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
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 attatches 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 Woloszyńska tenuissima; nomenclature as in Popovsky & Pfister, 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′-CCGGATCCTGATCCTTCTGCGAGTTCAACCTGGTTGATCCTGCCAGT) and 5′-GGCGTAATCTCCGCACTG). The remainder of the 5′ end of the gene was amplified using another eukaryote-specific primer (5′-CGAATCTCAACCTGGATCCTGCCAGT) and a primer designed to recognize only dinoflagellate SSU rRNA sequences (5′-ACTTACTCTTITCCGAC). Protein-coding genes for all organisms except Perkinsus marinus were amplified using the...
Table 1. Accession numbers for the new protein-coding gene sequences

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*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).

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 shape parameter was estimated from the data. Distance trees were constructed using weighted neighbour-joining using WEIGHBOOT (Bruno et al., 2000) and Fitch–Margoliash using FITCH (Felsenstein, 1993). One hundred bootstrap datasets were constructed using SEQBOOT and distances were calculated using PUZLEBOOT (by M. Holder and A. Roger; http://www.tree-puzzle.de) with the 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 Parvulucifera (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 herdmani, 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), α-tubulin (Fig. 3) and β-tubulin (Fig. 4) 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 Cryptothecodinium 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 $\Gamma$-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.
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
Perkinsus and Oxyrrhis (97–100 % bootstrap support). Lastly, in trees based on concatenated actin, z-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. 3. Phylogenetic tree constructed with weighted neighbour-joining from a Γ-corrected distance matrix of z-tubulin sequences (384 aa) from 50 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...
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 are 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 syndiniaeal (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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