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Multiple protein phylogenies show that *Oxyrrhis marina* and *Perkinsus marinus* are early branches of the dinoflagellate lineage

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Oxyrrhis marina and *Perkinsus marinus* are two alveolate species of key taxonomic position with respect to the divergence of apicomplexans and dinoflagellates. New sequences from *Oxyrrhis*, *Perkinsus* and a number of dinoflagellates were added to datasets of small-subunit (SSU) rRNA, actin, α-tubulin and β-tubulin sequences, as well as to a combined dataset of all three protein-coding genes, and phylogenetic trees were inferred. The parasitic *Perkinsus marinus* branches at the base of the dinoflagellate clade with high support in most of the individual gene trees and in the combined analysis, strongly confirming the position originally suggested in previous SSU rRNA and actin phylogenies. The SSU rRNA from *Oxyrrhis marina* is extremely divergent, and it typically branches with members of the Gonyaulacales, a dinoflagellate order where SSU rRNA sequences are also divergent. Conversely, none of the three protein-coding genes of *Oxyrrhis* is noticeably divergent and, in trees based on all three proteins individually and in combination, *Oxyrrhis* branches at the base of the dinoflagellate clade, typically with high bootstrap support. In some trees, *Oxyrrhis* and *Perkinsus* are sisters, but most analyses indicate that *Perkinsus* diverged prior to *Oxyrrhis*. Morphological characters have previously pointed to *Oxyrrhis* as an early branch in the dinoflagellate lineage; our data support this suggestion and significantly bolster the molecular data that support a relationship between *Perkinsus* and dinoflagellates. Together, these two organisms can be instrumental in reconstructing the early evolution of dinoflagellates and apicomplexans by helping to reveal aspects of the ancestors of both groups.

INTRODUCTION

The alveolates are a large and diverse assemblage of protists that include three major lineages: ciliates, dinoflagellates and apicomplexans. In addition to these three well-defined and relatively well-studied groups, there are also a number of species that display alveolate features (for example cortical alveoli, i.e. membranous sacs subtending the plasma membrane), but lack features that would ally them specifically with any one of the three subgroups. These organisms, called 'Protalveolata' by Cavalier-Smith (1998), are very likely to be paraphyletic or perhaps even polyphyletic, but we use the term protalveolates in a colloquial sense to simplify referring to this collection of organisms. Protalveolates are often regarded as intermediates between the major alveolate groups and are therefore potentially instrumental in reconstructing the evolutionary origin and history of the characteristics that define ciliates, dinoflagellates and apicomplexans. Here, we have examined the phylogenetic position of two such organisms, *Oxyrrhis marina* Dujardin 1841 and *Perkinsus marinus* (Mackin, Owen and Collier 1950) Levine 1978.

Oxyrrhis marina is a heterotrophic flagellate commonly found in marine and brackish near-shore waters, including rock pools, estuaries and marshes. The species has often been regarded as a dinoflagellate (e.g. Kofoid & Swezy, 1921; Dodge, 1984; Sournia, 1986), but has also been explicitly excluded from the group in other classification schemes (Fensome *et al.*, 1993), as it has a number of characters that are very different from those of true dinoflagellates. In *Oxyrrhis*, the mitotic spindle is intranuclear and originates from numerous plaques on the nuclear envelope (Triemer, 1982; Gao & Li, 1986); in dinoflagellates, the spindle is

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Abbreviation: SSU, small-subunit.

The GenBank/EMBL/DDBJ accession number for the SSU rRNA sequence of *Oxyrrhis marina* CCCM 534 is AF482425. The accession numbers for the actin, α-tubulin and β-tubulin gene sequences obtained in this study are AF482399–AF482424, as outlined in Table 1.

extranuclear and its microtubules are located within cytoplasmic channels that traverse the nucleus (Leadbeater & Dodge, 1967; Kubai & Ris, 1969; Ris & Kubai, 1974). The nuclear organization in *Oxyrrhis* is very atypical: it contains a large number of long, thin chromosomes, separated by numerous electron-dense bodies that could be small chromosome fragments (Dodge & Crawford, 1971). This organization is very different from the thick, continuously condensed, fibrillar chromosomes in the dinokaryon of typical dinoflagellates. Other differences between *Oxyrrhis* and true dinoflagellates are the lack of a girdle, a sulcus or pusules in *Oxyrrhis* (some dinoflagellates have secondarily lost the girdle and/or sulcus; Fensome *et al.*, 1993) and the presence of what could be histone proteins (Kato *et al.*, 1997); histones are absent in the nuclei of most dinoflagellates (the order Syndiniales could be an exception: Ris & Kubai, 1974; Sala-Rovira *et al.*, 1991).

Perkinsus marinus is the causative agent of 'dermo', an important disease of oysters and probably many other species of bivalves (Perkins, 1976). The taxonomic placement of *Perkinsus* has always been problematic: over the years, the genus has been considered to be a member of the fungi, labyrinthulids and haplosporidians. Eventually, ultrastructural data led to the conclusion that *Perkinsus* represents an early lineage of the apicomplexans (Levine, 1978; Perkins, 1996). This conclusion was based largely on the fact that its flagellated stage contains an apical organelle with similarities to the apicomplexan conoid, an apical structure composed of microtubular units arranged in a helical coil forming a truncated cone. In *Perkinsus*, however, this 'conoid' is open along one side, a feature that led to a reinterpretation of the significance of the structure for the taxonomy of the genus (Siddall *et al.*, 1997). The vegetative stage of *Perkinsus marinus* has, like dinoflagellates, two dissimilar flagella that insert ventrally; one of them with mastigonemes along one side (Perkins, 1996). Cell division in *Perkinsus* also appears to be dinoflagellate-like: the nuclear envelope remains intact during mitosis and deep channels are formed, continuous with the cytoplasm and lined by the nuclear membrane. The mitotic spindle runs through these channels and attaches to kinetochore-like structures on the nuclear envelope (Perkins, 1996). However, the interphase nuclear ultrastructure of this species is very unlike that of typical dinoflagellates: chromatin appears as electron-dense aggregates of varying density, not as the fibrillar structures of typical dinokaryons (Perkins, 1996). Most recently, *Perkinsus* was placed in its own alveolate phylum, the Perkinsozoa, together with a newly described parasite of dinoflagellates, *Parvilucifera infectans* (Norén *et al.*, 1999).

