J. Phycol. **40,** 341–350 (2004)

DOI: 10.1111/j.1529-8817.2004.03129.x

EARLY EVOLUTIONARY HISTORY OF DINOFLAGELLATES AND APICOMPLEXANS (ALVEOLATA) AS INFERRED FROM HSP90 AND ACTIN PHYLOGENIES[1]

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Three extremely diverse groups of unicellular eukaryotes comprise the Alveolata: ciliates, dinoflagellates, and apicomplexans. The vast phenotypic distances between the three groups along with the enigmatic distribution of plastids and the economic and medical importance of several representative species (e.g. *Plasmodium*, *Toxoplasma*, *Perkinsus*, and *Pfiesteria*) have stimulated a great deal of speculation on the early evolutionary history of alveolates. A robust phylogenetic framework for alveolate diversity will provide the context necessary for understanding the basic biological properties of the group and for developing appropriate strategies for management. We addressed the earliest stages of alveolate evolution by sequencing heat shock protein 90 (hsp90) genes from several ciliates, apicomplexans, and dinoflagellates, including key species thought to represent early diverging lineages: *Oxyrrhis marina*, *Perkinsus marinus*, *Cryptosporidium parvum*, and the eugregarine *Monocystis agilis*. Moreover, by sequencing the actin gene from *Monocystis*, we were able to examine the sister relationship between gregarines and cryptosporidians with a three-protein concatenated data set (hsp90, actin, and β-tubulin). Phylogenetic analyses of the hsp90 data set provided a robust topology for alveolate relationships: Alveolates were monophyletic and apicomplexans and dinoflagellates formed sister groups to the exclusion of ciliates. *Oxyrrhis* formed the earliest diverging sister lineage to the "core" dinoflagellates, and *Perkinsus* formed the earliest diverging sister lineage to the *Oxyrrhis*–dinoflagellate clade. This topology was strongly supported in all analyses and by a unique indel shared by *Oxyrrhis* and dinoflagellates. A sister relationship between *Cryptosporidium* and *Monocystis* was weakly supported by the hsp90 data set but strongly supported by the three-protein concatenated data set.

Key index words: **Alveolata; Apicomplexa; dinoflagellates; gregarines; heat shock protein 90; molecular phylogeny**

Abbreviations: **hsp90, 90-kDa heat shock protein gene family; SSU, small subunit**

The Alveolata is one of the most biologically diverse supergroups of eukaryotic microorganisms, consisting of ciliates, dinoflagellates, apicomplexans, and several minor lineages. Although molecular phylogenies unequivocally support the monophyly of alveolates, members of the group share only a few derived morphological features, such as distinctive patterns of cortical vesicles (syn. alveoli or amphiesmal vesicles) subtending the plasma membrane and presumptive pinocytotic structures, called "micropores" (Cavalier-Smith 1993, Siddall et al. 1997, Patterson 1999). Each of the three major subgroups of alveolates is highly derived along its own evolutionary trajectory. Ciliates, which are mostly active predators, are apomorphically defined by dimorphic nuclei and a distinctive cytoskeleton comprised of numerous short flagella (cilia) and associated root systems (Schlegel and Eisler 1996, Katz 2001). Dinoflagellates are biflagellates with diverse modes of nutrition (e.g. phagotrophy, parasitism, phototrophy, and mixotrophy) and are apomorphically defined by a dinokaryon (permanently condensed chromosomes thought to lack typical eukaryotic histones) and a distinctive flagellar apparatus consisting of a coiled transverse flagellum within a cingular groove or girdle and a posterior flagellum within a sulcal groove (Taylor 1987, Fensome et al. 1999). Apicomplexans are intracellular, largely nonflagellated parasites of animals and are apomorphically defined by a novel cell invasion apparatus (consisting mainly of rhoptries and a closed conoid) called the "apical complex" (Levine 1970, 1978, 1988, Ellis et al. 1998). The vast phenotypic differences between ciliates, dinoflagellates, and apicomplexans have made the earliest stages of alveolate evolution difficult to infer.

Molecular phylogenies strongly indicate that dinoflagellates and apicomplexans are more closely related to each other than to ciliates (Gajadhar et al. 1991, Cavalier-Smith 1993, Fast et al. 2002, Leander and Keeling 2003). The peculiar suite of features in apicomplexans (intracellular parasitic mode of life and vestigial plastids in some) and dinoflagellates (free-living predators with roughly half possessing photosynthetic plastids) combined with the medical and economic importance of representative species in both groups have stimulated a great deal of interest in their diversity and natural history. Confident inferences about the characteristics of the last common ancestor of dinoflagellates and apicomplexans and the

[1]Received 18 July 2003. Accepted 23 November 2003.
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subsequent transformations in both lineages would provide the necessary framework for understanding the functions and distributions of characters in more derived lineages. The ultrastructural characteristics and preliminary molecular phylogenetic positions of two key alveolate groups, perkinsids and colpodellids, provide considerable scaffolding to this framework (Leander and Keeling 2003). For instance, current evidence tentatively suggests that colpodellids, which are biflagellated predators of microeukaryotic prey, are the earliest sister lineage to "true" apicomplexans (Kuvardina et al. 2002, Leander et al. 2003b) and perkinsids, which are marine parasites of bivalves and microeukaryotes, are the earliest sister lineage to "true" dinoflagellates (Reece et al. 1997, Siddall et al. 1997, Saldarriaga et al. 2003b). This working topology combined with the very similar ultrastructural organization of colpodellids and perkinsid zoospores strongly suggests that the last ancestor of apicomplexans and dinoflagellates possessed all the character states shared by colpodellids and perkinsids, such as two heterodynamic flagella, an apical complex consisting of an open-sided conoid and rhoptries, and the ability to consume the cytoplasmic contents of eukaryotic prey (Kuvardina et al. 2002, Leander and Keeling 2003, Leander et al. 2003b).

