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A novel clade of protistan parasites near the animal–fungal divergence

(*Dermocystidium*/*Ichthyophonus*/*Psorospermium*/small-subunit ribosomal RNA/molecular phylogenetics)

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ABSTRACT **Sequences of nuclear-encoded small-subunit rRNA genes have been determined for representatives of the enigmatic genera *Dermocystidium*, *Ichthyophonus*, and *Psorospermium*, protistan parasites of fish and crustaceans. The small-subunit rRNA genes from these parasites and from the "rosette agent" (also a parasite of fish) together form a novel, statistically supported clade. Phylogenetic analyses demonstrate this clade to diverge near the animal–fungal dichotomy, although more precise resolution is problematic. In the most parsimonious and maximally likely phylogenetic frameworks inferred from the most stably aligned sequence regions, the clade constitutes the most basal branch of the metazoa; but within a limited range of model parameters, and in some analyses that incorporate less well-aligned sequence regions, an alternative topology in which it diverges immediately before the animal–fungal dichotomy was recovered. Mitochondrial cristae of *Dermocystidium* spp. are flat, whereas those of *Ichthyophonus hoferi* appear tubulovesiculate. These results extend our understanding of the types of organisms from which metazoa and fungi may have evolved.**

Identifying the group of protists from among which the animal lineage arose stands as one of the oldest and least tractable problems in zoology (1–3). Recent phylogenetic analyses based on protein (4) and rDNA (5, 6) sequences have identified the fungi (Eumycota) as the major group of multicellular organisms most closely related to animals. However, certain protistan groups appear to have arisen from the animal and fungal lineages subsequent to the animal–fungal dichotomy. Chytridiomycetes, which some authorities include among the protists (7), are widely considered to constitute the most basal branch within the fungal lineage. Choanoflagellates, likewise considered protists (8, 9), have been proposed on structural and biochemical grounds to constitute the most basal clade in the animal lineage (10); this proposal has received support from some analyses of rDNA sequence data (5), although in other rDNA trees the choanoflagellates diverge among the radiate animals (9) or even before green plants (11).

The enigmatic "rosette agent," an intracellular parasite of salmonids (12, 13), was recently shown by rDNA sequence analysis to be closely related to choanoflagellates and, thus, to animals (14). Investigations in our laboratories have now revealed that aquatic parasites of the enigmatic genera *Dermocystidium*, *Ichthyophonus*, and *Psorospermium* are specifically related to rosette agent.

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More than 20 species of *Dermocystidium* occur either as cysts in skin or gill cysts, or as systemic infections in carp, goldfish, salmonids, eels, newts, and frogs (15). The genus has been a depository for organisms that have a spherical stage with a large, refractile-inclusion-bearing vacuole that restricts the cytoplasm and nucleus to the cell periphery; at least some species have septate hyphae (15), uniflagellate zoospores (16), and flat mitochondrial cristae (Fig. 1*A*). *Dermocystidium* spp. have been considered to be haplosporeans (17) or fungi (15, 18), although the former assignment is untenable because their sporangium lacks the characteristic operculum. Some species originally described within *Dermocystidium* have since been recognized to be apicomplexans and reassigned to the genus *Perkinsus*. Patterson (19) includes *Dermocystidium* among his Residua, "genera with unclear identities and unplaceable within a phylogenetic classification."

I. hoferi causes systemic infections in more than 80 species of fish, both marine and freshwater, producing widespread mortalities and significant economic losses (20). The genus has variously been assigned to the Sporozoa (21), Haplosporidea (22), Myxosporidea (23), Oomycetes (24), Chytridiomycetes (25), Zygomycetes (26), Fungi Imperfecti (27), Fungi incertae sedis (28), and its own class Ichthyophonales (29) within the fungi. *I. hoferi* (25) resembles eumycota in possessing spindle pole bodies, but its mitochondria have tubulovesiculate, not flat, cristae (Fig. 1*B*); its cell walls are periodic acid/Schiff reagent-positive but lack both cellulose and chitin (29). Flagellate stages have not been observed.

Psorospermium haeckelii, a parasite occurring in connective tissue of freshwater crayfish, was first observed by Haeckel (30). It appears as an elongate or ovoid shell-bearing spore with internal globules; like *Dermocystidium* and *Ichthyophonus*, it has resisted culture *in vivo*, and its complete life history is unknown. It is widely distributed among several species and populations of crayfish, and has been linked to commercially significant decreases in crayfish stocks (31). Based on the limited information available, *P. haeckelii* has variously been considered to be a sporozoan (32) or the histopathogenic stage of a dimorphic fungus (33).

Herein we report, based on analysis of nuclear-encoded rDNA sequences, that the protists *Dermocystidium salmonis*,

Abbreviations: DRIPs, grouping of *Dermocystidium*, rosette agent, *Ichthyophonus*, and *Psorospermium*; ssu-rDNA, small-subunit rRNA genes; BP, bootstrap proportion; Ts/Tv ratio, transition/transversion ratio.

Data deposition: The sequences reported in this paper have been deposited in the GenBank data base (accession nos. U21337, U21336, U25637, U33180, and U33181).