Molecular data from the protalveolates are uncommon, but small-subunit (SSU) rRNA and actin sequences have been used to address the phylogenetic position of *Perkinsus*. Together, these two gene phylogenies provide fairly convincing evidence that *Perkinsus* is closely related to dinoflagellates, not apicomplexans (Goggin & Barker, 1993; Reece *et al.*, 1997). Nevertheless, the support for this position is sometimes not very strong in SSU rRNA trees (e.g. Siddall *et al.*, 1997), and other analyses of SSU rRNA have also shown *Perkinsus* and *Parvilucifera* branching at the base of the apicomplexans (Norén *et al.*, 1999). In actin phylogenies, the very divergent sequences of ciliates fall far from either dinoflagellates or apicomplexans (e.g. Keeling, 2001), making it difficult to draw any firm conclusions on the position of *Perkinsus* based solely on this gene. The phylogenetic position of *Oxyrrhis* has not been substantially investigated using molecular data. Only one report describes any sequence data from *Oxyrrhis* (Lenaers *et al.*, 1991), and here only 235 nucleotides from two domains of the large subunit rRNA gene were used: phylogenetic trees inferred from this sequence and that of 12 dinoflagellates and one ciliate placed *Oxyrrhis* basal to the dinoflagellates (no apicomplexan sequences were included). Recently, SSU rRNA sequences from a number of unidentified alveolates were obtained from environmental samples of marine picoplankton (López-García *et al.*, 2001; Moon-van der Staay *et al.*, 2001; Diez *et al.*, 2001) and one large group of sequences was proposed to be from relatives of *Oxyrrhis* (Moon-van der Staay *et al.*, 2001). This hypothesis has not been tested, as no SSU rRNA sequence for *Oxyrrhis* has been available.

To investigate the origins of *Oxyrrhis* and *Perkinsus* further, we have sequenced genes encoding SSU rRNA, actin, α-tubulin and β-tubulin from *Oxyrrhis marina*, α-tubulin and β-tubulin from *Perkinsus marinus* and actin, α-tubulin and β-tubulin from a variety of dinoflagellates. We inferred phylogenies of these genes individually and in combination to determine the relationships between *Oxyrrhis*, *Perkinsus* and other alveolates and to begin to reconstruct the nature of the ancestors of dinoflagellates and apicomplexans.

METHODS

Cultures of *Oxyrrhis marina* and all photosynthetic dinoflagellates examined were obtained from culture collections as listed in Table 1. Marine cultures were maintained in HESNW medium (Harrison *et al.*, 1980), while fresh-water organisms (*Peridinium willei* and *Woloszynskia tenuissima*; nomenclature as in Popovský & Pfiester, 1990) were cultured as recommended by NIES. Genomic DNA from *Perkinsus marinus* was a gift from R. Waller and G. McFadden (Melbourne, Australia). A second SSU rRNA sequence from a different isolate of *Oxyrrhis marina* (NIES 494) was recently released to GenBank (accession no. AB033717; K. Takishita, K. Aoi and A. Uchida, unpublished) and was also included in our analyses.

DNA was purified from all cultures using the Plant DNeasy kit (Qiagen). The SSU rRNA gene of *Oxyrrhis marina* was amplified using two sets of primers resulting in overlapping fragments. The 3′ end of the gene was obtained with primers designed to amplify all eukaryotic SSU rRNA genes (5′-CCGGATCCTGATCCTTCTGCAGGTTCACCTAC and 5′-GCGGTAATTCCAGCT). The remainder of the 5′ end of the gene was amplified using another eukaryote-specific primer (5′-CGAATTCAACCTGGTTGATCCTGCCAGT) and a primer designed to recognize only dinoflagellate SSU rRNA sequences (5′-ACTTACTCTTTTCAGGCAC). Protein-coding genes for all organisms except *Perkinsus marinus* were amplified using the

Table 1. Accession numbers for the new protein-coding gene sequences

Taxon	Actin	α-Tubulin	β-Tubulin
Amphidinium corpulentum UTEX LB1562	–	–	AF482405
Amphidinium herdmanii CCCM D532	–	AF482406, AF482407	–
Gyrodinium instriatum CCMP 431	–	–	AF482408
Heterocapsa rotundata CCCM D680	AF482409	AF482410	–
Heterocapsa triquetra CCMP 449	AF482411	AF482412	AF482413, AF482414
Karenia brevis CCMP 718	AF482415–AF482418	AF482419	–
Oxyrrhis marina CCCM 534*	AF482402	AF482403	AF482404
Peridinium willei NIES 304	AF482420	–	AF482421
Perkinsus marinus†	–	AF482399, AF482400	AF482401
Symbiodinium sp. CCMP 421	AF482423	–	AF482424
Woloszynskia tenuissima NIES 619	–	–	AF482422

*The accession number for the SSU rRNA sequence of *Oxyrrhis marina* CCCM 534 is AF482425.
†DNA was kindly provided by R. Waller and G. McFadden. Actin sequence data were taken from Reece *et al.* (1997).

following primers: 5′-GAGAAGATGACNCARATHATGTTYGA and 5′-GGCCTGGAARCAYTTNCGRTGNAC for actin, 5′-TCCGAATTCARGTNGGNAAYGCNGGYTGGGA and 5′-CGCGCCATNCCYTCNCCNACRTACCA for α-tubulin and 5′-GCCTGCAGGNCARTGYGGNAAYCA and 5′-TCCTCGAGTRAAYTCCATYTCRTCCAT for β-tubulin, all in PCRs using genomic DNA. α-Tubulin and β-tubulin were amplified from *Perkinsus marinus* DNA using a two-step nested PCR approach, since only small quantities of DNA were available. The initial 10 μl reaction used primers 5′-GGGCCCCAGGTCGGCAAYGCNTGYTGG and 5′-GGGCCCCGAGAACTCSCCYTCYTCCAT for α-tubulin and 5′-GCCTGCAGGNCARTGYGGNAAYCA and 5′-TCCTCGAGTRAAYTCCATYTCRTCCAT for β-tubulin. These reactions were used to seed a second 50 μl reaction using primers 5′-TCCGAATTCARGTNGGNAAYGCNGGYTGGGA and 5′-CGCGCCATNCCYTCNCCNACRTACCA for α-tubulin and 5′-CAGATCGGCGCGAARTTYTGGGARAT and 5′-CTCGTCCATGCCYTCNCCNGTRTACCA for β-tubulin. Sequences from *Heterocapsa triquetra* were obtained by RT-PCR. Total RNA was isolated with the RNeasy kit (Qiagen) and poly(A)$^+$ RNA was isolated with the Oligotex mRNA kit (Qiagen). Full-length RNAs were selected following the procedure of Suzuki *et al.* (1997) and Maruyama & Sugano (1994) and cDNA was synthesized with a poly(dT) primer using standard methods. An amplified cDNA pool served as template for degenerate PCRs using the primers described above.