Despite the fact that most dinoflagellates are either free-living predators or phototrophs, small subunit (SSU) rDNA sequences indicate that the earliest stages of dinoflagellate evolution appear to be dominated by parasitic lineages, such as perkinsids and syndinians (e.g. *Amoebophrya* and *Hematodinium*) (Gunderson et al. 1999, 2002, Lopez-Garcia et al. 2001, Moon-van der Staay et al. 2001, Saldarriaga et al. 2001). Certain nuclear features, such as the possible presence of histones (Ris and Kubai 1974), are consistent with the early phylogenetic position of syndinians in molecular trees. This trend, placed in the larger context of a close sister group between apicomplexans and dinoflagellates, forces one to entertain whether dinoflagellates are actually derived from parasitic ancestors. However, a better understanding of certain free-living predators, like *Oxyrrhis* (and colpodellids), may enlighten this perspective. *Oxyrrhis marina* is a marine biflagellate that feeds on eukaryotic microalgae, and although it lacks the diagnostic characters of dinoflagellates (e.g. pusules, a sulcus, a cingulum, a dinokaryon consisting of thick fibrillar chromosomes, and an extranuclear spindle with cytoplasmic channels), *Oxyrrhis* has several dinoflagellate-like features, such as a pattern of small polygonal alveoli; a similar, albeit more complex, flagellar apparatus; a similar peripheral microtubular cytoskeleton; and thin yet permanently condensed chromosomes (Dodge and Crawford 1971a,b, Roberts 1985, Höhfeld et al. 1994, Höhfeld and Melkonian 1998). Accordingly, the taxonomic history of *Oxyrrhis* has been indecisive: Some schemes include it within the dinoflagellates (Kofoid and Swezy 1921, Dodge 1984) and others do not (Fensome et al. 1993). Current evidence suggests that *Oxyrrhis* diverged early in dinoflagellate evolution (Saldarriaga et al. 2003b) but where it branches relative to perkinsids and syndinians remains uncertain.

The earliest stages of apicomplexan evolution involved the development of intracellular invasion of animal cells. This is inferred to have been a fairly straightforward transition from the "myzocytotic" mode of predation (i.e. withdrawing the cytoplasm of prey cells; Schnepf and Deichgraber 1984) used by colpodellids (Leander and Keeling 2003). It is widely suspected that the earliest diverging true apicomplexans are gregarine-like organisms, particularly so-called archigregarines such as *Selenidium* (Schrével 1968, 1971a, Leander et al. 2003c). Gregarines are restricted to a single host (monoxenous), and most of the life cycle occurs in the extracellular spaces of insects and marine invertebrates, such as the coelom, intestinal lumen, and reproductive vesicles. Like in colpodellids, some marine archigregarine trophozoites have been shown to use myzocytosis when feeding on cells of the host gut lining (Schrével 1968, 1971a). These apparently ancestral features indicate that the phylogenetic position and interrelationships of gregarines will play a critical role in any attempt to reconstruct the early evolutionary history of apicomplexans (Leander and Keeling 2003). Moreover, SSU rDNA and β-tubulin phylogenies weakly suggest that gregarines, in general, are closely related to cryptosporidians (Carreno et al. 1999, Leander et al. 2003a, 2003c), which are gut parasites of vertebrates, including humans. This implies either that cryptosporidians are a specialized lineage within or sister to a gregarine clade or that cryptosporidians diverged before gregarines as one of the earliest offshoots in apicomplexan evolution. Although the specific branching order between archigregarines, eugregarines, cryptosporidians, and other apicomplexans remains unknown, the close affinities between some gregarines and cryptosporidians could have a significant impact on how we understand the basic biological properties of cryptosporidians and on views about the parallel evolution of apicomplexans in vertebrate hosts.

We attempted to address the earliest stages of alveolate phylogeny by expanding a novel protein gene data set for the group, namely the 90-kDa heat shock protein gene family (hsp90). We sequenced hsp90 genes from several lineages of ciliates, dinoflagellates, and apicomplexans, including key species such as *Perkinsus marinus*, *Oxyrrhis marina*, *Cryptosporidium parvum*, and the eugregarine *Monocystis agilis*. Attempts to sequence the hsp90 gene from an archigregarine (*Selenidium*) and *Colpodella* were unsuccessful, so their respective sequences could not be included in the analyses. However, we were able to sequence the actin gene from *M. agilis* to address the relationship between cryptosporidians and eugregarines using a concatenated protein data set (hsp90, actin, and β-tubulin). The hsp90 gene was chosen for this comparative study because previous reports suggest that it is appropriately conserved yet variable enough to pro-

vide phylogenetic signal for the distant relationships of interest (Lindquist and Craig 1988, Ruef et al. 2000, Frankel et al. 2001, Fast et al. 2002, Simpson et al. 2002). Moreover, the gene has critical functions in all eukaryotic cells (Csermely et al. 1998, Rutherford and Lindquist 1998, Pearl and Prodromeau 2000) and has been sampled widely enough from other eukaryotes to include an adequate variety of nonalveolate taxa in the phylogenetic analyses.

MATERIALS AND METHODS

Acquisition of organisms. Cultures of *O. marina* (CCCM 534) and *Prorocentrum micans* (CCCM 818) were obtained from the Canadian Center for the Culture of Microorganisms (CCCM). A culture of *Heterocapsa triquetra* (CCMP 449) was obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP). Genomic DNAs from *Halteria grandinella*, *Blepharisma intermedium*, and *Tetrahymena bergeri* were gifts from D. Lynn (Guelph, Ontario, Canada) and J. Berger (Vancouver, British Columbia, Canada). *Lessardia elongata* was obtained as described previously (Saldarriaga et al. 2003a). Genomic DNA from *P. marinus* was a gift from R. Waller and G. McFadden (Melbourne, Australia), and genomic DNA from *C. parvum* was a gift from J. Keithly (Albany, New York, USA). The hsp90 sequence from the unidentified dinoflagellate A was obtained from marine water samples collected at low tide near Bamfield Marine Station (British Columbia, Canada) in April 2002 (Leander et al. 2002). Genomic DNA from approximately 50 gametocysts (filled with lemon-shaped oocysts) of *M. agilis* was obtained from the seminiferous vesicles of earthworms (*Lumbricus terrestris*) purchased from Berry's Bait and Tackle (Richmond, British Columbia, Canada). The bacteriotrophic chrysophyte, *Spumella uniguttata*, was the prey organism for a culture of *Colpodella edax* isolated from a freshwater pond near Borok (Yaroslavskaja, Russian Federation) in February 1980 (Leander et al. 2003b). Genes were surveyed from *S. uniguttata* in several attempts to sequence the *C. edax* homologues.

