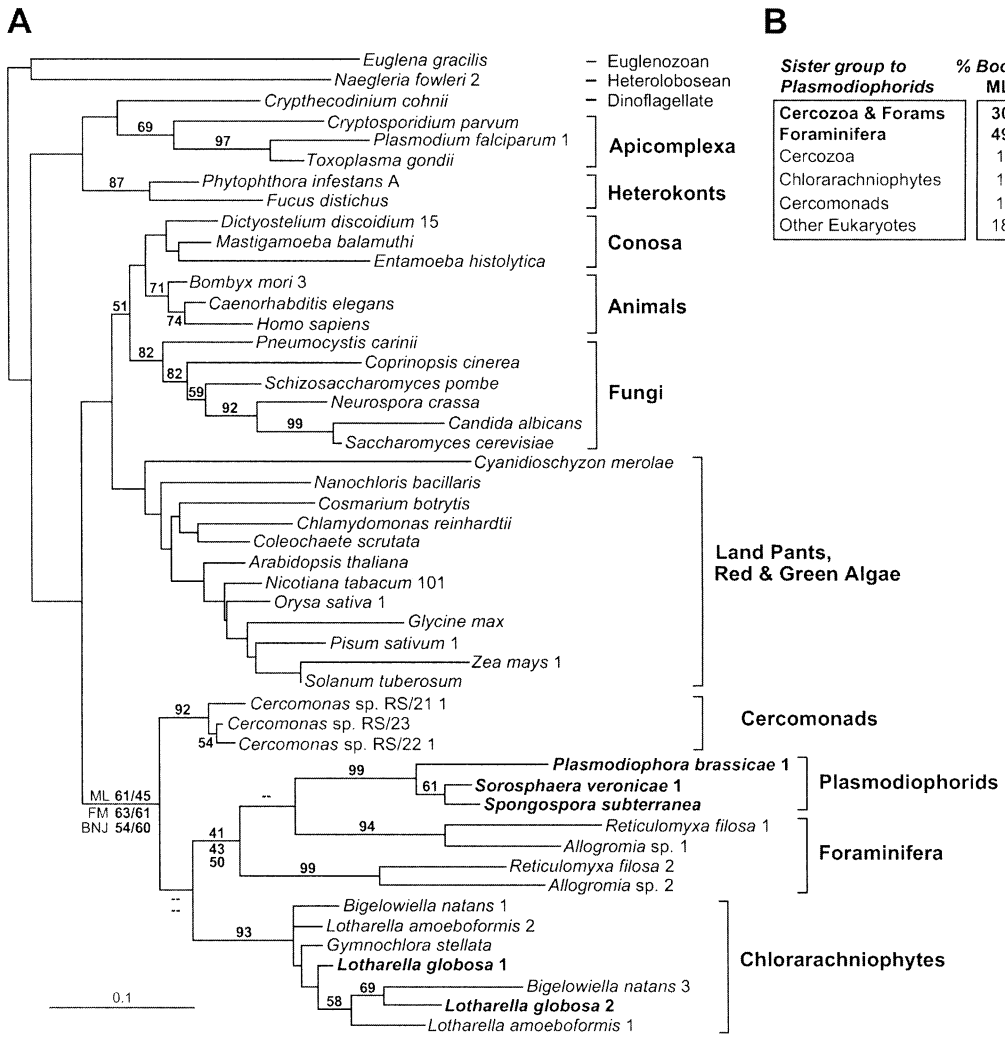

Fig. **2.** Evolutionary position of plasmodiophorids based on actin protein sequences. (**A**) Maximum likelihood (ML) phylogenetic tree ($-\ln L = 5269.67697$) constructed with PROML taking into account among-site rate heterogeneity (see text). The alignment contained 49 sequences and 232 amino acid positions. The plasmodiophorid and *Lotharella globosa* sequences determined in this study are in bold. PROML bootstrap values (100 replicates) are shown for all nodes where > 50%, and for specific nodes involving the placement of the plasmodiophorid sequences, ML-distance values are also provided if > 40% (ML, Fitch-Margoliash (FM), and BIONJ (BNJ), top-to-bottom). For the node uniting the Cercozoa, Foraminifera, and plasmodiophorids, the second set of bootstrap values corresponds to the results of analyses in which the foraminiferan sequences have been removed. The scale bar indicates the estimated number of amino acid substitutions per site. (**B**) Bootstrap partitions for the phylogeny shown in (A) for ML, Fitch-Margoliash (FM), and BIONJ (BNJ) methods. The numbers indicate the proportion of the 100 bootstrap trees for each method in which the plasmodiophorids branched as the sister group to Cercozoa and Foraminifera (Forams), Foraminifera, Cercozoa, Chlorarachniophytes, Cercomonads or another eukaryotic group. The ''Cercozoa & Forams'' partition included instances in which plasmodiophorids branched with some but not all of the cercozoan and foraminiferan sequences.

pology was not present in the PROML bootstrap consensus tree and was not observed in any of the ML-distance analyses and is therefore unlikely to be significant.

These phylogenetic trees (Fig. 1 and 2A) reveal that the foraminiferan actin genes, and to a lesser extent, those of plasmodiophorids, are somewhat divergent relative to chlorarachniophyte and cercomonad actins. To determine whether this divergence is influencing the position of the plasmodiophorid genes in the phylogenies, we performed analyses using an additional alignment in which the foraminiferan sequences had been removed. The support for the placement of the plasmodiophorids with the Cercozoa in these phylogenies was very similar (data not shown) to that obtained in analyses in which Foraminifera were included (Fig. 2A). This indicates