PCR products were cloned into vector pCR 2.1 using the TOPO TA cloning kit (Invitrogen) and several clones of each gene were sequenced on both strands. Protein-coding gene sequences were translated and added to existing alignments of eukaryotic sequences (Van de Peer *et al.*, 1998; Saldarriaga *et al.*, 2001; Keeling, 2001; Fast *et al.*, 2002). Only unambiguously aligned characters were used in the phylogenetic analyses, resulting in respective datasets of 1488, 244, 384 and 395 characters for SSU, actin, α-tubulin and β-tubulin. Phylogenetic trees were inferred both by using comprehensive alignments containing a large number of taxonomically diverse eukaryotes to confirm the alveolate nature of the *Oxyrrhis* and *Perkinsus* sequences and also with smaller subsets of these alignments that contain only alveolate taxa, so that more sophisticated analyses could be performed. In SSU rRNA trees, *Perkinsus* was used alone to represent all perkinsids, since the sequence from *Parvilucifera* is comparatively very divergent. In the larger, global analyses, *Oxyrrhis* and *Perkinsus* sequences were always related most closely to apicomplexans and dinoflagellates so, for most of the smaller datasets, ciliates were used as the outgroup. This was not the case in the actin dataset: ciliate actin sequences are known to be so divergent that they do not form a group with other alveolates (e.g. Keeling, 2001). In this case, heterokonts were used as outgroups, as these seem to be the nearest relatives to alveolates in actin phylogenies (e.g. Baldauf *et al.*, 2000; Keeling, 2001). In addition to the single-gene datasets, an alignment composed of concatenated sequences of actin, α-tubulin and β-tubulin was also produced (1023 amino acids). It contained only alveolate taxa for which the complete sequence of all three genes is known: three ciliates, three apicomplexans, *Oxyrrhis*, *Perkinsus* and the only dinoflagellate for which we could obtain all three genes, *Heterocapsa triquetra*.

Phylogenies from the single-gene and the concatenated datasets were inferred using distance and maximum-likelihood methods of tree reconstruction. Distance matrices were calculated with TREE-PUZZLE 5.0 (Strimmer & Von Haeseler, 1996) using the HKY substitution frequency matrix for nucleotides and the WAG substitution matrix for proteins. Nucleotide and amino acid frequencies, as well as transition/transversion ratios in the case of SSU, were estimated from the data. The among-site rate variation was modelled on a Γ distribution with invariable sites plus eight variable rate categories, and the α shape parameter was estimated from the data. Distance trees were constructed using weighted neighbour-joining using WEIGHBOR (Bruno *et al.*, 2000) and Fitch–Margoliash using FITCH (Felsenstein, 1993). One hundred bootstrap datasets were constructed using SEQBOOT and distances were calculated using PUZZLEBOOT (by M. Holder and A. Roger; http://www.tree-puzzle.de) with the α shape parameter, nucleotide or amino acid frequencies and transition/transversion ratio from the initial tree enforced on the 100 replicates. Protein maximum-likelihood trees were inferred using ProML (Felsenstein, 1993) with the JTT substitution frequency matrix, global rearrangements and 10 input-order jumbles. Site-to-site rate variation was modelled using the r option with the frequencies and rates calculated by TREE-PUZZLE. Protein maximum-likelihood bootstrapping was performed as above, with the rates and rate categories from the original dataset enforced on each replicate. Because of computational limitations, a dataset restricted to 30 species and 1581 nucleotides was used to infer maximum-likelihood trees of SSU rRNA sequences. These trees were inferred under an HKY model incorporating a discrete Γ distribution to correct for rate heterogeneity (invariable sites and eight variable rate categories, the shape parameter, nucleotide frequencies and transition/transversion ratio were estimated from the data, 10 input-order jumbles) using PAUP 4.0b8 (Swofford, 1999).

RESULTS AND DISCUSSION

SSU rRNA phylogeny of *Oxyrrhis*, *Perkinsus* and *Colpodella*

The taxonomic diversity of known alveolate SSU rRNA genes is quite broad, but data from *Oxyrrhis marina* were lacking, so as a first step in characterizing its phylogenetic position we amplified and sequenced the SSU rRNA gene from that species. The SSU rRNA sequence of *Oxyrrhis* proved to be extremely divergent, as can be seen in inferred trees (Fig. 1). In all phylogenetic analyses, the two *Oxyrrhis* isolates branched together with very high support (the two sequences are very similar). In analyses including all eukaryotes (Fig. 1), and in a more detailed analysis restricted to alveolates only (not shown), *Oxyrrhis* nearly always branched with the dinoflagellate order Gonyaulacales, and this relationship was generally relatively well supported by bootstrap (e.g. 81 and 76 % support uniting *Oxyrrhis* and the gonyaulacalean *Gonyaulax spinifera* in Fig. 1). The only analysis where this relationship did not appear was the maximum-likelihood tree of alveolates-only, where *Oxyrrhis* branches from within the dinoflagellates, but not specifically with Gonyaulacales (not shown). However, the divergent nature of the *Oxyrrhis* sequences makes it difficult to draw any firm conclusions about the phylogenetic placement of the species. Indeed, the *Oxyrrhis* branch lengths in the weighted neighbour-joining distance tree of Fig. 1, for example, are almost eight times as long as that of *Gonyaulax spinifera*. Accordingly, the SSU rRNA phylogeny must be considered very cautiously, especially as *Oxyrrhis* tends to branch with the Gonyaulacales, an otherwise morphologically coherent group with relatively divergent SSU rRNA sequences compared with other dinoflagellates (Saunders *et al.*, 1997; Saldarriaga *et al.*, 2001). Both the large eukaryotic dataset (Fig. 1) and the alveolate-only dataset (not shown) contained sequences from the unidentified group of marine alveolates proposed to be related to *Oxyrrhis* (Moon-van der Staay *et al.*, 2001), but in no tree did the *Oxyrrhis* sequence branch with that group. Although the divergent nature of the *Oxyrrhis* sequence makes any conclusions difficult, this analysis obviously does not support the notion that these unidentified organisms are closely related to *Oxyrrhis*. Nevertheless, as shown below, the position of *Oxyrrhis* in SSU rRNA phylogenies is likely false; its true position is at the base of the dinoflagellates. Accordingly, a possible relationship between *Oxyrrhis* and these picoplanktonic alveolates cannot be excluded.

