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Ellobiopsids of the Genus *Thalassomyces* are Alveolates

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ABSTRACT. Ellobiopsids are multinucleate protist parasites of aquatic crustaceans that possess a nutrient absorbing 'root' inside the host and reproductive structures that protrude through the carapace. Ellobiopsids have variously been affiliated with fungi, 'colorless algae', and dinoflagellates, although no morphological character has been identified that definitively allies them with any particular eukaryotic lineage. The arrangement of the trailing and circumferential flagella of the rarely observed bi-flagellated 'zoospore' is reminiscent of dinoflagellate flagellation, but a well-organized 'dinokaryotic nucleus' has never been observed. Using small subunit ribosomal RNA gene sequences from two species of *Thalassomyces*, phylogenetic analyses robustly place these ellobiopsid species among the alveolates (ciliates, apicomplexans, dinoflagellates and relatives) though without a clear affiliation to any established alveolate lineage. Our trees demonstrate that *Thalassomyces* fall within a dinoflagellate + apicomplexa + Perkinsidae + ''marine alveolate group 1'' clade, clustering most closely with dinoflagellates. However, the poor statistical support for branches within this region indicates that additional data will be needed to resolve relationships among these taxa.

Key Words. Alveolata, dinoflagellate, Ellobiopsidae, parasite, phylogenetic analysis, SSU rRNA, *Thalassomyces*.

WITHOUT a doubt, some of the strangest looking organisms are found among marine zooplankton. While most of the organisms brought up in plankton nets can be readily sorted into known plant, animal, fungal, or protist groups, some remain enigmatic—their phylogenetic affinities and taxonomy largely unknown. Various crustaceans often harbor visible infections by bizarre multinucleate parasites called ellobiopsids (Ellobiopsidae). These organisms appear as 'cysts' or 'tufts' of tissue on the mouthparts, antennae, or carapace of infected crustaceans, though closer scrutiny reveals that the parasites penetrate into the interior of the hosts to varying extents. The evolutionary history of these understudied parasites has been a subject of speculation for almost 100 years (see Galt and Whisler 1970 for review), but few cytological and no molecular studies have been conducted to establish their phylogenetic affiliation.

Other than distribution and abundance surveys, very little is known about ellobiopsids. There are five described genera: *Thalassomyces*, *Ellobiopsis*, *Parallobiopsis*, *Ellobiocytsis*, and *Rhizellobiopsis*. The best characterized of these are *Thalassomyces* and *Ellobiopsis*. The other three are epibionts that resemble *Ellobiopsis* in external morphology (Kane 1964) and one, *Rhizellobiopsis*, infects polychaete worms rather than crustaceans (Zachs 1923). The inclusion of *Parallobiopsis*, *Ellobiocytsis*, and *Rhizellobiopsis* in Ellobiopsidae may be regarded as provisional and their affinity to the group requires more thorough study and detailed taxonomic revision.

For the most part, *Thalassomyces* and *Ellobiopsis* infect a wide array of pelagic marine malacostracan crustaceans, including, shrimp, euphausiids, mysids, amphipods, and copepods (Shields 1994; Vader 1973). There are recent reports of infected freshwater copepods from North American, African and possibly European lakes (Bridgeman, Messick, and Vanderploeg 2000; Rayner and King 1986). Many ellobiopsid hosts are important planktivores that serve as primary prey for larger animals.

Both *Ellobiopsis* and *Thalassomyces* have 'roots' that penetrate through their host's cuticle and reside within the body cavity. The external portions of the parasite develop into reproductive structures (Fig. 1). The simple root of *Ellobiopsis* may cause localized tissue damage where it penetrates into the host. The more extensive internal absorptive network of *Thalassomyces* may cause more serious pathologies when infiltrating host nervous or gonadal tissues. Ellobiopsid infections can cause host sterility and/or alteration in behavior and endocrine function (Einarsson 1945; Hoffman and Yancey 1966; Mauchline 1966; Wickstead 1963). Unfortunately, the effects of parasitism on the crustacean-host populations are incompletely known, preventing an accurate assessment of this parasite's ecological and economic importance.