that the plasmodiophorids were likely not simply attracted to the Cercozoa/Foraminifera clade due to long branch attraction, and that the mediocre support for the entire clade was not solely due to the inclusion of foraminiferan sequences. In the absence of the foraminiferans, the plasmodiophorids showed no affinity for any particular cercozoan lineage, but instead branched as the deepest group in the Cercozoa (data not shown), consistent with SSU rRNA analyses (e.g. Bulman et al. 2001; Cavalier-Smith and Chao 1997; Cavalier-Smith and Chao 2003a).

To investigate the relationship between the plasmodiophorid actin sequences and those of Cercozoa and Foraminifera more closely, we examined the bootstrap partitions for the 100 ML and ML-distance trees obtained with the 49-sequence data set (Fig. 2B). The modest support for the plasmodiophorid/Cercozoa/Foraminifera clade seems in large part to be due to the unstable position of particular sequences. For example, while

the ML bootstrap support for this grouping is only 61%, only 18 of the 100 bootstrap trees showed plasmodiophorids branching with a eukaryotic group other than Foraminifera and/or Cercozoa, and no obvious pattern was observed in this subset of the data. Most of the bootstrap trees (49%) showed plasmodiophorids clustering with at least one of the two foraminiferan paralogs, and in 30 of the trees, plasmodiophorids formed a cluster with most or all of the foraminiferan and cercozoan sequences. Only in a single instance were the plasmodiophorid sequences positioned as a sister to chlorarachniophytes, cercomonads, or cercozoans as a whole. The apicomplexan, heterokont, and *Cyanidioschyzon merolae* sequences, both individually and in combination, had a tendency to interrupt the plasmodiophorid/Cercozoa/Foraminifera clade (data not shown), while the position of the actin-2 paralog of foraminiferans was somewhat unstable, with a tendency to move outside of the foraminiferan/cercozoan clade. A similar pattern of instability was observed when the results of ML-distance bootstrap analyses were examined closely (Fig. 2B). Overall, the results of the actin phylogenies presented above support the hypothesis that plasmodiophorids are specifically related to Cercozoa and Foraminiferans, although the relative branching order among the three groups is not resolved.