The position of *Perkinsus* in the SSU rRNA analysis (basal to dinoflagellates) is consistent with most results published previously (Goggin & Barker, 1993; Siddall *et al.*, 1997). However, this position is not well supported by bootstrap (e.g. 58 and <50 % in Fig. 1), and other analyses of SSU rRNA have found conflicting positions for *Perkinsus* and the related *Parvilucifera* (Norén *et al.*, 1999). The combination of poor bootstrap support and conflicting results indicates that SSU rRNA data are insufficient to resolve the position of perkinsids and other data must be sought. It is also noteworthy that the SSU rRNA sequence from *Colpodella*, another protalveolate unrelated to *Perkinsus* or *Oxyrrhis*, branches at the base of the apicomplexans, as previously seen in analyses based on SSU rRNA (Siddall *et al.*, 2001; Leander *et al.*, 2003).

Phylogenetic position of *Oxyrrhis* and *Perkinsus* based on actin, α-tubulin and β-tubulin sequences

Given the difficulties imposed by the divergent *Oxyrrhis* SSU rRNA sequence and the general lack of support for the topology of the SSU rRNA tree of dinoflagellates, we sought to examine the relationships between *Oxyrrhis*, *Perkinsus*, dinoflagellates and apicomplexans using three protein-coding genes: actin, α-tubulin and β-tubulin. Genes encoding all three proteins were amplified from *Oxyrrhis* and both α-tubulin and β-tubulin were amplified from *Perkinsus*. As no α-tubulin sequences and only three actin and one β-tubulin sequences have previously been characterized from dinoflagellates, we amplified those three genes from several members of the group (actin from five species, α-tubulin from four and β-tubulin from seven species; summarized in Table 1). In many species of dinoflagellates, we found more than one copy of a particular gene. For example, we sequenced two different copies of α-tubulin from *Amphidinium herdmanii*, two β-tubulin genes from both *Heterocapsa triquetra* and *Perkinsus marinus* and four distinct actin genes from *Karenia brevis*. We also found introns in two genes: one in the α-tubulin gene from *Heterocapsa rotundata* (107 bp) and one in the β-tubulin gene of *Symbiodinium* sp. CCMP 421 (120 bp).

In contrast to the SSU rRNA gene, none of the three protein-coding genes sampled from *Oxyrrhis* was found to be particularly divergent (neither were the new tubulin genes from *Perkinsus*). All of the large, taxonomically diverse phylogenetic trees based on actin (Fig. 2), α-tubulin (Fig. 3) and β-tubulin (Fig. 4) place both *Oxyrrhis* and *Perkinsus* within the alveolate clade, confirming their general taxonomic position as alveolates. However, unlike the SSU rRNA trees, the protein trees almost never show *Oxyrrhis* branching within the dinoflagellates: in the weighted neighbour-joining tree of actin (Fig. 2), the dinoflagellate *Crypthecodinium cohnii* branches below an *Oxyrrhis*/*Perkinsus* clade (without bootstrap support), but both maximum-likelihood and distance trees based on only alveolate sequences from all three proteins (Fig. 5a–c) place *Oxyrrhis* and *Perkinsus* earlier than all dinoflagellates. In general, actin phylogenies (Figs 2 and 5a) produce consistent and strong support for both *Oxyrrhis* and *Perkinsus* branching with the dinoflagellates, but little or no support for the node uniting dinoflagellates at the exclusion of *Oxyrrhis* and *Perkinsus*. We must also note that the actin sequence reported from *Colpodella* (Siddall *et al.*, 2001) branches at the base of the kinetoplastids in Fig. 2. This sequence was derived from a culture fed on *Bodo*, so this