Classification of Ellobiopsidae is hindered by the limited knowledge of their life histories and life cycles. A *Thalassomyces* infection initiates inside the host with the development of the rooting network (Mauchline 1966). Eventually the parasite ulcerates the host's carapace allowing development of reproductive structures. These consist of bifurcating, stem-like trophomeres that, when mature, terminate with a 'spore' forming gonomere (Fig. 1). *Ellobiopsis* species are presumed to initiate infection on the external carapace of their host (Fage 1936). Their rudimentary 'root' pierces the host carapace and anchors a single unbranched reproductive stalk from which 'spores' form at the distal end.

The 'spores' of *Ellobiopsis* do not appear to be flagellated (Hovasse 1951). However, the 'zoospores' of *Thalassomyces* develop into trophic, dispersive bi-flagellates (Galt and Whisler 1970). In *Thalassomyces marsupii* and *Thalassomyces boschmai*, these cells possess a trailing flagellum and a circumferential flagellum, similar to the typical dinoflagellate arrangement (Galt and Whisler 1970). Although these flagellates seemed to present a mode of parasite transmission, they failed to be infective to the host species that nurtured them (Galt and Whisler 1970). It is likely that not all of the life stages of even the best-studied ellobiopsid species are known.

The peculiarities of ellobiopsids have thwarted attempts to classify them. They have been variously linked with 'colorless algae', fungi (including the chytridiomycetes) (Grassé 1952; Jepps 1937; Kane 1964), proposed to be close relatives of dinoflagellates (Caullery 1910; Chatton 1920; Galt and Whisler

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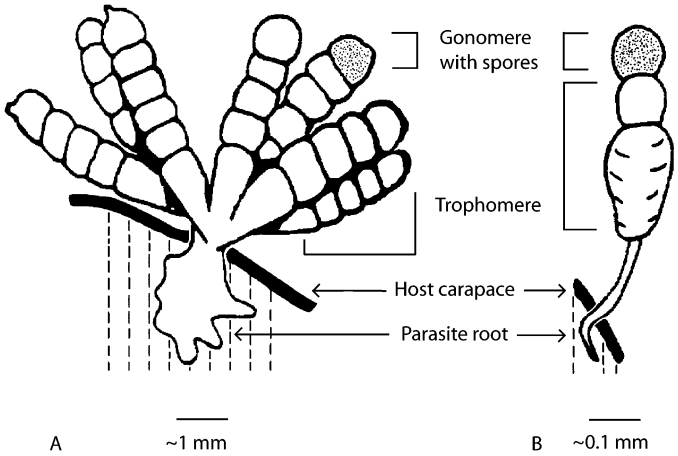

Fig. **1.** Schematic drawing of (**A**) *Thalassomyces* sp. and (**B**) *Ellobiopsis* sp. (adapted from Kane 1964).

1970), or aptly considered 'protists of unknown affiliation' (Boschma 1949; Collard 1964; McCauley 1962). The few ultrastructural studies reported (Galt and Whisler 1970; Whisler 1990) have not uncovered any structure that strongly suggests affinities with any particular eukaryotic lineage. This uncertainty led us to explore the phylogenetic placement of this enigmatic group of organisms with molecular sequence data. Here we report the small subunit ribosomal gene sequence (SSU rRNA) of two species of *Thalassomyces* and their phylogenetic affinities among eukaryotes.

MATERIALS AND METHODS

Organisms, DNA isolation, and gene sequencing. Zooplankton were captured at night in an Isaacs-Kidd net fished from the surface to 800 meters off the coast of Long Beach, California in March 2000 and May 2001 aboard the RV Yellowfin, owned and operated by the Southern California Marine Institute. Crustaceans were visually examined for parasites. A single specimen of an unidentified *Thalassomyces* species was obtained from the hyperiid amphipod host, *Cystisoma* sp., in March 2000. In May 2001, three representatives of *Thalassomyces fagei* were found infecting euphausiids (krill) provisionally identified as either *Nematobrachion flexipes* or *Nematoscelis difficilis*. The external, reproductive, portions of the parasites were removed with forceps and preserved in absolute ethanol. Genomic DNA was extracted from single parasites by pulverizing the tissue in the reagent DNAzol (GibcoBRL, Rockville, MD; Chomczynski et al. 1997), followed by centrifugation and ethanol precipitation. Small subunit ribosomal genes were amplified in vitro using eukaryotic specific primers (Medlin et al. 1988) and sequenced directly and completely in both orientations using internal primers.