Plasmodiophorid polyubiquitin genes. Ubiquitin is a 76-amino acid eukaryotic protein that plays a central role in a large number of fundamental cellular processes including endocytosis, signal transduction, and regulation of the cell cycle (reviewed by Hershko and Ciechanover 1998). Ubiquitin monomers are typically encoded as fusions with ribosomal protein genes (Ozkaynak et al. 1987; Redman and Rechsteiner 1989) or as multimers of head-to-tail ubiquitin coding units (polyubiquitins) (Ozkaynak, Finley, and Varshavsky 1984).

We recently showed that the Cercozoa and Foraminifera possess a highly unusual polyubiquitin structure in which extra amino acids have been inserted at the junction points between adjacent ubiquitin monomers (Archibald et al. 2003). In order to determine if the polyubiquitin genes of plasmodiophorids also have this characteristic, we amplified polyubiquitin gene fragments from *P. brassicae* and *S. subterranea*. Seven different polyubiquitin fragments were isolated from *P. brassicae*, all but one of which encoded proteins that are identical in amino acid sequence. For *S. subterranea*, two distinct sequences were obtained, and the *S. subterranea* polyubiquitin 2 gene contains a 51-nucleotide intron that is absent in polyubiquitin 1 and all of the *P. brassicae* sequences. Polyubiquitin genes were amplified from *S. veronicae* gDNA samples, but these sequences were found to be highly similar to plant polyubiquitins and were thus likely amplified from host cell DNA. Plant polyubiquitins were also obtained during *S. subterranea* amplifications, but were easily distinguished from the plasmodiophorid genes.

The plasmodiophorid sequences contain an insertion of a single threonine residue at the monomer-monomer boundary, immediately following the carboxy-terminal glycine residue at position 76 (G76) (Fig. 3). All of the polyubiquitin genes isolated from *P. brassicae* and *S. subterranea* encode proteins with a threonine insertion, although this residue differs from the serine, alanine, serine/glycine or serine/alanine insertions found in the same region of the cercozoan and foraminiferan polyubiquitins. It is, however, significant that threonine and serine have very similar biochemical properties and can readily substitute for one another in proteins. As discussed previously (Archibald et al. 2003), the evolutionary significance of these insertions lies in the fact that ubiquitin is an extremely highly conserved protein and no length variation exists in any known ubiquitin, other than those of Cercozoa and Foraminifera. The monomer-monomer boundary region is extremely important: by definition, it is involved in the processing of ubiquitin polyproteins into free monomers and G76 is known to be critical for the conjugation of ubiquitin monomers to target substrates (Hershko and Ciechanover 1998; Pickart 2001). Therefore, while there is clearly length and sequence heterogeneity in the plasmodiophorid, cercozoan, and foraminiferan polyubiquitin insertions, it is extremely unlikely that they occurred independently in the three groups. This unusual molecular character is very likely a derived feature shared by plasmodiophorids, Foraminifera, and Cercozoa.

