'Total evidence' refutes the inclusion of *Perkinsus* species in the phylum Apicomplexa

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SUMMARY

The phylogenetic affinities of the oyster pathogen *Perkinsus marinus* were investigated with morphology, 18S-like rDNA data and actin sequence data. Morphological investigations revealed that *Perkinsus* species do not have a conoid and that other criteria which have been used to place them in the Apicomplexa are general to alveolates. When considered separately, 18S-like rDNA and actin data sets each support a closer affinity for *Perkinsus marinus* with the dinoflagellates. However, each of these separate analyses possess their own biases and weaknesses. Use of the phylogenetic principle of 'total evidence' in which data sets are combined in simultaneous analysis yielded a more robust hypothesis that is stable both to character and taxonomic sampling. The resulting cladogram strongly corroborates the placement of *Perkinsus* species with the Dinoflagellida and not with the Apicomplexa.

Key words: congruence, Dinoflagellida, *Perkinsus*, phylogeny, Apicomplexa.

INTRODUCTION

Perkinsus marinus was described by Mackin, Owen & Collier (1950) under the name *Dermocystidium marinum*. Although the colloquial name 'dermo' for the disease of oysters caused by this protistan has persisted, there has been considerably less agreement regarding the taxonomic and phylogenetic position of the parasite. Since its discovery in the Gulf of Mexico and initial placement in the fungal genus *Dermocystidium*, the taxon has been placed variously with the Ascomycetales, the Entomophthorales, the Saprolegniales, and even the Haplosporidia (Mackin, 1951; Ray, 1954; Mackin & Boswell, 1956; Perkins & Menzel, 1967). Mackin & Ray (1966) considered their observations of 'mucoid tracks' in cultures to be sufficient grounds for removing the parasite from the genus *Dermocystidium* and so renamed it *Labyrinthomyxa marina*.

The first suggestion of a phylogenetic affinity with the apicomplexans came with Perkins & Menzel (1967) commenting that the presence of more than 1 limiting membrane was exceptional among protists and is more typical of gregarines and the malaria parasites. Later, Perkins (1969) discovered a pinocytic structure in some developmental stages and drew comparisons with the micropores of apicomplexans. In spite of some reservations regarding life-history patterns, Perkins (1976) argued for the placement of this parasite in the recently established phylum Apicomplexa citing the presence of the subpellicular membrane, the micropore and the presence of what he called a 'conoid'. Levine (1978) established the genus *Perkinsus* and formally placed the taxon in the phylum Apicomplexa. Although the suggested apicomplexan affinity for *Perkinsus* species was widely accepted, Vivier (1982) urged caution regarding Perkins' (1976) ultrastructural interpretations. In particular, the trilaminar pellicle and micropores were known not to be unique to the apicomplexans, and the so-called conoid only superficially resembled the corresponding structure of coccidians and gregarines.

Most authors have retained the genus *Perkinsus* in the Apicomplexa. Perkins (1996) has recognized some similarities with the dinoflagellates, but still considered the parasites to be apicomplexans and expressed the desire for 'more molecular and morphological information ... before a revision of the classification of *Perkinsus* sp. is attempted'. Phylogenetic analysis of the 18S-like rDNA from a variety of alveolates has already indicated that *Perkinsus* species are more closely related to dinoflagellates than to apicomplexans (Fong *et al*. 1993; Goggin & Barker, 1993; Siddall, Stokes & Burreson, 1995; Flores, Siddall & Burreson, 1996). Identical relationships were revealed in phylogenetic analyses using an actin gene fragment (Reece *et al*. 1997).

The issue of corroboration and the relative combinability of data sets currently is being debated in the phylogenetic literature (e.g. Kluge & Wolf, 1993; Wheeler, Cartwright & Hiyashi, 1993; Farris *et al*. 1994; De Queiroz, Donoghue & Kim, 1995; Miyamoto & Fitch, 1995; Huelsenbeck, Bull &

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Cunningham, 1996) but has received little attention in protozoological phylogenetics. Kluge (1989) characterized the combination of all available data in simultaneous analysis as being logically and philosophically consistent with the principle of 'total evidence'. Specifically, with respect to the phylogenetic affinities of the genus *Perkinsus*, what remains unknown is whether or not the combined information of the 18S-like rDNA and actin genes, as well as that of morphology, provides more corroboration of relationships than any of these data sets alone.