DNA extraction, amplification, cloning, and sequencing. Genomic DNA was extracted using a standard hexadecyltrimethylammonium bromide extraction protocol (Zolan and Pukkila 1986). PCR amplifications consisted of an initial denaturing period (95° C for 2 min); 35 cycles of denaturing (92° C for 45 s), annealing (48° C for 45 s), and extension (72° C for 1.5 min); and a final extension period (72° C for 5 min). In most cases, hsp90 sequences were amplified as a single fragment with the following primers: HSP90F1–5′ ACGTTYTAYWSNAAYAARGARAT 3′ or HSP90F4–5′ CGG CACGTTCTACWSNAAYAARGA 3′ and HSP90R2–5′ CGCC TTCATMATNCSYTCCATRTTNGC 3′. A partial fragment from *M. agilis* (1132 bases) was amplified using HSP90F4 and HSP90R3–5′ GATGACYTTNARDATYTTRTTYTGYTG 3′. The hsp90 sequence from *H. triquetra* was obtained by N. M. Fast by RT-PCR as described previously (Saldarriaga et al. 2003b). Actin sequences from *M. agilis* and *S. uniguttata* were amplified as a single fragment with the following primers: ACTF5–5′ GAGAAGATGACNCARATHATGTTYGA 3′ and ACTR3–5′ GGCCTGGAARCAYTTNCGRTGNAC 3′. PCR products corresponding to the expected size were gel isolated and cloned into the pCR2.1 using the TOPO TA cloning kit (Invitrogen, Burlington, Ontario, Canada). Two to four clones from each organism were partially sequenced with a big-dye reaction mix (ABI, Piscataway, NJ, USA) using the vector primers and one exact match internal primer for hsp90 genes. Introns were absent in all genes sequenced, except in the hsp90 gene of *P. marinus*, where two canonical introns near the 5′ end of the template sequence were present between residues 28 and 77 (48 bases) and residues 177 and 228 (50 bases). Sequences examined in this study are listed in Table 1.

Phylogenetic analyses. Inferred amino acid sequences from hsp90 and actin were added to existing alignments consisting of a wide variety of eukaryotes (Fast et al. 2002). Unambiguously aligned sequences were confirmed by eye. The hsp90 alignment (excluding the partial sequence from *M. agilis*) consisted of 36 taxa (18 alveolates) and 526 unambiguously aligned amino acid characters. The actin alignment consisted of 15 ingroup taxa (alveolates), 6 outgroup taxa (stramenopiles), and 206 unambiguously aligned amino acid characters; ciliates were excluded from the actin alignment because of their highly divergent nature (Keeling 2001, Saldarriaga et al. 2003b). The partial hsp90 sequence from *M. agilis* was added to the hsp90 alignment, producing an alignment with 37 taxa and 332 characters. Two alignments comprised of different protein sequences were also analyzed: an hsp90/actin alignment (12 taxa and 491 characters) and an hsp90/actin/β-tubulin alignment (11 taxa and 845 characters).

Maximum likelihood distances for all protein data sets were calculated with TREE-PUZZLE 5.0 using the WAG substitution matrix (Strimmer and Von Haeseler 1996). Site-to-site variation was modeled on a Γ distribution with an invariable sites parameter and eight variable rate categories producing estimates for the fraction of invariable sites and the α shape parameter. Distance trees were constructed with weighted neighbor-joining using WEIGHBOR (Bruno et al. 2000). After creating 100 bootstrap data sets with SEQBOOT (Felsenstein 1993), distances for each bootstrap data set were generated with the α shape parameter estimated from the original data set using the shell script "puzzleboot" (M. Holder and A. Roger, *www.tree-puzzle.de*). Bootstrap distance trees were constructed with WEIGHBOR.

Protein maximum likelihood analysis was conducted on all alignments using ProML (Felsenstein 1993). Trees were searched with eight random taxon additions followed by global rearrangements. For the actin and hsp90 data sets, site-to-site rate variation was modeled using the "R" option with five categories of substitution rates (four rates plus invariable sites), using the probabilities and frequencies estimated by TREE-PUZZLE. For the concatenated protein data sets, nine categories of substitution rates were used (eight rates plus invariable sites). ProML bootstrapping was also performed for 100 resampled data sets (from SEQBOOT) using the same settings but with only one input order jumble.

We also analyzed each data set with parsimony. Amino acids were treated as independent unordered character states of equal weight, and gaps were treated as missing data. A heuristic search was performed using PAUP* 4.0 (Swofford 1999) with ACCTRAN character state optimization, tree bisection reconnection branch swapping, random step-wise addition of taxa (10 input order jumbles), and MULTREES on. Bootstrap values from 500 replicates were generated to evaluate the robustness of each node on the most parsimonious tree(s).

RESULTS AND DISCUSSION

Phylogeny of alveolates as inferred from hsp90 protein sequences. Phylogenetic analyses of hsp90 amino acid sequences produced a strongly supported internal topology for alveolates (Fig. 1). The Alveolata was shown to be monophyletic with strong bootstrap support (bootstrap percentages = 98, 98, and 81; Fig. 1), and dinoflagellates and apicomplexans were more closely related to each other than to ciliates. The closest, albeit weakly supported, sister group to alveolates was a stramenopile clade consisting of a

TABLE 1. GenBank accession numbers for the protein sequences used in this study.