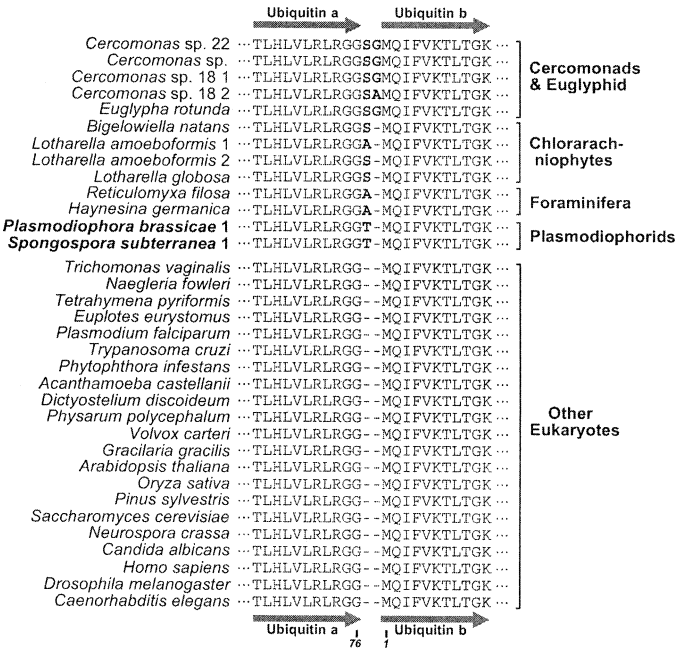

Fig. **3.** Evidence from ubiquitin protein sequences that plasmodiophorids are related to Cercozoa and Foraminifera. The figure shows the highly conserved ubiquitin monomer-monomer junction in polyubiquitin proteins from plasmodiophorids, Cercozoa (chlorarachniophytes, cercomonads, and a euglyphid) and Foraminifera aligned with those of other eukaryotes. The plasmodiophorid proteins possess a threonine (T) residue (highlighted in bold) inserted at the same position as the single or double amino acid insertions in the cercozoan and foraminiferan sequences. The canonical methionine (M) residue at position one is labeled, as is glycine 76 (G76), a residue known to play an essential role in the ubiquitin conjugation reaction.

Are the plasmodiophorids Cercozoa? In early SSU rRNA phylogenies that included sequences from plasmodiophorids, the lineage branched robustly as a sister to the cercomonads, chlorarachniophytes, thaumatomonads, and euglyphid amoebae, but did not show a strong affinity for any particular cercozoan group (Bulman et al. 2001; Cavalier-Smith and Chao 1997). More recent SSU rRNA phylogenies are similarly unresolved and have complicated matters further by indicating that a number of other groups are also members of, or closely related to the Cercozoa, including the haplosporidian parasites, Heliozoa, Radiolaria, Apusozoa, and *Gromia* (Burki, Berney, and Pawlowski 2002; Cavalier-Smith and Chao 2003a; Cavalier-Smith and Chao 2003b). While foraminiferan SSU rRNA sequences are too divergent to be reliably used in global eukaryotic phylogenies (Pawlowski et al. 1997), analyses of actin and polyubiquitin have shown the Foraminifera to be part of this radiation as well (Archibald et al. 2003; Keeling 2001). The actin trees presented here are suggestive of a specific relationship between plasmodiophorids and foraminiferans, but the support for this relationship is very weak and the topology is at odds with the

distribution of a single nucleotide deletion in a conserved region of SSU rRNA, present in ''classical'' Cercozoa (chlorarachniophytes, cercomonads, euglyphid amoebae, thaumatomonads), *Gromia*, haplosporidians, and plasmodiophorids, but not in Heliozoa, Radiolaria, Apusozoa or Foraminifera (Cavalier-Smith and Chao 2003a). In sum, while molecular analyses have shown convincingly that plasmodiophorids are not fungi, they are at present unable to identify their closest relatives within the cercozoan / foraminiferan radiation. Protein sequence data from more diverse members of both groups will hopefully resolve this issue, and one promising candidate is the largest subunit of RNA polymerase B (Longet et al. 2003). The ubiquitin insertion character also has the potential to be particularly important for inferring the evolutionary history of Cercozoa and Foraminifera, as a lineage found to be related to this group but to lack the insertion would be an excellent candidate for an early-diverging member of the group.

ACKNOWLEDGMENTS

We thank Simon R. Buhlman, James P. Braselton, and Shinichi Ito for the plasmodiophorid DNA samples used in this study and Ken-ichiro Ishida for a culture of *Lotharella globosa*. We also thank Brian S. Leander for critical review of this manuscript. This work was supported by a grant (227301-00) from the Natural Science and Engineering Research Council of Canada (NSERC) to PJK. JMA is supported by postdoctoral fellowships from the Canadian Institutes of Health Research (CIHR) and the Killam Foundation (University of British Columbia). PJK is a scholar of the Canadian Institute for Advanced Research, CIHR, and the Michael Smith Foundation for Health Research.

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Received 06/10/03; accepted 10/30/03