MATERIALS AND METHODS

Morphological characteristics of the genus *Perkinsus* were reconsidered by reassessing published electron micrographs (Perkins & Menzel, 1967; Perkins, 1969, 1976; Azevedo, 1989; Azevedo, Corral & Cachola, 1990). Similarly, ultrastructural data were examined from other alveolate taxa for the presence of putative homologues in apicomplexans and in dinoflagellates.

The choice of taxa for this study was dictated by the intersection of the set of available alveolate 18S-like rDNA sequences and the set of available actin sequences for alveolate genera: *Amphidinium*, *Prorocentrum*, *Toxoplasma*, *Plasmodium*, *Cryptosporidium*, *Oxytricha*, *Euplotes* and *Tetrahymena*. The protein-coding data set was the more limiting and actin sequences have only recently become available for the dinoflagellates and for *Perkinsus marinus* (Reece *et al*. 1996). Except for *Plasmodium falciparum*, there is only 1 full length sequence for the 18S-like rDNA gene for each of the included taxa. For *P. falciparum* we used the asexually expressed sequence in combined analyses. Both for *Oxytricha nova* and for *P. marinus* there are 2 actin sequences available. These were each combined with the same 18S-like rDNA sequence for those taxa. Actin data sets comprised approximately 625 bases corresponding to nucleotides 426 though 1048 in *Homo sapiens* alpha actin (GenBank accession #J05192) which represents approximately half of the gene. GenBank accession numbers for actin sequences are as follows: U10429, M13939, X05195, M22480, U06071, J01163, J04533, U84287, U84288, U84289 and U84290. It should be noted that for *Toxoplasma gondii*, this region contains an intron from position 1333 to 1760 in the deposited sequence, which needed to be removed from the sequence prior to alignment. Full-length 18S-like rDNA data sets were used, with GenBank accession numbers as follows: L13719, L16996, M14590, M14649, M19172, M98021, X03948 M14601, X56165, X75429, X75762.

For 18S-like rDNA multiple alignment, Wheeler & Gladstein's (1993) Malign was used on a SPARC5 SUN-OS platform specifying the following parameters: changecost 1, internal (gap cost) 3, leading (and) trailing (gapcosts) 100, iter, build, alignaddswap, alignswap, alignnodeswap, treeaddswap, treeswap, keeptrees 100, keepaligns 20. Multiple alignment for the actin data set was accomplished by eye with reference to inferred amino acid sequences. Amino acid translations of the aligned data set were generated with MacClade (Maddison & Maddison, 1992) with 1 caveat: MacClade is, of course, unaware that certain ciliates use codons for glutamine and glutamic acid that are otherwise 'universally' used as stop codons (Harper & Jahn, 1989). For these, the appropriate amino acid was inserted after conversion. The first base in the nucleotide sequence is in third-position serial homology and was necessarily discarded in translations.

Except for amino acid data sets, parsimony analyses were conducted with Hennig86 (Farris, 1988). Those data sets containing amino acid translations were analysed with PAUP (Swofford, 1993). In all analyses, irrespective of composition or combination, all aligned sites were considered to be of equal weight and transversional weighting using Sankoff matrices was not employed.

Various phylogenetic randomization tests were conducted with Random Cladistics (Siddall, 1994). Six tests of data set combinability were investigated with the incongruence length difference test (Farris *et al*. 1994) using ARNIE, specifying 'cc-.;mh;bb;' and 99 randomizations. These were: 18S-like rDNA versus all 3 positions of actin nucleotides, 18S-like rDNA versus first and second position actin nucleotides, 18S-like rDNA versus actin amino acids, 18S-like rDNA versus first position versus second position versus third position actin nucleotides, 18S-like rDNA with first and second position actin nucleotides versus third position actin nucleotides, and first and second position actin nucleotides versus third position actin nucleotides. Character bootstrapping (Felsenstein, 1985) and taxon jackknifing (Siddall, 1995) were accomplished with HEYJOE and LANYON respectively and specifying 'cc-.;mh;bb;' for each. Character parsimony jackknifing (Farris *et al*. 1996) was performed by first converting Hennig86 data files with TORA and then analysing these data with MM (Farris, 1995) specifying 1000 jackknives.