Sequence alignments, taxon selection and phylogenetic analyses. The two *Thalassomyces* SSU rRNA sequences were initially aligned in a 42-taxon data set comprising representatives from most major eukaryotic lineages, rooted with two archaebacterial SSU rRNA sequences. Secondary structure motifs aided in aligning the primary nucleotide sequences and only those positions of unambiguous alignment were utilized for phylogenetic analyses (1,096 sites). Based on the results of these analyses, an additional data set was constructed to critically evaluate the relationship of *Thalassomyces* among various alveolates. This 'refined' data set encompassed the breadth of alveolate phylogenetic diversity and consisted of 53 taxa with 1,393 aligned sites.

The branching of *Thalassomyces* SSU rRNA sequences varied with the composition of the alveolates in the data set, but was not particularly sensitive to the outgroup (non-alveolate) sequences employed. In an attempt to prevent analysis artifacts, fast evolving sequences (i.e. long-branches) were omitted where possible. Sequences omitted from analyses included those from *Oxhyrris marina*, *Haplozoon axiothellae* (whose gross morphology is reminiscent of ellobiopsids), *Parvilucifera infectans*, gregarines, haplosporidians, *Colpodella*, and *Plasmodium*, after preliminary analyses determined that none was likely to be very closely related to those of *Thalassomyces*. The divergent SSU rRNA sequences from the parasitic Syndiniales dinoflagellates *Amoebophrya* and the partial sequences of *Hematodinium* were not used. These parasites formed a strongly supported clade with the environmental sequences from 'marine alveolate group 2' (nomenclature of López-Garcia et al. 2001) which served as surrogate (labeled '*Amoebophrya*-clade' in Fig. 3, data not shown). Long branched gonyaulacales dinoflagellate sequences (e.g. *Crypthecodinium cohnii*, *Gonylaux spinifera*, and *Fragilidium subglobosum*) destabilized tree topologies and were omitted from analyses. *Thalassomyces* tended to attract the single most divergent gonyaulacalid, drawing it away from the others, outside the rest of the dinoflagellates; *Crypthecodinium cohnii* was especially problematic in this respect (data not shown).

Phylogenetic trees were inferred with maximum likelihood (ML), distance (minimum evolution), and parsimony criteria using PAUP* 4.10b (Swofford 2000). For each data set, the best-fitting model of nucleotide evolution, as determined by hierarchical nested likelihood ratio tests implemented in Modeltest version 3.06 (Posada and Crandall 1998), was a general time reversible model of substitution, incorporating a gamma distribution for among-site rate variation (4 discrete rate categories) plus an estimate of invariable sites (GTR + Γ + I). This model was employed in ML and maximum likelihood–distance (ML-distance) tree reconstructions. Heuristic tree searches were conducted with 100 random taxon additions in parsimony analyses, 10 in the broad-scale ML analysis, and four in the refined ML analyses, followed by 'tree bisection-reconnection' branch rearrangements. One-thousand bootstrap re-sampled data sets were analyzed by parsimony and distance methods, and 352 and 212 re-sampled data sets were analyzed by ML for the broad-scale and refined data sets, respectively.

The difference in log-likelihood scores (ΔlnL) among trees in which *Thalassomyces* sequences branched exclusively with 1) dinoflagellate, 2) *Perkinsus* + relatives, 3) apicomplexan, 4) ciliate, or 5) outgroup sequences (either by forced constraints and re-optimization, or as found in optimal trees) was assessed by the Shimodaira-Hasegawa (SH), Kishino-Hasegawa (KH), approximately unbiased (AU), and the expected likelihood weights (ELW) tests (Kishino and Hasegawa 1989; Shimodaira 2002; Shimodaira and Hasegawa 2001; Shimodaira and Hasegawa 1999; Strimmer and Rambaut 2002). For the ELW method, a set of 220 unique trees was examined, comprising the best trees found in the 212 ML bootstrap replicates, plus the optimum ML, ML-distance, parsimony, and constrained trees. The 95% confidence interval, given this set of trees was calculated using 1,000 bootstrap replications with substitution model parameters re-estimated for each replicate of a Jukes-Cantor distance-corrected neighbor-joining topology using the PERL script ELW.pl (http://hades.biochem.dal.ca/Rogerlab/Software/software.html).