Taxon	Hsp90	Actin	β-Tubulin
Stramenopiles			
Achlya ambisexualis	AAM90675	—	—
Achlya bisexualis	—	P26182	—
Costaria costata	—	S24409	—
Fucus distichus	—	P53502	—
Phytophthora megasperma	—	P13363	—
Pythium irregulare	—	S49007	—
Spumella uniguttata	AY391254[a]	AY391263[a]	—
Ciliates			
Blepharisma intermedium	AY390395[a]	—	—
Halteria grandinella	AY391253[a]	AAF00923	—
Paramecium tetraurelia	AAG00569	—	S25182
Tetrahymena bergeri	AY391257[a]	—	—
Tetrahymena pyriformis	AAG00567	—	—
Tetrahymena thermophila	AAD41357	—	S41470
Dinoflagellates			
Amphidinium carterae	—	AAB62063	—
Crypthecodinium cohnii	AAM02974	AAM02969	AAM02970
Gymnodinium varians	—	AA049352	—
Heterocapsa triquetra	AY391255[a]	AF482411	AA049342
Karenia brevis	—	AF482415	—
Lessardia elongata	AY391256[a]	—	—
Oxyrrhis marina	AY391258[a]	AF482402	AF482404
Peridinium willei	—	AF482420	—
Prorocentrum micans	AY391260[a]	—	—
Prorocentrum minimum	—	AAB62066	—
Unidentified dinoflagellate A	AY391261[a]	—	—
Unidentified dinoflagellate B	—	BAC44870	—
Perkinsid			
Perkinsus marinus	AY391259[a]	AAB62064	AF482401
Apicomplexans			
Babesia bovis	AAF61428	AAM77563	Q04709
Cryptosporidium parvum	AY423866[a]	P26183	P14643
Eimeria tenella	AAB97088	From ESTs	AAB41262
Leidyana migrator[b]	—	—	AF457131
Monocystis agilis	AY391262[a]	AY391264[a]	—
Plasmodium falciparum	CAD52775	P10988	P14643
Theileria parva	AAA30132	—	—
Toxoplasma gondii	—	P53476	—
Other eukaryotes			
Bodo saltans	AAM93754	—	—
Diplonema papillatum	AAM93745	—	—
Trypanosoma cruzi	P06660	—	—
Dictyostelium discoideum	P54651	—	—
Guillardia theta nucleomorph	AAF24209	—	—
Caenorhabditis elegans	NP_506626	—	—
Drosophila auraria	O02192	—	—
Gallus gallus	HHCH90	—	—
Homo sapiens	P07900	—	—
Candida albicans	P46598	—	—
Neurospora crassa	AAF34607	—	—
Saccharomyces cerevisiae	NP_013911	—	—
Schizosaccharomyces pombe	S51795	—	—
Arabidopsis thaliana	NP_200411	—	—
Triticum aestivum	AAD11549	—	—
Zea mays	Q08277	—	—

[a]Sequence derived from this study.
[b]Used as part of the "combined gregarine" in the concatenated phylogenetic analysis presented in Figure 4d.

chrysophyte (*S. uniguttata*) and an oomycete (*Achlya ambisexualis*), which is consistent with previous reports using different genes (van de Peer and de Wachter 1997, Baldauf et al. 2000, Fast et al. 2001). Core dinoflagellates (*P. micans*, *Crypthecodinium cohnii*, *L. elongata*, *H. triquetra*, and the sequence from unidentified dinoflagellate A) formed a well-supported clade to the exclusion of *Oxyrrhis* and *Perkinsus*. *Oxyrrhis* formed the nearest sister lineage to the core dinoflagellates, lending credence to previous interpretations that it is an early diverging lineage with several character states that are intermediately developed along the dinoflagellate stem lineage (e.g. unarmored theca, coiled transverse flagellum, and