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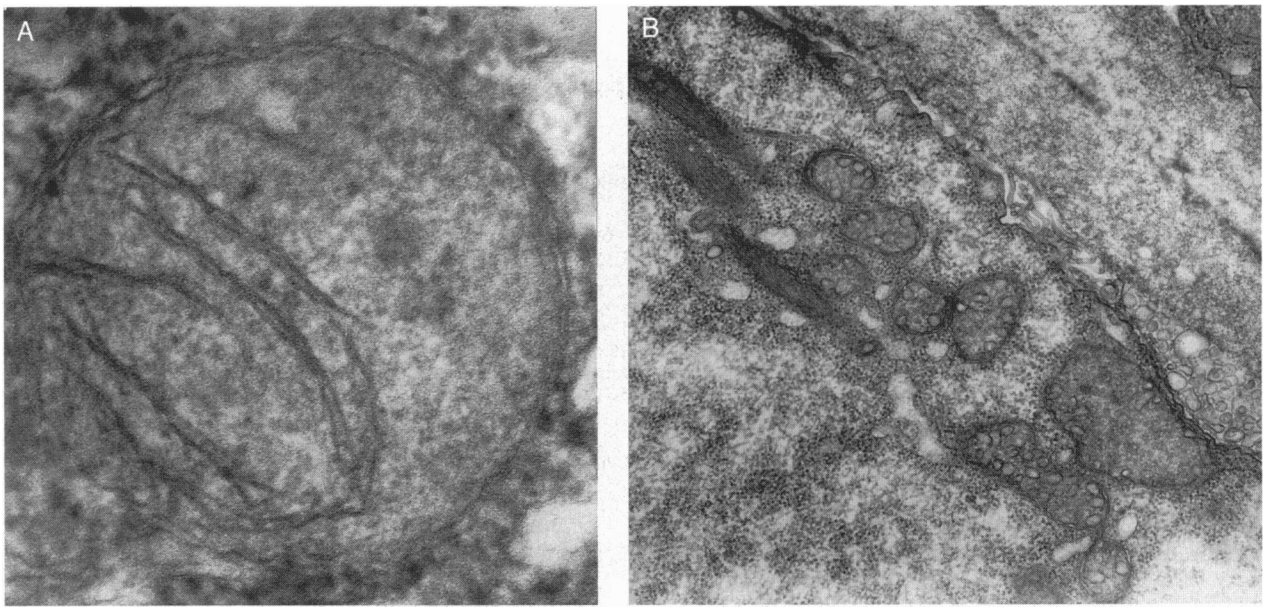

FIG. 1. Electron micrographs of mitochondria of (*A*) schizont of *Dermocystidium* sp. from brook trout and (*B*) *Ichthyophonus hoferi*, showing morphology of cristae. Widths of the fields shown are 0.65 mm (*A*) and 2.2 mm (*B*).

Dermocystidium sp., *I. hoferi*, and *P. haeckelii* are specifically related to rosette agent to the exclusion of choanoflagellates. This unanticipated and as-yet-unnamed grouping of *Dermocystidium*, rosette agent, *Ichthyophonus*, and *Psorospermium*, which we provisionally call the DRIPs clade, is part of the "eukaryotic crown assemblage," and in our best phylogenetic inferences appears (although without definitive statistical support) as the deepest branch within the animal kingdom.

MATERIALS AND METHODS

Cysts of *D. salmonis* were obtained by microsurgery from gills of the chinook salmon *Onchorhynchus tschawytscha* (Trask State Fish Hatchery, OR), and cysts of *Dermocystidium* spp. were obtained from gills of the brook trout *Salvelinus fontinalis* (Fraser's Mills Hatchery, St. Andrews, NS Canada). Cysts were crushed by vigorous grinding in microfuge tubes at −196°C and incubated with Proteinase K and 4% SDS. *I. hoferi* was isolated from liver lesions of the yellowtail flounder *Limada ferruginea*, collected from Sable Island Bank and Brown's Bank, northwest Atlantic Ocean. Inocula were plated onto Petri dishes containing EFS medium (34), and sporangiophores growing onto the surface of the medium were excised. *P. haeckelii* was isolated, by centrifugation through a Percoll cushion, from subcarapacial connective tissue of the crayfish *Astacus astacus* (35), collected from Lake Aspen, Södermanland, Sweden by Erland Mählström. DNA was purified by phenol-chloroform extraction and ethanol precipitation. Nuclear small-subunit rRNA genes (ssu-rDNAs) were amplified by PCR and either sequenced directly (*Dermocystidium* spp.) or cloned in pAmp1 (GIBCO/BRL) for sequencing (36, 37).

The rDNAs of *D. salmonis, Dermocystidium* sp., *I. hoferi*, and *P. haeckelii* were aligned with those of 40 other higher eukaryotes selected to represent all major eukaryotic "crown" taxa, avoid ssu-rDNAs highly divergent in branch length or nucleotide composition, and minimize (so far as possible) lengths of internal edges in the inferred trees, and thereby to avoid topological artifacts that can arise from unequal apparent rates of acceptance of mutations (38). Alignment was based, to the extent possible, on conservation of secondary- and higher-order structures as revealed by covariation of nucleotides (39, 40). Every position was identified as unpaired, involved in a short-range base pair (e.g., stem position), or long-range base paired in the folded rRNA (Fig. 2), and in some analyses these assignments were used either directly or to aid Hidden Markov Model-based assignment of rate categories.