New SSU rRNA gene sequences have been deposited in GenBank (accession numbers AY340590 and AY340591).

RESULTS AND DISCUSSION

The placement of *Thalassomyces* in the eukaryotic tree. The SSU rRNAs from *Thalassomyces* sp. and *T. fagei* are ex-

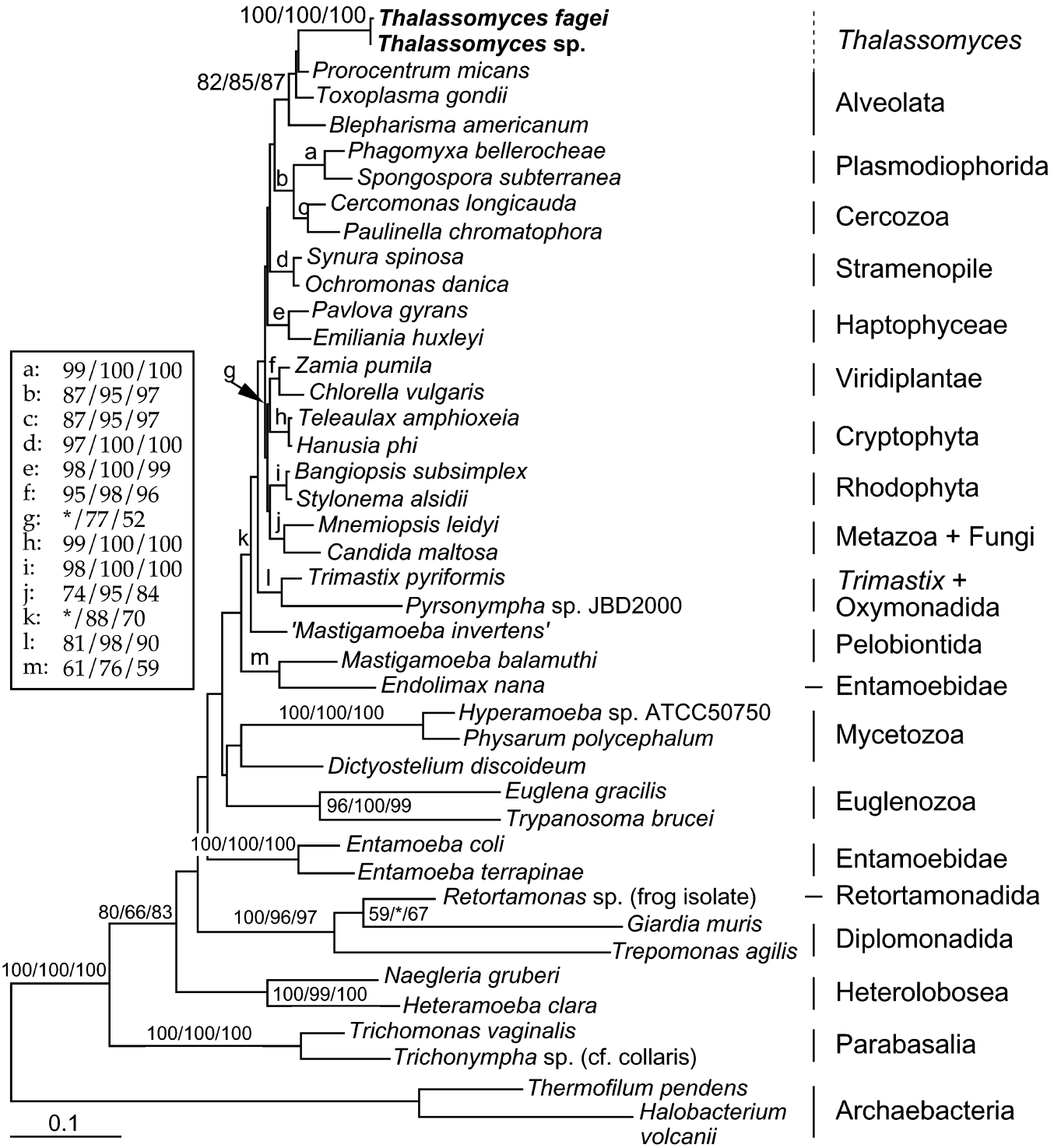

Fig. **2.** Maximum likelihood tree (GTR + Γ + I) based on small subunit rRNA sequences from 42 taxa placing *Thalassomyces* within the Alveolata. Model parameters estimated from the data include the proportion of invariant sites (I) = 0.128 and a gamma shape distribution for site-to-site rate variation (4 rate categories) α = 0.731. Bootstrap values over 50% for ML, ML-distance, and parsimony, respectively, are shown above selected nodes. An asterisk (*) represents bootstrap proportions under 50%. The scale bar represents 10 changes per 100 positions.