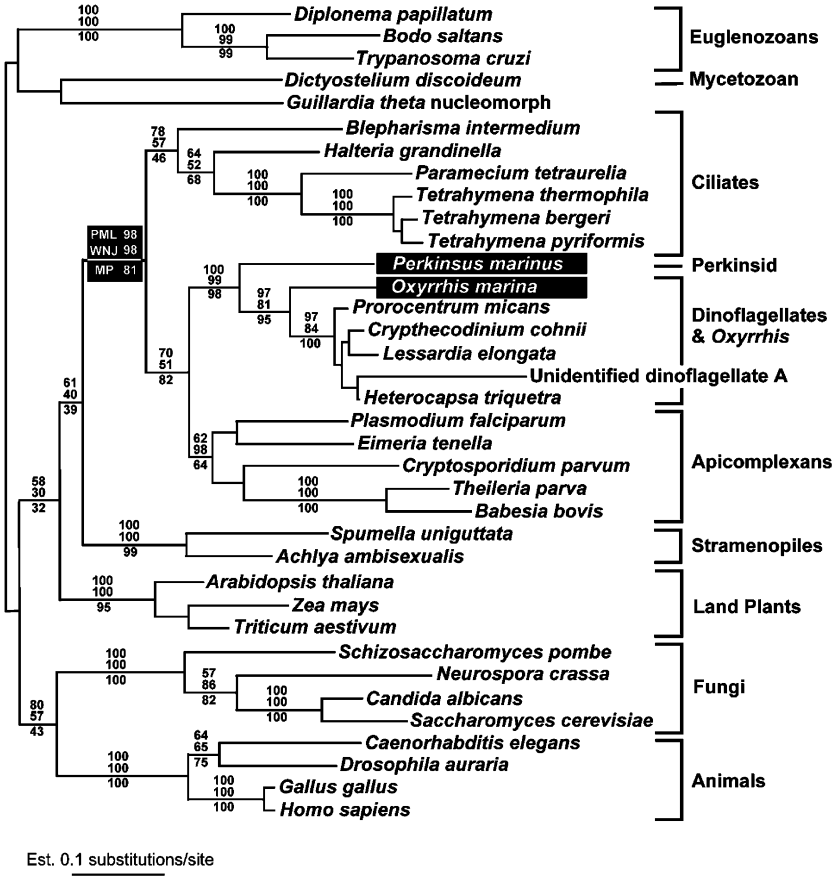

FIG. 1. Γ-Corrected protein maximum likelihood tree of 36 hsp90 amino acid sequences (526 sites) showing the phylogenetic positions of *Perkinsus marinus* and *Oxyrrhis marina* within the alveolate clade (ciliates, dinoflagellates, and apicomplexans). Ciliates, dinoflagellates, and apicomplexans each formed monophyletic groups in all analyses, where dinoflagellates and apicomplexans were more closely related to each other than either was to ciliates. Stramenopiles consistently diverged as the closest sister group to alveolates but with weak statistical support. Numbers at the internodes correspond to bootstrap proportions for protein maximum likelihood (PML), weighted neighbor joining (WNJ), and maximum parsimony (MP).

condensed chromosomes) (Kofoid and Swezy 1921, Dodge and Crawford 1971a, Dodge 1983, Fensome et al. 1993, Loeblich 1984).

Although several alternative relationships have been proposed (Ray 1954, Mackin and Ray 1966), perkinsids were long considered apicomplexan-like flagellates (Perkins and Menzel 1967, Perkins 1969, 1976, Levine 1978, Norén et al. 1999, Perkins et al. 2002), because they possess an apical complex reminiscent of those in apicomplexans and colpodellids (Perkins 1976, 1996, Azevedo et al. 1990, Myl'nikov 1991, Brugerolle 2002). Analyses of hsp90 sequences, however, place *P. marinus* as the nearest sister lineage to the clade comprised of *Oxyrrhis* and core dinoflagellates. Bootstrap analyses using Γ-corrected protein maximum likelihood, Γ-corrected distance, and parsimony indicated that the relative topology of *Perkinsus*, *Oxyrrhis*, and core dinoflagellates was strongly supported by the hsp90 data. Moreover, a novel deletion at amino acid position 511 and a glutamine residue at position 431 (using the *G. theta* nucleomorph sequence as a reference—AAF24209) was present in the core dinoflagellates and in *Oxyrrhis* but was absent in all other eukaryotes, including *Perkinsus* (Fig. 2). The sister relationship of perkinsids with dinoflagellates sheds considerable light onto the biological properties of the most recent ancestor of apicomplexans and dinoflagellates. This ancestor must have possessed the character states shared by perkinsids and apicomplexans, most significantly an apical (eukaryotic) cell invasion apparatus comprising of a (open) conoid, micronemes, and rhopteries (Leander and Keeling 2003).

The hsp90 data set provides the strongest evidence yet for both the early diverging position of *Oxyrrhis* among dinoflagellates and the sister relationship between perkinsids and the *Oxyrrhis*–dinoflagellate clade. Thus, it can be more confidently inferred that the absence of pusules, a cingulum, a sulcus, and permanently condensed fibrillar chromosomes are ancestral character states in *Oxyrrhis* (Fig. 3). Moreover, although examples of convergent evolution (reversals) would not be surprising, the presence of small polygonal alveoli in *Oxyrrhis* and perkinsids strongly suggests that this cortical pattern in many lineages of dinoflagellates (e.g. athecate species such as "*Gymnodinium*") is a plesiomorphic character state (Dodge 1983). This is further supported by the presence of small polygonal alveoli in colpodellids (Leander et al. 2003b), which appear to be the earliest diverging sister group to true apicomplexans (Kuvardina et al. 2002, Leander et al. 2003b). Other characters in *Oxyrrhis* are clearly autapomorphic, including external scales (Clarke and Pennick 1976), an intranuclear mitotic spindle originating from plaques on the nuclear envelope (Triemer 1982, Gao and Li 1986), and perhaps several structures associated with the flagellar root system (Dodge and Crawford 1971b, Roberts 1985, Höhfeld et al. 1994) (Fig. 3).

	495 519
***Guillardia theta* nucleomorph**	GKKLVCATKEGLDLGDSENDKKVKE
Achlya ambisexualis	GKKLICATKEGLKMEETEDEKKSFE
Spumella uniguttata	GKKLVCATKEGLKINESEDEKKSFE
Blepharisma intermedium	GKKLKNCTKEGLELDEGEEEKKKFE
Halteria grandinella	GKKLKSCSKEGLDLEETEEEKKQKE
Tetrahymena bergeri	GKKLKNCTKEGLDLDQTEDEKKKFE
Tetrahymena thermophila	GKKLKNCSKEGLELEQSEDEKKSFE
Paramecium tetraurelia	GKKLKNCSKEGLELESTEDEKKKFE
Tetrahymena pyriformis	GKKLKNCSKEGLELEQTEDEKKKFE
Babesia bovis	GKKLRCCTKEGLTLEETAEEKEAFE
Plasmodium falciparum	GKKLKCCTKEGLDIDDSEEAKKDFE
Eimeria tenella	NHKLRCCTKEGLEIDESEEEKKKFE
Theileria parva	GKKLKCCTKEGLDLDEGEDEKKSFE
Cryptosporidium parvum	GKKLKCCTKENLELEDTEEERKNFE
Unidentified dinoflagellate A	GKKLKSTTKEGLDIED-EDEKKKLE
Heterocapsa triquetra	GKKLQLTTKAGLDLED-EDEKKKLE
Prorocentrum micans	GKKLKSTTKEGLDLED-EDEKKKLE
Crypthecodinium cohnii	GKKLKSVTKEGLDIAD-EDEKKKLE
Lessardia elongata	GKKLKSTTKEGLDIED-EDEKKKLE
Oxyrrhis marina	GKKLKSCTKEGLDLED-EDEKKSME
Perkinsus marinus	GHKLKSITKEGLDLNESDEEKKAFE
Dictyostelium discoideum	GKKLVSITKEGLKLDETEDEKKKAE
Diplonema papillatum	DKKFRCVTKEGLKFEETEDEKKKHE
Trypanosoma cruzi	DKKFACLTKEGVHFEETEEEKKQRE
Arabidopsis thaliana	GKKLVSATKEGLKLEETDDEKKKKE
Zea mays	GKKLVSATKEGLKLDDEDDEKKRRE
Homo sapiens	GKTLVSVTKEGLELPEDEEEKKKQE
Caenorhabditis elegans	GKKLVSVTKEGLELPETEEEKKKFE
Drosophila auraria	GKQLVSVTKEGLELPEDDAEKKKRE
Neurospora crassa	GKKLVDITKD-FELEETEEEKKQRE
Candida albicans	DKKLVDITKD-FELEESDEEKAARE
Saccharomyces cerevisiae	GKTLVDITKD-FELEETDEEKAERE