Columns of data corresponding to PCR primers (47 nucleotide positions) and the most sparsely populated alignment positions were removed to yield "full" matrix A (44 species × 1983 positions). Three additional matrices were produced and differed in the degree to which ambiguously aligned positions

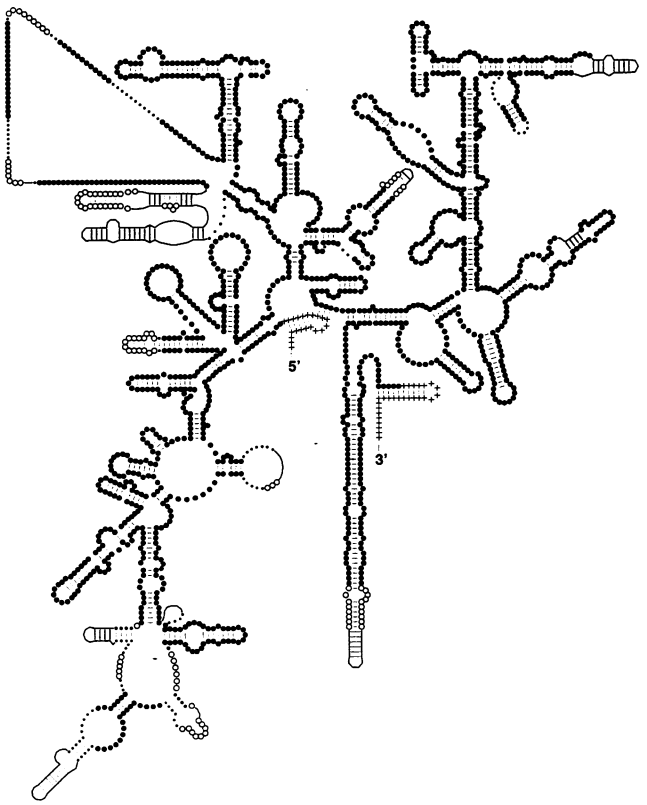

FIG. 2. Maskings for phylogenetic analyses of nucleotide positions, displayed on a folded ssu-rRNA. •, Most conservative positions, included in all analyses; ·, positions excluded from matrix C; ○, positions excluded from matrices C and E; solid lines, positions excluded from matrices C, E, and B; +, PCR primers, excluded from all analyses.

were removed (Fig. 2). Removal of the more ambiguously aligned positions yielded matrix B (44 × 1622 positions); rigorous removal gave matrix E (44 × 1461 positions); and deletion of all positions for which a secondary structure could not be unambiguously assigned yielded the "most conservative" matrix C (44 × 1370 positions). Third and subsequent gap positions were coded as unknown characters for parsimony analyses.

Phylogenetic trees were inferred by maximum likelihood using FASTDNAML version 1.0.6 (41) and an alpha version of DNAML from PHYLIP (version 4.0) (42) with random addition order and global (42-level) optimization. In some analyses, base paired positions were assigned higher relative rates of evolution to effect downweightings similar to those sometimes employed in parsimony analysis (43–45). Parsimony trees were inferred using DNAPARS in PHYLIP (version 3.53c) (42); multiple (n = 500 or 1000) iterations were made to increase the likelihood of finding maximally parsimonious solutions, and a majority-rule consensus tree was calculated from the equally parsimonious solutions using CONSENSE (42). Distance matrices corrected for superposed substitutions were calculated under a generalized Kimura two-parameter model using DNADIST, and neighbor-joining trees were derived using NEIGHBOR (42). Analyses were bootstrapped (n = 500 or 1000) by sequential use of the PHYLIP programs SEQBOOT, DNAPARS (parsimony) or DNADIST and NEIGHBOR (neighbor-joining), and CONSENSE (46). Alternative topologies were subjected to the nonparametric Templeton–Felsenstein test under parsimony (47) and the Kishino–Hasegawa test under likelihood (48) using DNAPARS (version 3.53c) and DNAML (version 4.0), respectively. The decay index (49) and tree statistics were computed using PAUP (version 3.1) (50) on a Macintosh IIci; most other computation was done on a Sun 10/61 Unix workstation.

RESULTS

Single products corresponding to intron-free nuclear rDNAs were amplified and sequenced from *D. salmonis*, *Dermocystidium* sp., *I. hoferi*, and *P. haeckelii* (Table 1). Alignment with ssu-rDNAs of 40 other selected eukaryote nuclear rDNAs was straightforward, yielding (upon progressive removal of PCR-primer regions, sparsely populated sites and ambiguously alignable regions) a series of matrices. Among these, only matrices C and E are unambiguously aligned (i.e., provide unambiguous homology statements), the former fully supported by secondary and higher-order structure, the additional 91 positions of the latter based on extensive primary-sequence identity.

Maximum-likelihood (Fig. 3), parsimony, and neighbor-joining analyses revealed an unanticipated grouping of ssu-rDNAs of the two *Dermocystidium* spp., *I. hoferi* and *P. haeckelii*, and rosette agent. The consistency index of this DRIPs clade ranged from 0.893 (full matrix A) to 0.957 (most conservative matrix C). With the more conservative matrices C and E, bootstrap proportions (BPs) for integrity of the DRIPs clade were 60–79% under parsimony and 76–99% under distance. Simulations indicate that BPs are highly conservative estimates of accuracy (51, 52); although extrapolation to real-life trees remains problematic, here, where internodal distances are short (51) and the number of species is large (52), the probability that the DRIPs clade does not appear in the true tree can be estimated to range from much less than 15% (for BPs = 60%) to essentially 0 (for BPs ≥ 80%). In contrast, the greatest BP for any individual grouping inconsistent with holophyly of the DRIPs clade [the flagellate *Apusomonas proboscidea* (53, 54) grouping with one or more DRIPs ssu-rDNAs; see below] was 10%, and BPs for alternative topologies in which one or more DRIPs group specifically with the two choanoflagellates ranged from 10% (matrix C, parsimony) to 0.9% (matrix E, parsimony); in simulations, BPs of 10% correspond to near-zero probabilities (51, 52) and are similar to the support observed for polyphyly of choanoflagellates, i.e., background noise. The decay index (49) for the DRIPs clade is >6 steps in heuristic analysis of matrix C, likewise indicating good support. Within the DRIPs clade, the two *Dermocystidium* spp. group stably together (BP = 100%), as do *I. hoferi* and *P. haeckelii* (BP = 92–99%), and the two *Dermocystidium* spp. with rosette (BP = 81–100%).