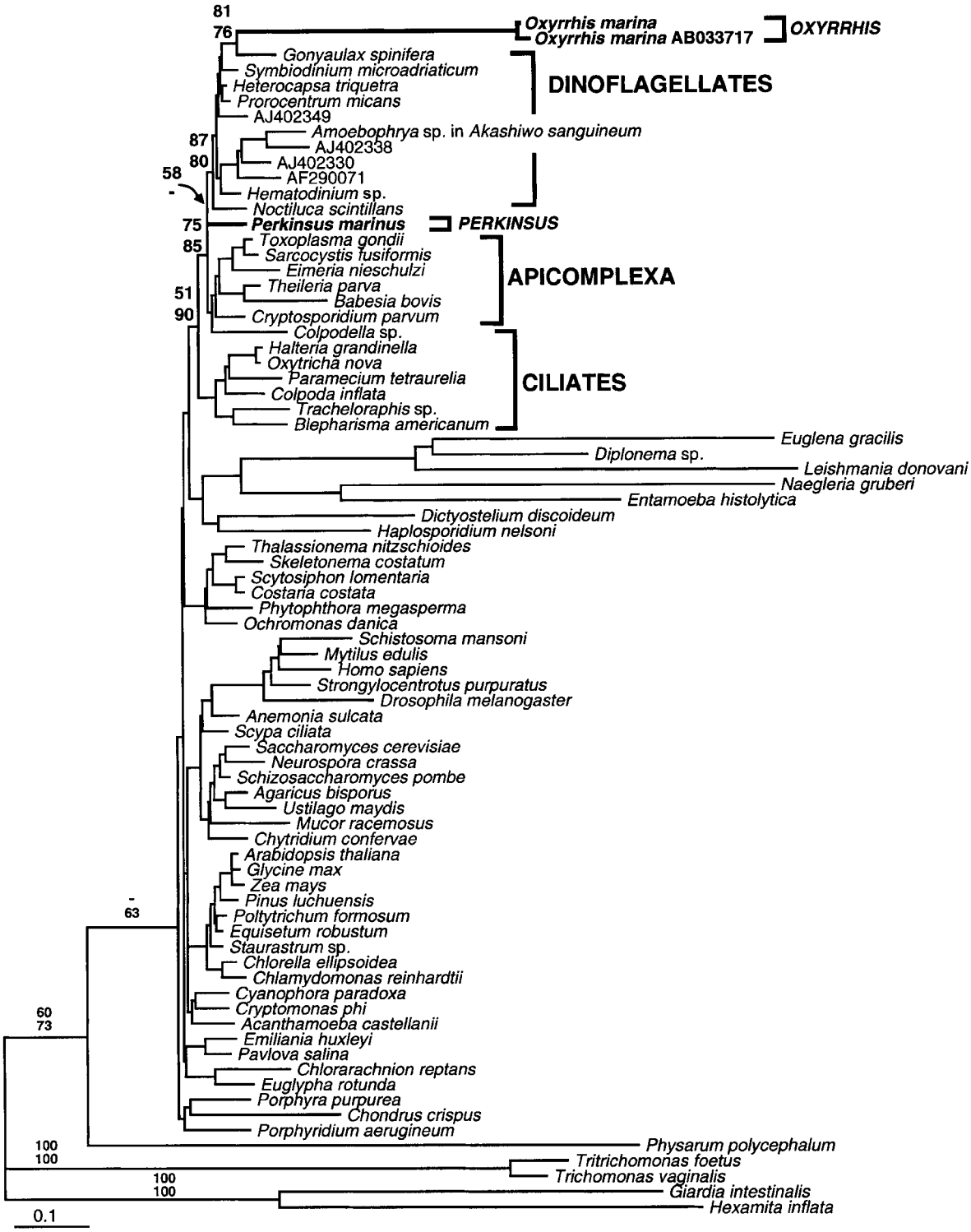

Fig. 1. Phylogenetic tree constructed with weighted neighbour-joining from a Γ-corrected distance matrix of SSU rRNA sequences (1488 nt) from 78 phylogenetically diverse eukaryotic species. Bootstrap values, based on weighted neighbour-joining (top) and Fitch–Margoliash (bottom), are shown above selected internodes. Alveolate groups are marked. Accession numbers are given for sequences from environmental samples of undetermined taxonomic identity (López-García *et al.*, 2001; Moon-van der Stay *et al.*, 2001) and for the Japanese isolate of *Oxyrrhis marina*.

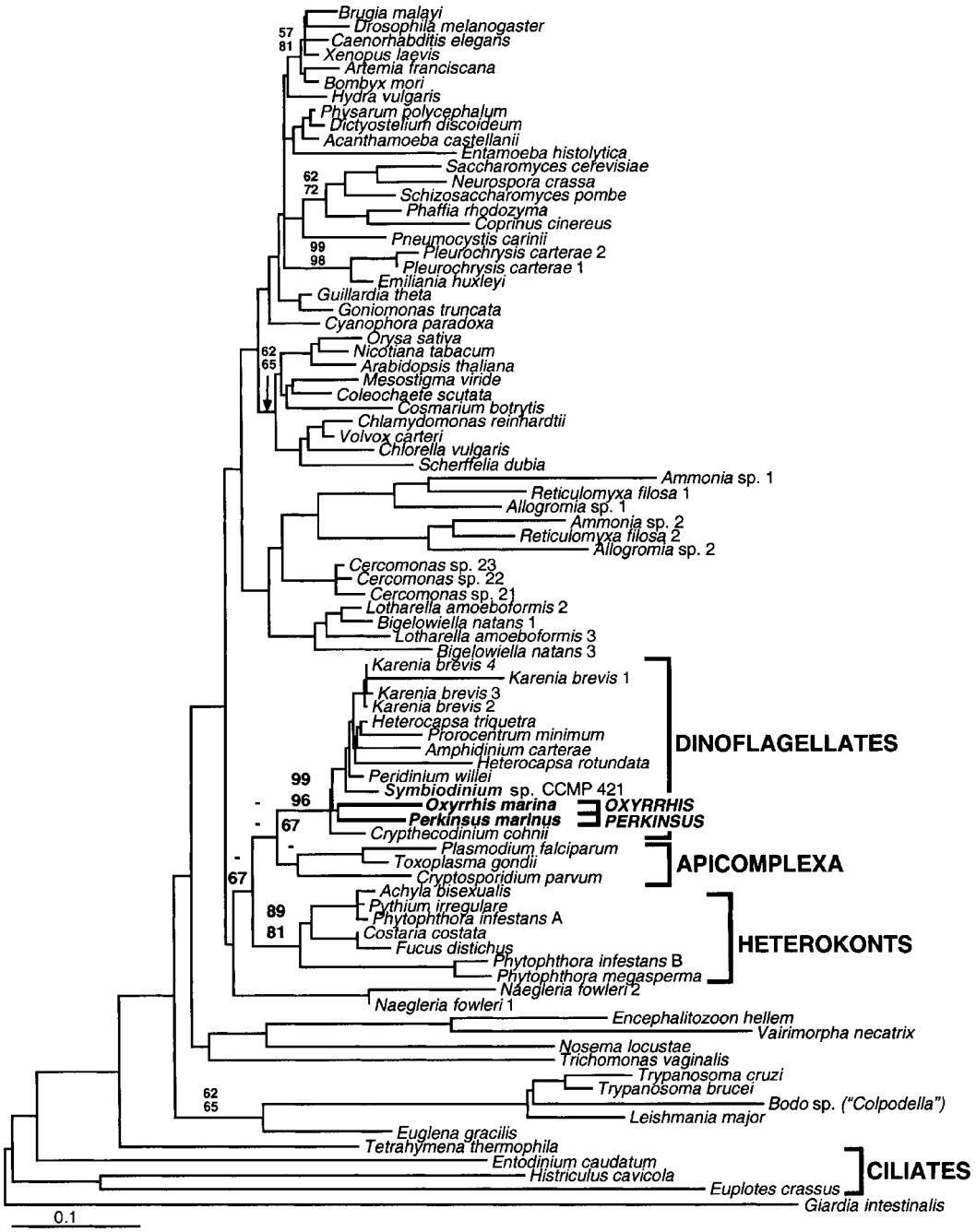

Fig. 2. Phylogenetic tree constructed with weighted neighbour-joining from a Γ-corrected distance matrix of actin sequences (244 aa) from 85 phylogenetically diverse eukaryotic species. Bootstrap values, based on weighted neighbour-joining (top) and Fitch–Margoliash (bottom), are shown above selected internodes. Alveolate groups and heterokonts are marked.