FIG. 2. A relatively conserved region of an alignment of hsp90 amino acid sequences showing a novel indel present in dinoflagellates and *Oxyrrhis marina* (highlighted by the box) but absent in all other eukaryotes, including *Perkinsus marinus*. This molecular character bolsters the phylogenetic topology presented in Figure 1, showing *O. marina* as the closest free-living sister lineage to "true" dinoflagellates (i.e. species having a dinokaryon and/or a distinctive flagellar apparatus consisting of a transverse flagellum within a cingular groove and a posterior flagellum within a sulcal groove). Reference numbers correspond to the hsp90 sequence from the nucleomorph in *G. theta*.

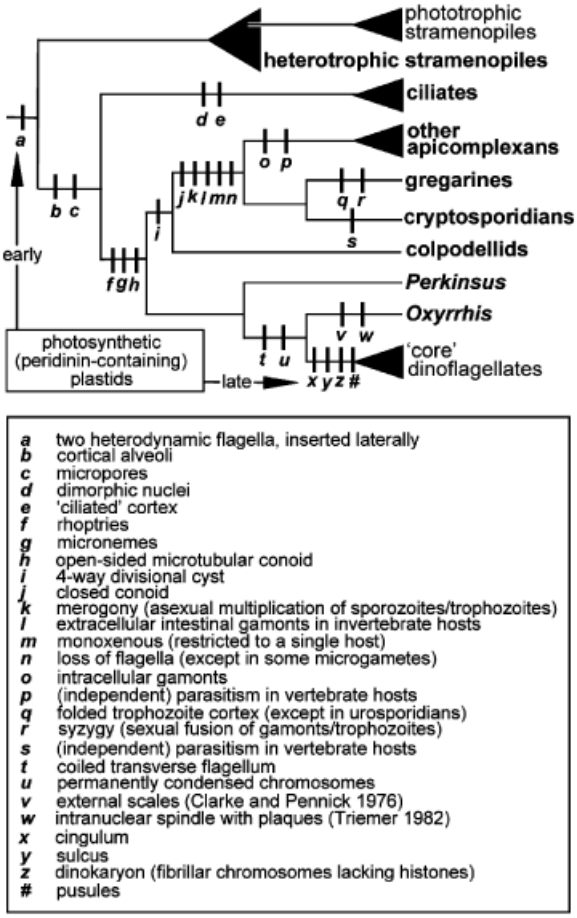

FIG. 3. Synthetic phylogeny of alveolates derived from the results of this study and previous reports (Carreno et al. 1999, Leander et al. 2003a,b,c, Saldarriaga et al. 2003b). Characters of interest are designated by letters and parsimoniously mapped onto the topological framework (see text for discussion). Names in bold indicate obligate heterotrophs. The two alternative views on the origin of the photosynthetic (peridinin-containing) plastids of dinoflagellates are marked as "early" and "late." The plastids-early view suggests that the photosynthetic (peridinin-containing) plastid of dinoflagellates is vertically homologous to the photosynthetic plastids of "chromists": stramenopiles (a.k.a. heterokonts), haptophytes, and cryptomonads. The plastids-late view suggests that the photosynthetic plastids of dinoflagellates arose by either a second-secondary endosymbiosis with a red alga or a tertiary endosymbiosis from a haptophyte prey cell. Because the results from this study have no direct impact on the origin of known vestigial plastids, the figure does not address when the non-photosynthetic "apicoplast" of apicomplexans arose or its potential relationships to the plastids of "chromists" and dinoflagellates.

The early phylogenetic positions of *Perkinsus* and *Oxyrrhis* (obligate heterotrophs) also provide context for arguments about the endosymbiotic origin of the photosynthetic (peridinin-containing) plastid of many dinoflagellates (Fig. 3). It is widely recognized that photosynthesis arose only once in the history of life and has subsequently either been secondarily lost or horizontally transferred via several types of endosymbioses (Archibald and Keeling 2002). Views on the origin of the peridinin-containing plastids of dinoflagellates can be conveniently divided into two camps: the *early* endosymbiosis camp advocates the relatively early origin of photosynthetic plastids in dinoflagellates (Cavalier-Smith 1999, Zhang et al. 2000, Fast et al. 2001, Harper and Keeling 2003), whereas the *late* endosymbiosis camp advocates the relatively recent origin of the photosynthetic plastids in dinoflagellates, perhaps via a plastid replacement event (Whatley and Whatley 1981, Taylor 1987, Yoon et al. 2002) (Fig. 3). Both camps can explain the tendency for heterotrophy among the earliest branches of the dinoflagellate lineage. The early endosymbiosis camp would infer that the photosynthetic (peridinin-containing) plastids of dinoflagellates were vertically inherited from the last ancestor of "chromists" (haptophytes, stramenopiles, and cryptomonads) and alveolates (collectively the "chromalveolates") and subsequently there were multiple independent losses of photosynthesis (or entire