Table 1. Nuclear-encoded ssu-rDNAs of *D. salmonis*, *Dermocystidium* spp. from brook trout, *I. hoferi*, and *P. haeckelii*

Organism	Length*	G+C, %	GenBank accession no.
D. salmonis	1780	43.8	U21337
Dermocystidium sp.	1821	43.6	U21336
I. hoferi	1808	43.8	U25637
P. haeckelii†	1792	44.1	U33180

*Length in nucleotides of PCR-amplified ssu-rDNAs, including PCR primer regions.

†The ssu-rDNA sequence of the crayfish host, *Astacus astacus*, has been deposited in the GenBank data base (accession no. U33181).

Phylogenetic analysis in this region of the eukaryotic ssu-rDNA tree is complicated by the volatility of ssu-rDNAs of *Cyanophora paradoxa*, *Acanthamoeba* spp., and especially *A. proboscidea*. These sequences have a tendency to migrate (with poor bootstrap support) within the tree in response to minor changes in model parameters, choice of alignment regions, or presence of attractor sequences. In parsimony and maximum-likelihood analyses of the two least stably aligned matrices, A and B, *A. proboscidea* tends to intrude into the DRIPs clade to group with *Dermocystidium* spp. The attraction between *A. proboscidea* and *Dermocystidium* ssu-rDNAs involves transversions as well as transitions, and does not appear to be purely a compositional (G+C) effect (data not shown). As analyses of matrix A yielded conflicting, often biologically unreasonable trees with low BPs even for well-established clades (i.e., 21% for metazoa), we interpret the appearance of *A. proboscidea* within the DRIPs clade (above) as an artifact of, or exacerbated by, the alignment of nonhomologous or highly diverged sites in regions that show no common secondary-structural helices or obvious primary-structural similarity among these ssu-rRNAs.

All analyses of all matrices (except some parsimony analyses of the least conservative matrices, A and B) showed the DRIPs clade (sometimes with *A. proboscidea*) to diverge either immediately after or immediately basal to the animal–fungal dichotomy. Maximum-likelihood and parsimony analyses of the best-supported matrices, C and E, resolved the DRIPs clade as the deepest branch within the animal lineage (Figs. 3 and 4, tree I). With matrix C, tree I was recovered under maximum likelihood at all eight investigated Ts/Tv ratios between 0.50 and 1.70 and all seven between 2.05 and 10.00, but tree II (Fig. 4), in which the DRIPs clade is the sister group to animals+fungi, was most likely when the Ts/Tv ratio was between 1.75 and 2.00. Tree I, however, showed the greatest overall likelihood (−16846.07632 at a Ts/Tv ratio of 1.50), more than 40 log-likelihood units greater than the best value (−16886.90976 at a Ts/Tv ratio of 1.75) observed for tree II. BPs for positioning the DRIPs clade as the deepest branch within animals, however, were only 37–53%.

The robustness of this result was examined in extensive maximum-likelihood analyses of matrices C and E. In some series, unpaired, stem, and long-range-paired positions were assigned different relative rates of change (1, 0.5–5.0, and 0.6–3.0, respectively), hence, in effect, different weights; in other series, positions were assigned to rate categories (values as above, sometimes with a fourth, zero-rate category for unvarying positions) using a Hidden Markov Model (55). The