actin gene is almost certainly derived from *Bodo* and not from *Colpodella*. α-Tubulin trees (Figs 3 and 5b) are probably the most robust of the three protein-coding genes and consistently show very high support for both *Oxyrrhis* and *Perkinsus* branching at the base of dinoflagellates. In β-tubulin phylogenies of alveolates, it has previously been shown that ciliates are paraphyletic (Fast *et al.*, 2001), and the same is found here (Figs 4 and 5c). Nevertheless, *Oxyrrhis* branches with the dinoflagellates with variable levels of support in different analyses and *Perkinsus* also branches at the base of the dinoflagellates in analyses restricted to alveolates (in the large weighted neighbour-joining tree, Fig. 4, it also branches at the base of dinoflagellates, but as sister to a ciliate, *Stylonychia*). Moreover, in all β-tubulin trees, the dinoflagellates form a very strongly supported clade to the exclusion of both

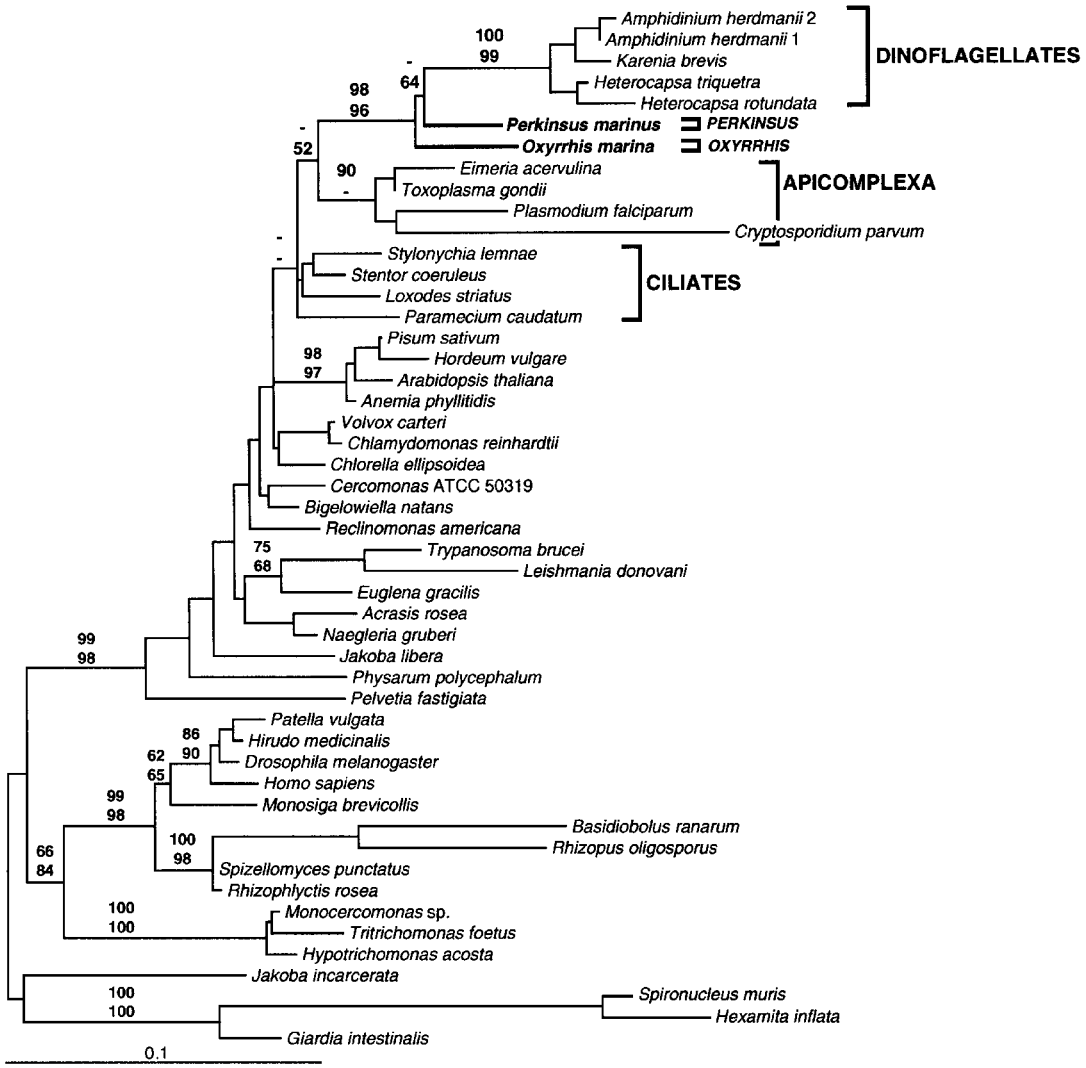

Fig. 3. Phylogenetic tree constructed with weighted neighbour-joining from a Γ-corrected distance matrix of α-tubulin sequences (384 aa) from 50 phylogenetically diverse eukaryotic species. Bootstrap values as in Fig. 2. Alveolate groups are marked.

Perkinsus and *Oxyrrhis* (97–100 % bootstrap support). Lastly, in trees based on concatenated actin, α-tubulin and β-tubulin sequences (Fig. 5d), there is very strong support for a clade containing *Perkinsus*, *Oxyrrhis* and dinoflagellates (100 % bootstrap support), but this dataset could not address whether either taxon branched within the dinoflagellates, since only one dinoflagellate was represented.

The phylogenies described above consistently support the conclusion that both *Perkinsus* and *Oxyrrhis* diverged from early ancestors of the dinoflagellates, but the order in which *Perkinsus*, *Oxyrrhis* and the true dinoflagellates branch is highly inconsistent. Among the trees, examples can be found where *Oxyrrhis* branches earlier than *Perkinsus* and the dinoflagellates (Fig. 3), where *Perkinsus* branches earlier than *Oxyrrhis* and the dinoflagellates (Fig. 5a, c) or even where *Perkinsus* and *Oxyrrhis* are sisters (Figs 2 and 5b). In cases where *Perkinsus* and *Oxyrrhis* are sisters, there is little support for the node uniting them. Similarly, there is typically little support for the node separating them in other analyses, although trees placing *Perkinsus* deeper tend to enjoy slightly higher bootstrap support. The concatenated dataset proved to be very useful for addressing this question, since the relative branching order of *Perkinsus*, *Oxyrrhis* and dinoflagellates could still be discerned from these trees even if only one dinoflagellate is represented. In this case (Fig. 5d), there is consistent and relatively high bootstrap support for *Perkinsus* branching first, followed by *Oxyrrhis* and the dinoflagellate, *Heterocapsa*. This most common branching order is most likely to be correct, but further data on this point are required.