plastids). Conversely, the late endosymbiosis camp would infer that the earliest lineages diverging from the dinoflagellate stem-lineage are plesiomorphically non-photosynthetic (which does not exclude the possible presence of a vestigial plastid, perhaps similar to the "apicoplast" of apicomplexans), and the photosynthetic (peridinin-containing) plastid arose (independently) later in dinoflagellate evolution via a second-secondary or tertiary endosymbiosis (Yoon et al. 2002) (Fig. 3). These two alternatives will likely be distinguishable once there is considerably more data from plastid genes from dinoflagellates. If dinoflagellate and apicomplexan plastid genes are generally closely related and if dinoflagellate plastid-targeted proteins are not, as a group, found to be related to homologues from a specific type of algae, then we would conclude that the extant plastids in apicomplexans and dinoflagellates are related by descent. If this is borne out by future data, it paints an intriguing picture of their common ancestor as a phototroph with a complex apparatus for feeding on other eukaryotes.

The ability for dinoflagellates to both gain and lose plastids (Chesnick et al. 1997, Schnepf and Elbraechter 1999, Tengs et al. 2000, Saldarriaga et al. 2001) makes distinguishing between these alternative viewpoints challenging. However, as illustrated in Figure 3, an unambiguous trend in alveolate evolution remains: The photosynthetic plastids of dinoflagellates are nested deeply within a very diverse group of heterotrophic organisms (e.g. ciliates, apicomplexans, perkinsids, *Oxyrrhis*, and perhaps several other minor lineages like *Acrocoelus*, *Rastrimonas* [a.k.a. *Cryptophagus*], *Colponema*, and some species of *Colpodella*). Moreover, it is important to note that this trend is bolstered by the early phylogenetic positions of syndinians (non-photosynthetic parasitic dinoflagellates that share several nuclear characteristics with *Oxyrrhis*) and *Noctiluca* in most SSU rDNA phylogenies (Saldarriaga et al. 2001, Gunderson et al. 1999, 2002).

The phylogenetic positions of cryptosporidians and gregarines. Cryptosporidians are intestinal parasites of vertebrates that have traditionally been allied with coccidians (e.g. *Toxoplasma*, *Eimeria,* and *Isospora*). *Cryptosporidium parvum* infects humans and has been implicated in widespread outbreaks of chronic intestinal disorders and acute diarrhea in patients suffering from acquired immunodeficiency syndrome. Some molecular phylogenies indicate that cryptosporidians are not as closely related to coccidians as previously thought (Morrison and Ellis 1997, Zhu et al. 2000) and are actually more closely related to a gregarine clade (Carreno et al. 1999, Leander et al. 2003a, 2003c).

We set out to test the interrelationships between cryptosporidians, coccidians, and gregarines by gathering protein sequences from hsp90 and actin genes from key taxa, specifically the eugregarine *M. agilis* and *C. parvum*, and phylogenetically analyzing them in the context of our emerging hsp90 data set and in concatenated protein data sets. Phylogenetic analysis of hsp90 amino acid sequences placed *M. agilis* and *C. parvum* together with weak support (bootstrap percentages = 58, 51, and 53; Fig. 4a). By contrast, actin phylogenies failed to recover a sister relationship between *M. agilis* and *C. parvum*, possibly due to artifacts associated with taxon sampling and long-branch attraction (the actin sequences from *M. agilis* had an inordinately long branch; Fig. 4b). An alignment of concatenated sequences from hsp90 and actin recovered the sister relationship between *M. agilis* and *C. parvum* with somewhat better statistical support than with the hsp90 sequences alone (Fig. 4c);, however, this might also reflect a taxon sampling effect.

A previous study used β-tubulin amino acid sequences from the eugregarine *Leidyana migrator* to examine the gregarine–cryptosporidian affinity (Leander et al. 2003a). This study demonstrated a sister relationship between gregarines and cryptosporidians, albeit with very weak support. We concatenated our protein sequences from *M. agilis* with the β-tubulin sequence from *L. migrator*, which provided a surrogate sequence for the group shown as "combined gregarine" in Figure 4d. This three-gene analysis recovered the sister relationship between *M. agilis* and *C. parvum* with strong statistical support (bootstrap percentages = 88, 81, and 79; Fig. 4d). Although consistent with previous analyses using SSU rDNA and β-tubulin, this is perhaps the strongest evidence so far that cryptosporidians and some gregarines are more closely related to each other than to coccidians (or any other group of apicomplexans). However, there is potential for misleading results when using concatenated genes from nonidentical taxa (Malia et al. 2003), so the statistical support in Figure 4c should be weighed accordingly.

Phylogenies derived from SSU rDNA suggest that cryptosporidians might be specifically related to some of the earliest diverging gregarines known: the archigregarines (Leander et al. 2003c). Archigregarines (e.g. *Selenidium*/*Selenidoides*) are exclusively found in marine environments and infect the intestines of a variety of invertebrates, such as sipunculans, polychaetes, and urochordates (Schrével 1968, 1971a,b, Levine 1971). Similarities between cryptosporidians and archigregarines include a monoxenous life cycle with extracellular gamonts/trophozoites, oocysts with four sporozoites, and occupancy in the host intestine (Fig. 3). Like most coccidians, however, cryptosporidians infect the intestines of vertebrates and possess very small gamonts and oocysts that exit the host via the feces (Fayer et al. 1997). Therefore, a gregarine ancestry for cryptosporidians would 1) represent an unanticipated example of convergent exploitation of vertebrate hosts by both coccidians and cryptosporidians and 2) have a significant impact on how we understand and deal with the basic biology of cryptosporidians. It has already been shown, for instance, that cryptosporidians are insensitive to most anticoccidial drugs (Woods et al. 1996, Coombs 1999). We suspect that improved understanding of gregarine