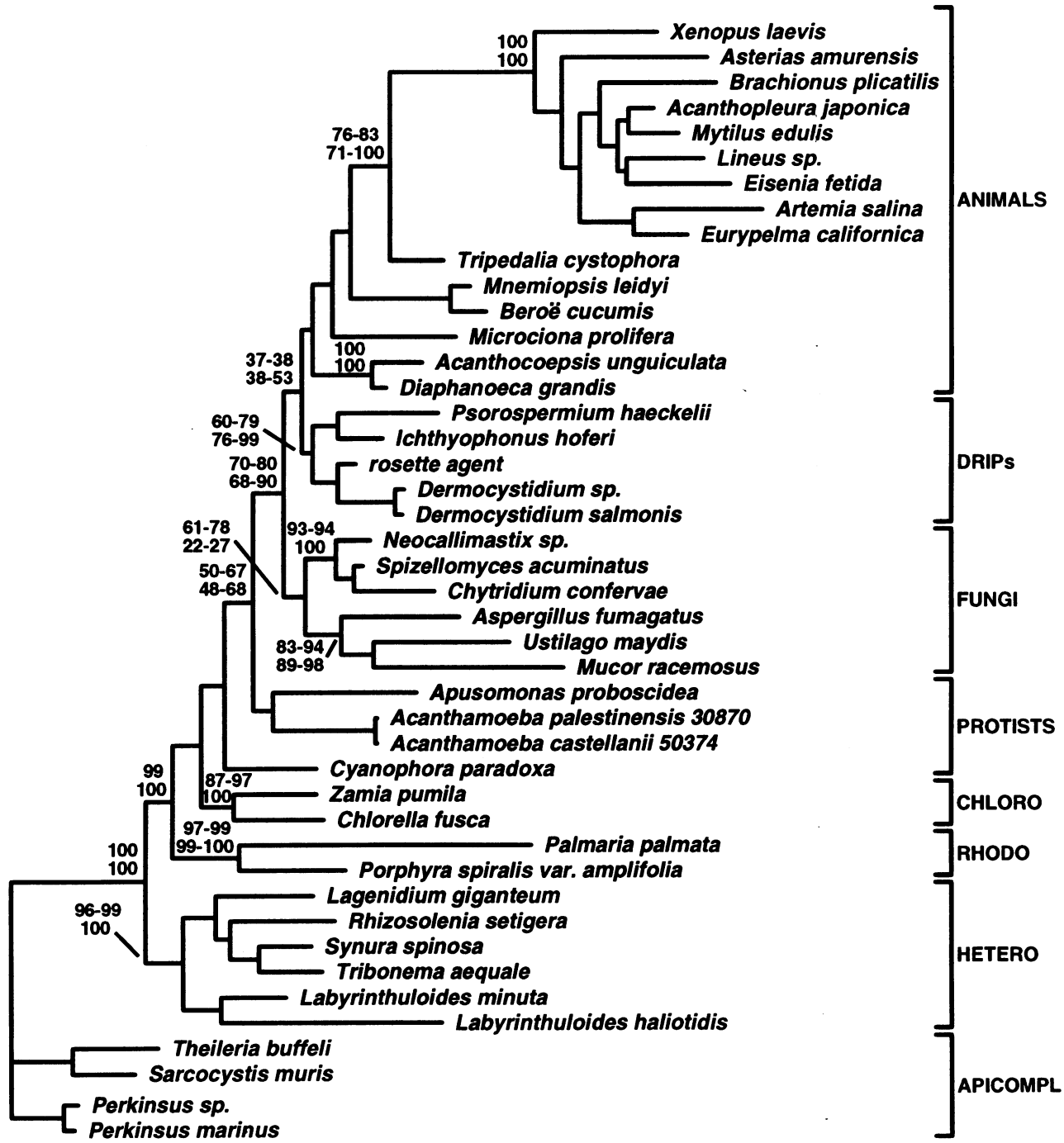

FIG. 3. Maximum-likelihood tree inferred from ssu-rDNAs of *Dermocystidium* sp., *D. salmonis*, *I. hoferi*, *P. haeckelii*, and 40 other eukaryotes, based on matrix C (1370 positions) at a transition/transversion (Ts/Tv) ratio of 1.50. In the topologically identical, most parsimonious tree inferred from matrix C, L = 2838 steps, ci = 0.400, rc = 0.222, and ri = 0.555; 521 positions are "informative." The upper and lower numbers give the bootstrap support (percent of 500 replicates) in parsimony and neighbor-joining analyses, respectively, of matrices C and E. GenBank accession numbers, from top to bottom: animals: X04025, D14358, U29235, X70210, L24489, X79878, X79872, X01723, X13457, L10829, L10826, D15068, L10825, L10823, and L10824; DRIPs: U33180, U25637, L29455, U21336, and U21337; fungi: M59761, M59759, M59758, M60300, X62396, and X54863; protists: L37037, U07411, U07413, and X68483; chlorophytes: M20017 and X74002; rhodophytes: Z14142 and L26177; heterokonts: X54266, M87329, M87336, M55286, L27634, and U21338; apicomplexa: Z15106, M64244, L07375, and X75762.

greatest overall likelihood was always associated with tree I, although in each series, tree II was locally more likely within a narrow (although variable) range of Ts/Tv ratios. Thus, these two topologies (tree I and, less favorably, tree II) are robustly inferred from the most stably aligned nucleotide positions under a wide range of biologically reasonable models.

To examine further the relative support for these two (and other possible) topologies, alternatives were tested by the method of Templeton and Felsenstein under parsimony, and by the method of Kishino and Hasegawa under maximum likelihood (Fig. 4). Under these nonparametric tests, alternative topologies are rejectable at 95% confidence if they require >1.96 SD more steps than the most parsimonious tree, or are >1.96 SD less likely than the most likely tree; the corresponding value for 90% confidence is >1.645 SD. Groups stable in the above-mentioned analyses were maintained intact, but their relative branching positions were permuted, exhaustively for the DRIPs clade and nearby lineages, for more remote