tremely similar to one another (97% identical) and branch together in all phylogenetic analyses (Fig. 2, 3). The broad-scale phylogenetic reconstruction, including sequences from representatives of most major eukaryotic lineages, convincingly placed *Thalassomyces* within the alveolates (Fig. 2). Bootstrap support (BS) was high for all methods of analyses employed (82 / 85 / 87 for ML, ML-distance, parsimony, respectively). These molecular data are supported by preliminary ultrastructure studies indicating that 'zoospores' of *Thalassomyces* possess typical alveoli (i.e. 'flattened vesicles and microtubules'

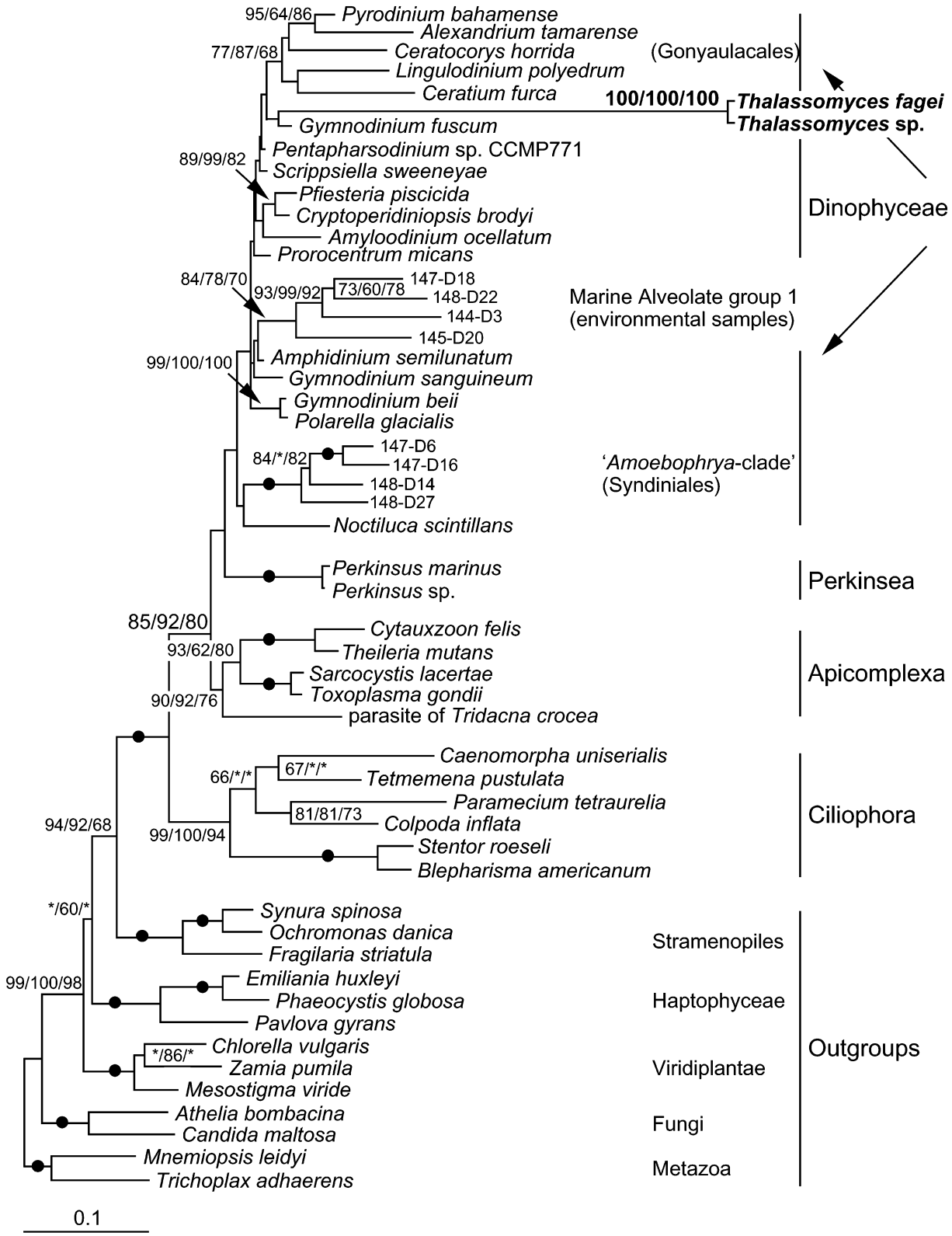

Fig. **3.** Maximum likelihood tree (GTR + Γ + I) based on small subunit rRNA sequences from 40 taxa representing a diverse assemblage of alveolates and outgroups. Bootstrap values over 60% from ML, ML-distance, and parsimony, respectively, are shown above the nodes. An asterisk (*) represents bootstrap proportions under 60%, and a closed circle (●) represents 100% bootstrap support for all methods of analyses. The scale bar represents 10 changes per 100 positions.

underlying the plasma membrane; Whisler 1990). Contrary to earlier hypotheses, *Thalassomyces* sequences do not branch with fungi (or opisthokonts in general), viridiplantae (green plants and algae) or 'colorless algae' (i.e. amongst lineages such as the stramenopiles, cryptopytes, or haptophytes) (Fig. 2).

In an attempt to find the closest relatives of *Thalassomyces*, the sequence alignment was refined to exclude very distantly related sequences and to include the breadth of alveolate diversity, along with numerous outgroup sequences from stramenopiles, haptophytes, plants, fungi, and metazoans (Fig. 3). The alveolates, including *Thalassomyces*, formed a monophyletic assemblage with 100% bootstrap support with all methods. Consistently and robustly, *Thalassomyces* branched in a clade composed of dinoflagellates, perkinsids, 'marine alveolate group 1' (terminology of López-Garcia et al. 2001) obtained from environmental sequences (López-Garcia et al. 2001; Moon-van