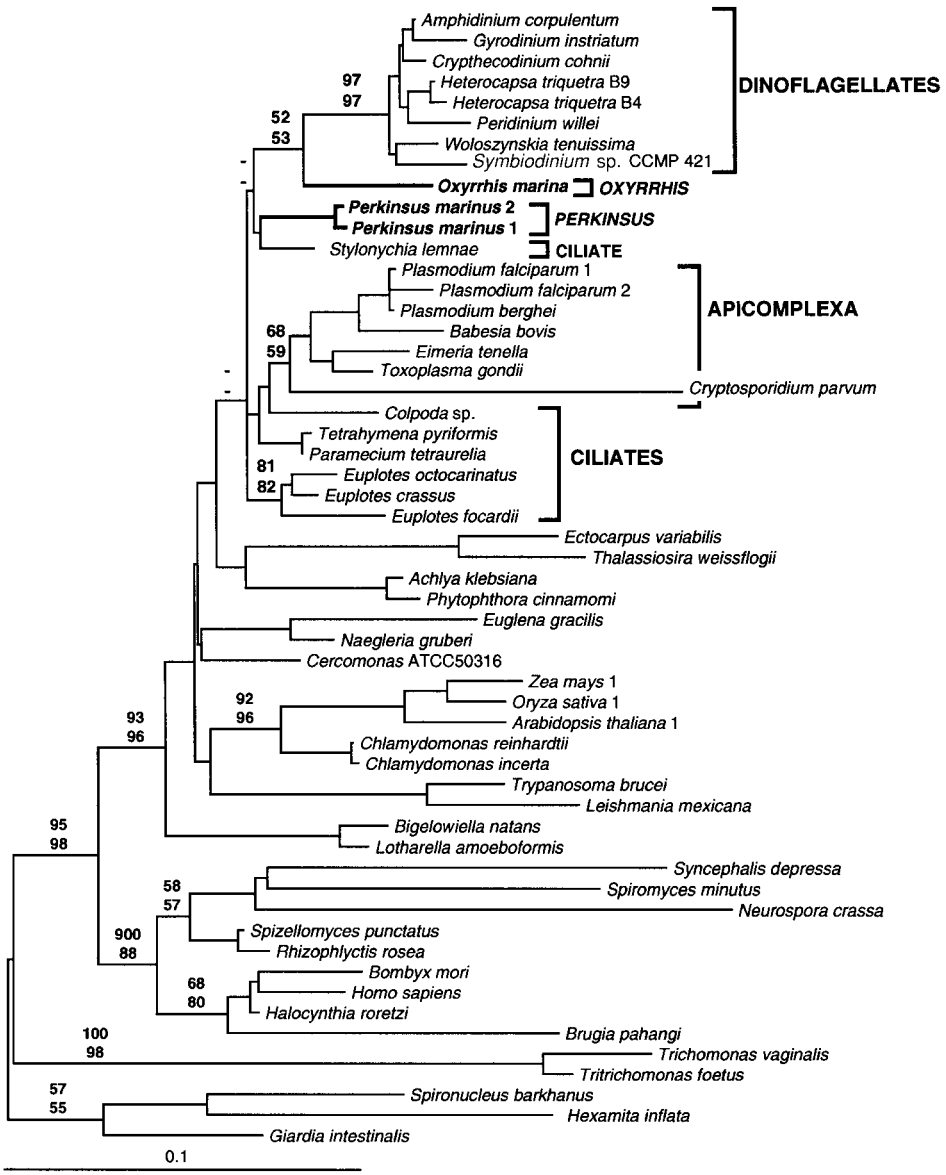

Fig. 4. Phylogenetic tree constructed with weighted neighbour-joining from a Γ-corrected distance matrix of β-tubulin sequences (395 aa) from 56 phylogenetically diverse eukaryotic species. Bootstrap values as in Fig. 2. Alveolate groups are marked.

Conclusions – the early evolution of dinoflagellates and apicomplexans

The results obtained from the SSU rRNA phylogenetic trees are not congruent with those obtained from any of the protein-gene trees: while in SSU rRNA-based trees, *Oxyrrhis marina* appears to have evolved from within the Gonyaulacales, in trees based on any of the three protein-genes, it branches as a sister taxon to all dinoflagellates. The highly divergent nature of the *Oxyrrhis marina* SSU rRNA sequences is likely causing them to branch artificially with the Gonyaulacales, since they too have divergent SSU rRNA genes compared with other dinoflagellates (e.g. Saunders *et al.*, 1997; Saldarriaga *et al.*, 2001). In contrast, the *Oxyrrhis* protein-coding gene sequences are generally no more or less derived than the dinoflagellate homologues and produce congruent phylogenetic trees that strongly support *Oxyrrhis* branching at the base of the dinoflagellates.

The phylogenetic position of the protalveolate *Colpodella* is also affected by these data. Previously, it was shown that SSU rRNA trees often placed *Colpodella* at the base of apicomplexans, but trees combining SSU rRNA and actin place it at the base of ciliates (Siddall *et al.*, 2001). It seems clear, however, that the actin gene used in this study is from *Bodo*, not *Colpodella* (see Fig. 2). Combining the *Colpodella* SSU rRNA with the *Bodo* actin could have