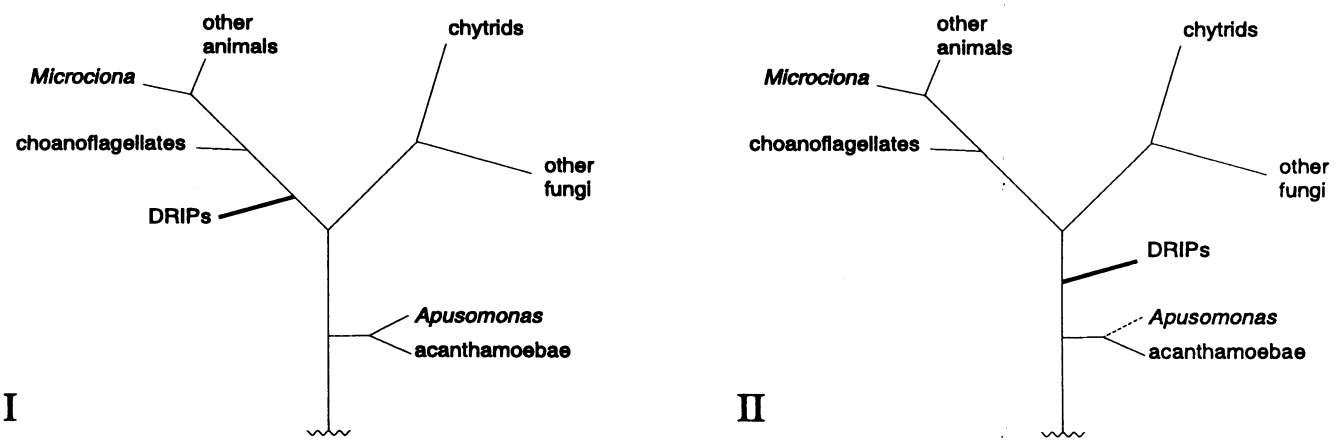

FIG. 4. Alternative relationships among the DRIPs clade and other eukaryote lineages not rejectable under the Templeton–Felsenstein and Kishino–Hasegawa tests. Tree I, most likely and most parsimonious topology based on matrices C and E (from Fig. 3); tree II, alternative topology inferred from matrix C when the Ts/Tv ratio was 1.75–2.00. Dashed line to *Apusomonas* ssu-rDNA denotes instability.

lineages only if initial tests showed a significant influence upon the position or length of the edge joining the DRIPs ssu-rDNAs to the rest of the tree. Of the hundreds of alternative topologies screened, 59 were selected in this way for more rigorous analysis.

With the most conservative matrix, matrix C, variances were relatively large for both tests, and about 40% of these alternative topologies could not be ruled out. Most of the acceptable alternatives involved rearrangements among lower invertebrates (*Acanthocoepsis unguiculata*, *Diaphanoeca grandis*, *Microciona prolifera*, *Mnemiopsis leidyi*, and *Beroë cucumis*), and/or migrations of the three volatile lineages mentioned above; but in a minority, the DRIPs clade was repositioned as the deepest clade within fungi, or immediately basal to the animal + fungal clade. Tests on matrix E were much more discriminatory; all but three alternatives to tree I could be rejected at 95% under parsimony, all but two at 90% under likelihood, and none was acceptable under both tests. With matrix B, all alternatives to tree I (but with *A. proboscidea* joining the DRIPs clade as a sister group to *Dermocystidium* spp.) were rejected at 95% under both tests.

DISCUSSION

The analyses presented herein clearly indicate the existence of an unanticipated and as-yet-unnamed clade of eukaryotic protists, all recognized members of which are parasites of aquatic animals, including fish (*Dermocystidium*, *Ichthyophonus*, rosette), amphibians (*Dermocystidium*), and crustaceans (*Psorospermium*). No complete life-history is known for any of these organisms, and none has been successfully cultured *in vivo*; thus, it is premature to propose a concept, archetype, or *bauplan* for this clade, or to speculate upon its likely phyletic delimitation. We provisionally refer to this group as the DRIPs clade after its presently known members.

Based on known life-history forms, *Dermocystidium* (15, 18), *Ichthyophonus* (25–29), *Psorospermium* (33), and the rosette agent (12) have all been proposed to have fungal affinities, although without satisfactory assignment to any existing fungal taxon. "Remarkable" similarities in gross pathology, intracellular location, and ultrastructure between the rosette agent and systemic *Dermocystidium* infections have already been noted (56). Moreover, the encysted resting stages of *P. haeckelii* and *Ichthyophonus* appear to be very similar in gross morphology, and an amoeboid stage in *P. haeckelii* (57) may correspond to the amoeboid (infective?) stage of *Ichthyophonus*.

Our analyses indicate that the DRIPs organisms diverged near the animal–fungal dichotomy, although the precise position could not be conclusively resolved. In the most likely and most parsimonious trees inferred from the most stably aligned ssu-rDNA regions, the DRIPs clade diverges subsequent to the animal–fungal dichotomy, as the most basal branch within the animal lineage. In their initial description of the ssu-rDNA sequence of rosette agent, Kerk *et al.* (14) reported that ssu-rDNAs of rosette agent and choanoflagellates form a monophyletic group with a BP of 81–94%. As described above, addition of ssu-rDNAs from *Dermocystidium*, *Ichthyophonus*, and *Psorospermium* dissolves this association and resolves choanoflagellates on the second-deepest branch within the animal lineage, thereby further strengthening molecular support for the hypothesis that metazoa arose from a flagellated protozoan (58) similar to modern choanoflagellates (5, 10).