der Staay, De Wachter and Vaulot 2001), and apicomplexans. Bootstrap support for this large grouping was moderate to strong (BS = 85/92/80 for ML, ML-distance, parsimony, respectively, Fig. 3), yet there was little resolution among major lineages within this radiation. The ML tree shows *Thalassomyces* branching within dinoflagellates, but with no strong affinity for any particular organism. An alternative clustering was seen in ML-distance and parsimony analyses in which *Thalassomyces* branched with *Perkinsus*, though with little bootstrap support (BS = 36/27 ML-distance, parsimony, respectively; data not shown). In these latter analyses, moderate bootstrap support separated *Perkinsus* + *Thalassomyces* from the dinoflagellates + marine alveolate group 1 (BS = 78/70, ML-distance, parsimony, respectively). These alternative trees suggest that Ellobiopsidae, as represented by *Thalassomyces*, may represent an entirely new alveolate lineage separate from both the dinoflagellates and *Perkinsus*. However, there appears to be insufficient phylogenetic information in these data to draw a strong conclusion about their precise affiliation.

The SSU rRNA gene sequences of both *Thalassomyces* species are highly divergent despite being of typical eukaryotic lengths, and only slightly biased in base composition (1,755 bp, 42.4–41.5% G+C, for *T.* sp and *T. fagei*, respectively). This is reflected in the extremely long branch joining them to other taxa (Fig. 2, 3). Because long branches are well known to confound phylogenetic inference (Philippe and Laurent 1998; Stiller and Hall 1999), great care must be taken not to over-interpret *Thalassomyces*' specific position in these trees. In addition to being highly divergent in primary sequence, the stem-loop region designated 'E10_1' (nomenclature of Wuyts et al. 2000) is truncated and differs in structure from all other examined eukaryotes. This secondary structural idiosyncrasy is presumably a derived character that provides no specific information about the alveolate lineage to which *Thalassomyces* may be most closely related.