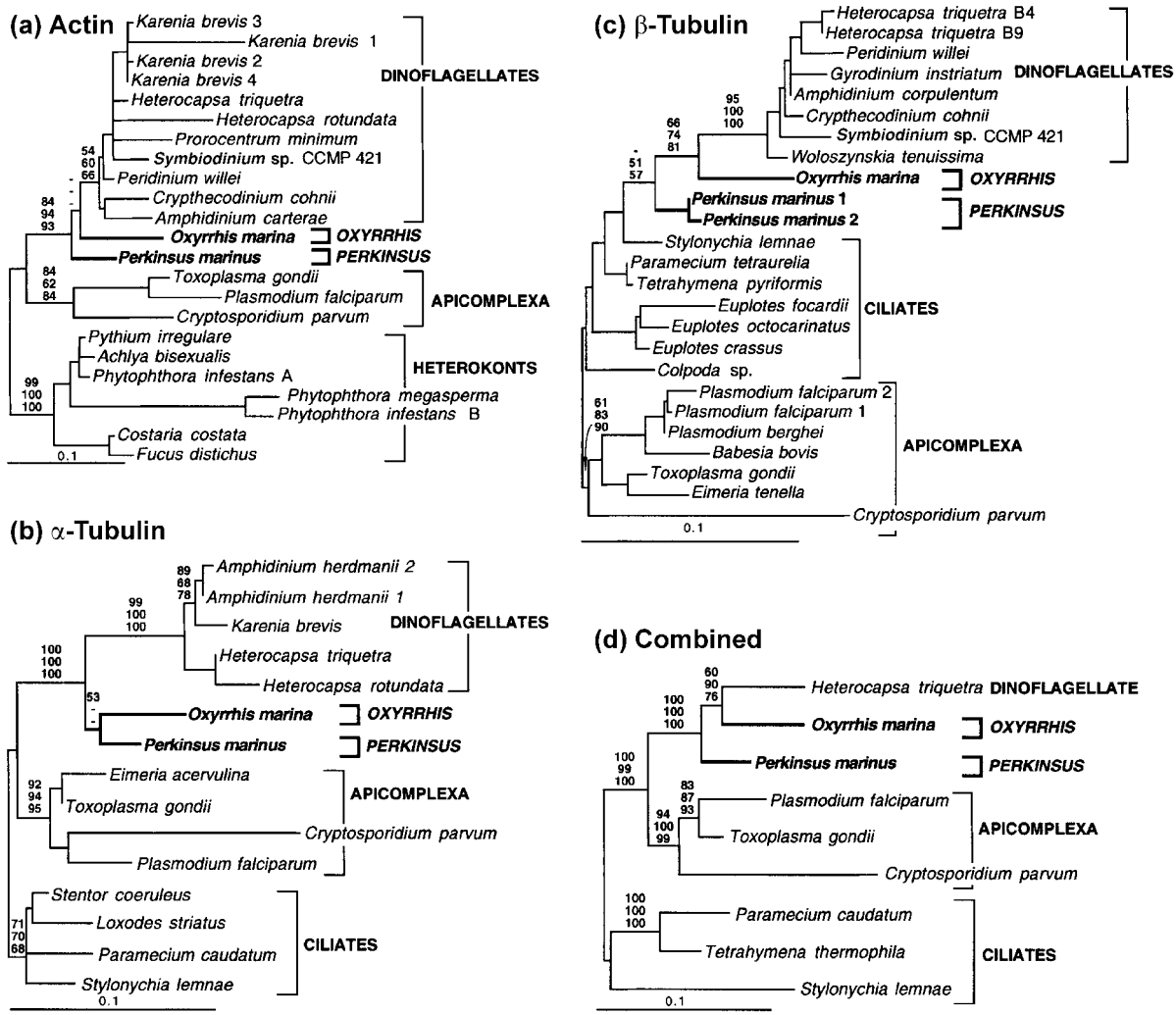

Fig. 5. Γ-Corrected protein maximum-likelihood phylogenetic trees based on actin (a), α-tubulin (b) and β-tubulin (c) genes and a concatenated dataset of actin, α- and β-tubulin sequences (d) from alveolates using ciliates as an outgroup. In (a), heterokonts are included instead of ciliates (see text for explanation). Bootstrap values based on protein maximum-likelihood (top), weighted neighbour-joining (centre) and Fitch–Margoliash (bottom) are shown above selected internodes.

artificially forced '*Colpodella*' to branch deeper in the alveolates in these analyses, especially since the ciliate actins are very divergent and do not branch with other alveolates in actin phylogenies (e.g. Keeling, 2001).

Determining the phylogenetic position of protalveolates like *Perkinsus*, *Oxyrrhis* and *Colpodella* may prove to be very useful for determining the evolutionary history of a number of morphological characters in alveolates. In the dinoflagellate lineage, for example, it now seems likely that lateral or ventral insertion of flagella originated soon after the divergence from the apicomplexan lineage, since it is a character also present in *Perkinsus* (Perkins, 1996) and in *Oxyrrhis* (Dodge & Crawford, 1971). In *Colpodella*, the site of flagellar insertion varies in the different species (Simpson & Patterson, 1996), and apicomplexans either lack flagella completely (gregarines) or have microgametes with apical flagella (e.g. coccidians; Levine, 1985). Similarly, a number of the features that characterize the dinoflagellate transverse flagellum (a feature used to define the group; Fensome *et al.*, 1993) also seem to be present in *Perkinsus* and *Oxyrrhis* (Roberts, 1985; Perkins, 1996; Dodge & Crawford, 1971). On the other hand, the dinokaryotic nuclei of dinoflagellates is one feature that most likely originated within the true dinoflagellates: *Oxyrrhis* has a nuclear organization that is significantly different from that of both dinokaryotic and syndinialean (i.e. histone-containing) dinoflagellates. It also has scales (Clarke & Pennick, 1976), an unusual cytoskeleton (Höhfeld & Melkonian, 1998) and a flagellar root system (Roberts, 1985, 1991) that, although reminiscent of and clearly related to those of dinoflagellates, also present important differences. Whether these differences were already present in the common ancestor of *Oxyrrhis* and the dinoflagellates or they arose in

the *Oxyrrhis* lineage is still unclear. In the case of the apicomplexan lineage, it appears that some elements of the apical complex of apicomplexa probably originated prior to the divergence of that group from the dinoflagellate lineage. A few of these structures seem to be present in modified forms in *Perkinsus* (Perkins, 1996) and may well be homologous to structures surrounding the apical pore of dinoflagellates (Siddall *et al.*, 1997).

In order to answer these kinds of questions with any certainty, more molecular and ultrastructural data are needed. By identifying the evolutionary position of the various protalveolates, we can begin to piece together the evolutionary history of some of the important characters that define the major alveolate groups. However, it is also necessary to expand the molecular phylogenetic data for some of the most poorly studied protalveolates (e.g. *Colpodella*, *Colponema*, ellobiopsids), as well as basal dinoflagellates and apicomplexans (e.g. syndinians and gregarines).

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