As detailed above, one alternative hypothesis for the position of the DRIPs ssu-rDNAs within the eukaryote tree cannot, based on our data, be ruled out statistically, although it is less likely and less parsimonious; in this hypothesis, the origin of the DRIPs clade is immediately basal to the animal–fungal dichotomy (tree II). This alternative is compatible with the ssu-rDNA data and, like the most parsimonious and most likely tree I, is consistent with monophyly of animals and of true fungi. The relative strengths of these two inferences depend to some degree on which ssu-rDNAs are included in the analysis; for example, removal of sequences diverging immediately basal to the animal–fungal dichotomy (*Acanthamoeba* spp., *A. proboscidea*, and *C. paradoxa*) diminishes the acceptability of tree II, whereas removal of choanoflagellate and sponge ssu-rDNAs resolves the DRIPs clade onto the fungal branch (results not shown). We have minimized arbitrariness by including representatives of all major eukaryotic crown groups meeting the branch-length and compositional criteria described above, retaining even volatile lineages such as *A. proboscidea*. However, ssu-rDNA sequence comparisons (5, 6, 9, 11, 14) have not yet converged on a stable phylogeny of the crown groups, and the relationships derived herein are unlikely to constitute the final word on the origin of animals. Phylogenetic analyses of protein sequences and comparison of intron positions in protein-coding genes (4, 5) should help select among these competing hypotheses.

Like almost all animals and eumycota, the *Dermocystidium* sp. from brook trout has flattened mitochondrial cristae. However, the mitochondrial cristae of *I. hoferi* appear tubulovesiculate under a variety of fixation and embedding protocols (Fig. 1). For technical reasons (e.g., thick spore walls), cristal morphology is unknown in rosette (D. Kerk, personal communication), *D. salmonis* (R. E. Olson, personal communication), and *P. haeckelii* (unpublished data). If (as in our best inferences) the DRIPs clade diverged after the animal–fungal dichotomy, the tubulovesiculate appearance of cristae in *I.*

hoferi must, like the tubular cristae of mesozoa, represent a reversion from the flat form typical of animals and true fungi. Shape of mitochondrial cristae is in general an excellent guide to phylogenetic position (19), but exceptions are known (59, 60).

It was recently suggested (61) that animal phyla do not contain protist species; the almost simultaneous discovery, based on analysis of ssu-rDNAs, that myxozoa are closely related to bilateral animals (11) does not provide a firm counterexample, as the classification of myxozoa among protists had long been controversial. The results presented herein strongly suggest that the two earliest branches within the animal kingdom include protists, as do early branches within the other two crown kingdoms (62), Fungi and Plantae. The appearance of an additional group of protists near the point of divergence of the animal and fungal lineages (4–6) casts new light on the nature of the ancestral animal, points to the need for additional study of these enigmatic organisms, and reminds us again that protist refers at best to a grade, not a clade, of eukaryotic organisms.