Has Ellobiopsidae been recovered from environmental DNA sequences? Sampling of SSU rRNA genes from microorganisms smaller than 3–5 μm from deep Antarctic waters (López-Garcia et al. 2001), shallow equatorial waters (Moon-van der Staay, De Wachter, and Vaulot 2001), and hydrothermal vent sediments (López-Garcia et al. 2003) uncovered a number of novel phylogenetic entities and lineages that showed an affiliation with the alveolates. Analyses by López-Garcia et al. (2001) and Moon-van der Staay et al. (2001) placed marine alveolate group 1 basal to the dinoflagellates and not specifically related to any other known sequences. Though slightly larger than the picoplankton filtrates analyzed, it was possible that these environmental DNAs could be derived from ellobiopsids (the 'zoospores' of *Thalassomyces* have an average diameter of 7.5 μm, Galt and Whisler 1970; see Díez, Pedrós-Alió, and Masana 2001 for discussion on the efficiency of size exclusion filtration of biological samples). In all our analyses (Fig. 3), the environmental isolates form a robust, highly supported clade. As in previous analyses (López-Garcia et al. 2001; Moon-van der Staay, De Wachter, and Vaulot 2001), marine alveolate group 1 branched basal to the dinoflagellates in the optimal ML-distance tree (data not shown), but emerged from within the dinoflagellate grouping in our optimal ML tree. No significant difference in the likelihood scores between these two tree topologies was observed by SH, KH, or AU tests ($p > 0.1$). Most significantly though, the *Thalassomyces* sequences never branched with this environmental clade in any optimal tree (ML, ML-distance, parsimony). In only 3% of the ML bootstrap partitions did these sequences cluster (Fig. 4) to the exclusion of all others. Thus, this environmental lineage does not appear to be specifically related to *Thalassomyces* and their identity remains to be determined.

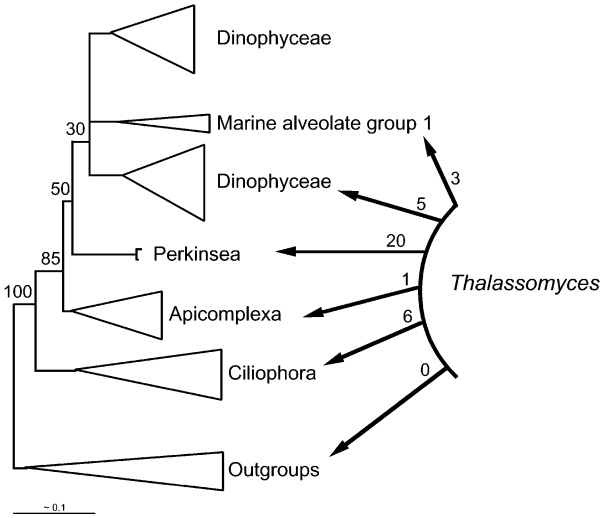

Fig. **4.** Maximum likelihood backbone tree topology of Fig. 3 showing ML bootstrap partitions for *Thalassomyces* branching with specific lineages. The numbers above the arrows are the percentages of ML bootstrap replicates in which *Thalassomyces* sequences branched with specific lineages to the exclusion of all others (212 replicates).

ML Bootstrap partitions—branching preferences of *Thalassomyces* among the alveolates. The maximum likelihood bootstrap partitions were examined in the refined, alveolate-rich data set, to determine whether alternative branching positions of *Thalassomyces* were favored or disfavored by the data. As seen in Fig. 3, 4, the backbone ML topology supports *Thalassomyces* branching within the alveolates in 100% of the ML-bootstrap replicates. In 85% of the bootstrap replicates, *Thalassomyces* branch in a group along with the Apicomplexa, *Perkinsus*, Dinophyceae, and the marine alveolate group 1. This large group is also reasonably well supported in distance and parsimony bootstrap analyses (Fig. 3). Thus, with confidence we can infer that *Thalassomyces* branches somewhere amongst these lineages.