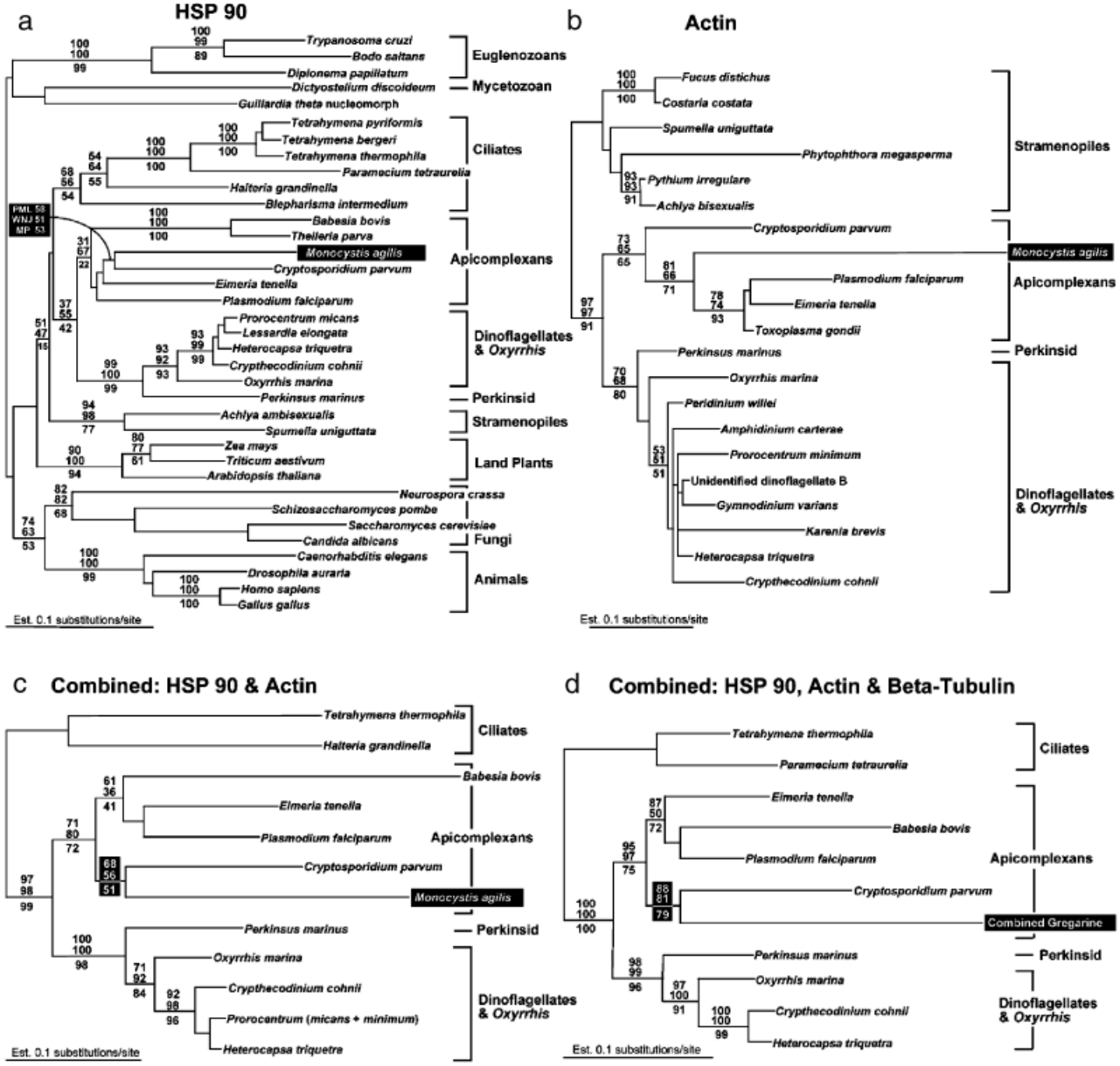

FIG. 4. Γ-Corrected protein maximum likelihood trees showing the phylogenetic position of the eugregarine *Monocystis agilis* as inferred from (a) an alignment of hsp90 amino acid sequences (332 sites), (b) an alignment of actin amino acid sequences (206 sites), (c) a concatenated data set of hsp90 and actin amino acid sequences (491 sites), and (d) a concatenated data set of hsp90, actin, and β-tubulin amino acid sequences (845 sites). In d, the concatenated sequence for the "combined gregarine" is derived from the hsp90 and actin genes from *M. agilis* and the β-tubulin gene from *Leidyana migrator* (Leander et al. 2003a). Phylogenetic trees based on β-tubulin sequences show a weak sister relationship between *Cryptosporidium parvum* and *L. migrator* (Leander et al. 2003a). Except in actin trees, gregarines, represented by *M. agilis* and the "combined gregarine" (highlighted in bold), formed the sister lineage to *C. parvum* with moderate to strong statistical support (highlighted in bold). Numbers at the internodes correspond to bootstrap proportions for protein maximum likelihood (PML), weighted neighbor joining (WNJ), and maximum parsimony (MP).

diversity will shed significant light onto the fundamental properties of not only gregarines but also cryptosporidians.

We thank J. M. Archibald and two anonymous reviewers for helpful comments and D. Lynn, J. Berger, R. Waller, and J. Keithly for providing genomic DNA. This work was supported by a grant to B. S. Leander from the National Science Foundation (Postdoctoral Research Fellowship in Microbial Biology, USA) and to P. J. Keeling from the Canadian Institutes for Health Research (MOP-42517). B. S. Leander and P. J. Keeling are Scholars of the Canadian Institute for Advanced Research (Program in Evolutionary Biology); P. J. Keeling is also a new investigator of the Michael Smith Foundation for Health Research and the Canadian Institutes for Health Research.

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