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1. Hyman, L. H. (1940) *The Invertebrates* (McGraw–Hill, New York), Vol. 1, pp. 248–253.
2. Cuénot, L. (1952) in *Traité de Zoologie,* ed. Grassé, P.-P. (Masson, Paris), Tome I, Fasc. I, pp. 1–33.
3. Brusca, R. C. & Brusca, G. J. (1990) *Invertebrates* (Sinauer, Sunderland, MA), pp. 112–116.
4. Baldauf, S. L. & Palmer, J. D. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 11558–11562.
5. Wainright, P. O., Hinkle, G., Sogin, M. L. & Stickel, S. K. (1993) *Science* **260,** 340–342.
6. Ragan, M. A. & Gutell, R. R. (1995) *Bot. J. Linn. Soc.* **118,** 81–105.
7. Margulis, L., Corliss, J. O., Melkonian, M. & Chapman, D. J., eds. (1990) *Handbook of Protoctista* (Jones & Bartlett, Boston).
8. Leadbeater, B. S. C. (1977) *J. Mar. Biol. Assoc. U.K.* **57,** 285–301.
9. Cavalier-Smith, T. (1993) *Microbiol. Rev.* **57,** 953–994.
10. Cavalier-Smith, T. (1987) in *Evolutionary Biology of the Fungi,* eds. Rayner, A. D. M., Brasier, C. M. & Moore, D. (Cambridge Univ. Press, Cambridge, U.K.), pp. 339–353.
11. Smothers, J. F., von Dohlen, C. D., Smith, L. H., Jr., & Spall, R. D. (1994) *Science* **265,** 1719–1721.
12. Harrell, L. W., Elston, R. A., Scott, T. M. & Wilkinson, M. T. (1986) *Aquaculture* **55,** 249–262.
13. Elston, R. A., Harrell, L. & Wilkinson, M. T. (1986) *Aquaculture* **56,** 1–21.
14. Kerk, D., Gee, A., Standish, M., Wainright, P. O., Drum, A. S., Elston, R. A. & Sogin, M. L. (1995) *Mar. Biol.* **122,** 187–192.
15. Dyková, I. & Lom, J. (1992) *J. Appl. Ichthyol.* **8,** 180–185.
16. Olson, R. E., Dungan, C. F. & Holt, R. A. (1991) *Dis. Aquat. Org.* **12,** 41–48.
17. Caullery, M. (1953) in *Traité de Zoologie,* ed. Grassé, P.-P. (Masson, Paris), Tome I, Fasc. II, pp. 922–934.
18. Allen, R. L., Meekin, T. K., Pauley, G. B. & Fujihara, M. P. (1968) *J. Fish. Res. Board Can.* **25,** 2467–2475.
19. Patterson, D. J. (1994) in *Progress in Protozoology,* eds. Hausmann, K. & Hülsmann, N. (Fischer, Stuttgart), pp. 1–14.
20. Sindermann, C. J. (1970) *Principal Diseases of Marine Fish and Shellfish* (Academic, New York), pp. 22–25.
21. Hofer, B. (1893) *Allg. Fischwirtschaftsztg.* **18,** 168–171.
22. Caullery, M. & Mesnil, F. (1905) *Arch. Zool. Exp. Gén. Ser. 4* **4,** 101–180.
23. Cox, P. (1916) *Contrib. Can. Biol. Fish.* **1914–1915,** 81–85.
24. Sproston, N. G. (1944) *J. Mar. Biol. Assoc. U.K.* **26,** 72–98.
25. Plehn, M. & Mulsow, K. (1911) *Zentralbl. Bakteriol. Parasitenkd. Orig.* **59,** 63–68.
26. Johnstone, J. (1913) *Proc. Trans. Liverpool Biol. Soc.* **27,** 196–218.
27. Chauvier, G. (1979) *Ann. Parasitol. (Paris)* **54,** 105–111.
28. Alderman, D. J. (1982) in *Microbial Diseases of Fish,* ed. Roberts, R. J. (Academic, London), pp. 189–242.
29. Rand, T. G. (1994) *Dis. Aquat. Org.* **18,** 21–28.
30. Haeckel, E. (1857) *Arch. Anat. Physiol. Med. (Müller's Arch.)* **24,** 469–568.
31. Söderhäll, K. & Cerenius, L. (1992) *Annu. Rev. Fish Dis.* **2,** 3–23.
32. Alderman, D. J. & Polglase, J. L. (1988) in *Freshwater Crayfish Biology, Management and Exploitation,* eds. Holdich, D. M. & Lowery, R. S. (Croom Helm, London), pp. 167–212.
33. Nylund, V., Westman, K. & Lounatmaa, K. (1983) *Freshwater Crayfish Pap. Int. Symp.* **5,** 307–314.
34. Rand, T. G. & Cone, D. (1990) *J. Wildl. Dis.* **26,** 323–328.
35. Cerenius, L., Henttonen, P., Lindqvist O. V. & Söderhäll, K. (1991) *Aquaculture* **99,** 225–233.
36. Sogin, M. L. (1990) in *PCR Protocols,* eds. Innis, M. A., Gelfand, D. H., Sninsky, J. J. & White, T. J. (Academic, San Diego), pp. 307–314.
37. Bird, C. J., Rice, E. L., Murphy, C. A. & Ragan, M. A. (1992) *Phycologia* **31,** 510–522.
38. Hendy, M. D. & Penny, D. (1989) *Syst. Zool.* **38,** 297–309.
39. Gutell, R. R. (1993) in *The Translational Apparatus,* eds. Nierhaus, K. H., Franchschi, F., Subramanian, A. R. & Erdmann, V. A. (Plenum, New York), pp. 477–488.
40. Gutell, R. R., Larsen, N. & Woese, C. R. (1994) *Microbiol. Rev.* **58,** 10–26.
41. Olsen, G. J., Matsuda, H., Hagstrom, R. & Overbeek, R. (1994) *Comput. Appl. Biosci.* **19,** 41–48.
42. Felsenstein, J. (1989) *Cladistics* **5,** 164–166.
43. Wheeler, W. C. & Honeycutt, R. L. (1988) *Mol. Biol. Evol.* **5,** 90–96.
44. Dixon, M. T. & Hillis, D. M. (1993) *Mol. Biol. Evol.* **10,** 256–267.
45. Bakker, F. T., Olsen, J. L., Stam, W. T. & van den Hoek, C. (1994) *Mol. Phylogenet. Evol.* **3,** 365–382.
46. Felsenstein, J. (1985) *Evolution* **39,** 783–791.
47. Templeton, A. R. (1983) *Evolution* **37,** 221–244.
48. Kishino, H. & Hasegawa, M. (1989) *J. Mol. Evol.* **29,** 170–179.
49. Donoghue, M. J., Olmstead, R. G., Smith, J. F. & Palmer, J. D. (1992) *Ann. Mo. Bot. Gard.* **79,** 333–345.
50. Swofford, D. L. (1993) PAUP, Phylogenetic Analysis Using Parsimony (Illinois Natural History Survey, Champaign, IL), Version 3.1.
51. Hillis, D. M. & Bull, J. J. (1993) *Syst. Biol.* **42,** 182–192.
52. Sitnikova, T., Rzhetsky, A. & Nei, M. (1995) *Mol. Biol. Evol.* **12,** 319–333.
53. Vickerman, K., Darbyshire, J. F. & Ogden, C. G. (1974) *Arch. Protistenkd.* **116,** 254–269.
54. Cavalier-Smith, T. & Chao, E. E. (1995) *Proc. R. Soc. London B* **261,** 1–6.
55. Felsenstein, J. & Churchill, G. A. (1996) *Mol. Biol. Evol.* **13,** 93–104.
56. Hedrick, R. P., Friedman, C. S. & Modin, J. (1989) *Dis. Aquat. Org.* **7,** 171–177.
57. Grabda, E. (1934) *Mém. Acad. Pol. Sci. Cracovie B* **6,** 123–142.
58. Haeckel, E. (1874) *Jena. Z. Naturwiss.* **8,** 1–55.
59. Patterson, D. J. (1990) *J. Mar. Biol. Assoc. U.K.* **70,** 381–393.
60. O'Kelly, C. J. (1993) *J. Eukaryotic Microbiol.* **40,** 627–636.
61. Corliss, J. O. (1994) *Acta Protozool.* **33,** 1–51.
62. Knoll, A. H. (1992) *Science* **256,** 622–627.