Reciprocally, we can infer unsupported relationships by their low ML-bootstrap partitions. For instance, *Thalassomyces* exclusively branches within an otherwise apicomplexan or ciliate-clade in only 1% and 6% of the ML bootstrap replicates respectively. Therefore it is unlikely that ellobiopsids are specifically related to either of these groups. *Thalassomyces* branches exclusively with *Perkinsus* in 20% of the ML-bootstrap replicates, which is the highest support linking *Thalassomyces* with any single lineage. But this affinity should be interpreted cau-

tiously since long-branch attraction may be mistakenly drawing these most divergent sequences (in the data set) together. *Thalassomyces* specifically branches with Dinophyceae in only 5% of the ML bootstrap replicates to the exclusion of all other lineages but support for clustering *Thalassomyes* in a group composed the Dinophyceae and marine alveolate group 1 is higher (30%). In our analyses, *Thalassomyces* species do not robustly form a clade with any particular organism or lineage.

Statistical testing of alternative topologies within a likelihood framework. We optimized separate ML trees that constrained the *Thalassomyces* sequences to branch with each alveolate lineage and also to branch outside of the alveolates. Then the log-likelihood of these trees along with the optimized ML, ML-distance, and parsimony trees were compared to determine if any of these branching alternatives could be rejected using statistical tests. SH, KH, AU, and ELW tests did not detect significant differences in the log-likelihood scores between the best ML tree (with *Thalassomyces* branching within the dinoflagellates) and trees optimized with *Thalassomyces* constrained to branch exclusively with any alveolate lineage (e.g. *Perkinsus*, apicomplexa, ciliates, marine alveolate group 1, or with the dinoflagellates exclusive of this latter lineage). The only topology that was rejected (α-level < 0.05) was the one in which *Thalassomyces* was constrained to branch with the non-alveolate sequences (e.g. outgroups). These likelihood tests were unable to reject alternative affiliations among *Thalassomyces* and the rest of the alveolates despite the fact that *Thalassomyces* rarely clustered with either the ciliates or apicomplexans in bootstrap analyses (Fig. 3, 4). The difficulty in establishing a robust affiliation of *Thalassomyces* with any particular alveolate group may be exacerbated by the observation that the SSU rRNA sequences of Dinophyceae are likely too conserved to yield well-resolved phylogenies within the phylum and among close relatives.

Speculation based on independent data. Our SSU rRNA analyses do not resolve whether *Thalassomyces* belongs to a described phylum (e.g. Perkinsozoa or Dinophyceae) or represents a novel phylogenetic entity within the alveolates. The lack of pertinent information regarding morphology confounds attempts to further classify ellobiopsids, but a specific affinity with Perkinsozoa seems unlikely—*Perkinsus* and the related parasite *Parvilucifera infectans* (infecting the dinoflagellate *Dinophysis*) have an anterior and a posterior directed flagellum supported by an extensive cytoskeletal investment (Azevedo 1989; Norén, Moestrup and Rehnstam-Holm 1999; Siddall et al. 1997). Although information is currently lacking on the microtubular organization of the *Thalassomyces* 'zoospore' flagellar apparatus, they possess a trailing flagellum and a circumferential flagellum, much like the dinoflagellate arrangement (Galt and Whisler 1970). Additionally, the Perkinsozoa anterior flagellum is decorated with simple tubular hairs whereas the flagella of *Thalassomyces* lack ornamentation (Whisler 1990).

Arguments for or against a specific affiliation with the dinoflagellates are less compelling, partly due to the heterogeneous nature of morphological and cytological features in parasitic forms (Shields 1994). However, it is particularly intriguing to speculate on a dinoflagellate affiliation based on the unusual centriolar complexes found outside the nucleus, residing in folds of the nuclear membrane in *Thalassomyces* and the parasitic Syndiniales dinoflagellate *Syndinium* (Ris and Kubai 1974; Whisler 1990). However, the unique and characteristic feature of most dinoflagellates—the highly organized, continuously condensed chromosomes of the 'dinokaryon' nucleus—has not been observed in any ellobiopsid to date (Galt and Whisler 1970; Taylor 1990), although it is possible that this nuclear morphology may be present in a yet unobserved developmental stage.

Presently, molecular analyses based on SSU rRNA sequences broadly depict a phylogenetic affiliation of *Thalassomyces* to a diverse group comprised of Dinophyceae, marine alveolate group 1, *Perkinsus* and relatives. Precise determination of the phylogenetic position of these and other ellobiopsids will require detailed morphological studies at the gross and ultrastructural levels from all life phases of diverse ellobiopsid lineages and additional molecular data from these organisms, especially from protein-coding genes.

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