RESULTS AND DISCUSSION

The putative conoid

Perkins (1976, 1996) has suggested that *P. marinus* has a conoid with coiled microtubules, and remarked that these form 'an open sided' truncated cone. Re-examination of published micrographs does not support the use of apicomplexan terminology for *Perkinsus*. The microtubular ribbon, which has been

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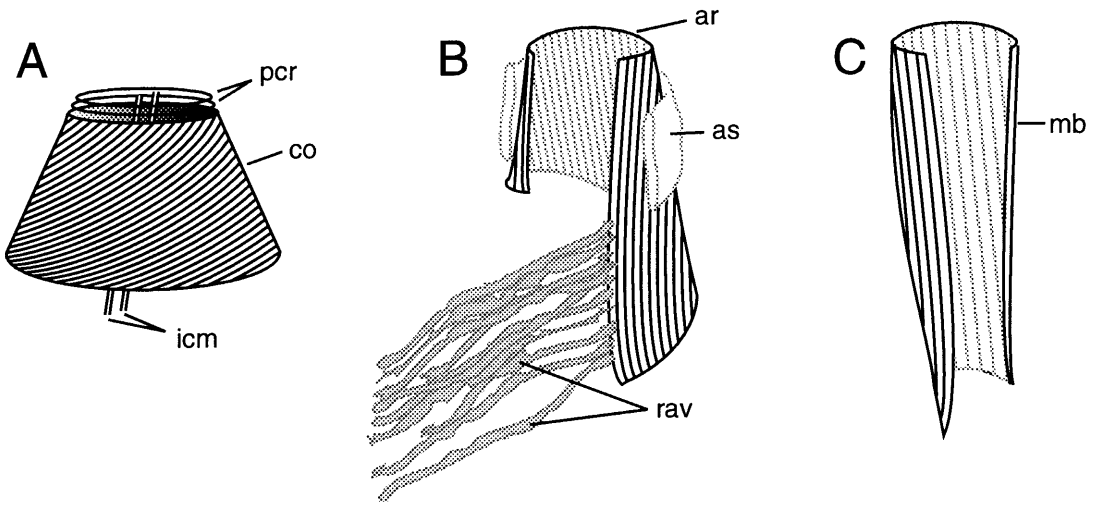

Fig. 1. Ultrastructural characteristics of (A) the apicomplexan conoid (co) which has transversely coiled tubules forming a complete truncated cone, a pair of preconoidal rings (pcr) and intraconoidal microtubules (icm), compared with (B) the open-sided apical ribbon (ar) in *Perkinsus* spp. which has more longitudinally arranged tubules, is surrounded by an alveolar sheath (as) and has associated vesicular structures (rav), and with (C) the microtubular basket (mb) of the feeding peduncle of *Amphidinium poecilochroum*. Redrawn after various sources.

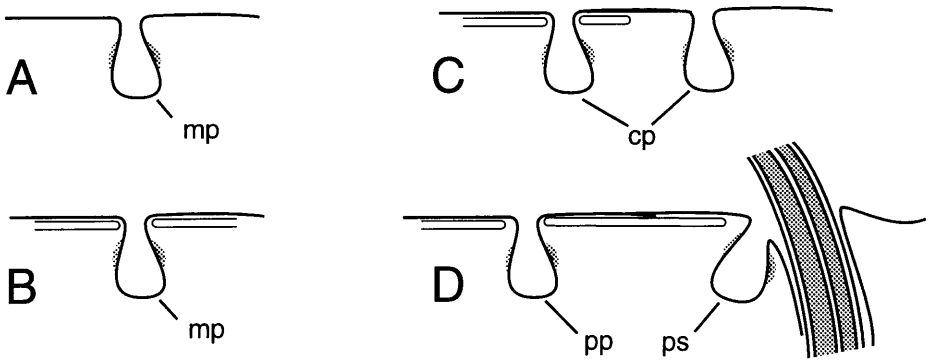

Fig. 2. Ultrastructural characteristics of pinocytic structures in the Alveolata including micropores (mp) of (A) apicomplexans and (B) *Perkinsus* species as well as (C) the collared pits (cp) of dinoflagellates and (D) the pellicular pores (pp) and parasomal sacs (ps) of ciliates.

called a conoid, is never found in a complete truncated cone form (Fig. 1). Nor are the microtubules found in a lateral coil as they are in the apicomplexans. Instead, there is a strongly arced ribbon of tubules arranged parallel to the long axis of the cell which recurves gradually and elongates on the right edge to the posterior (Fig. 1B). An apicomplexan conoid cut in longitudinal section invariably reveals the constituent tubules in cross-section whereas the tubular ribbon in *Perkinsus* spp., when cut in longitudinal section, reveals tubules in longitudinal section. The objects labelled 'conoid' in Fig. 6 of Azevedo *et al*. (1990) are the so-called 'conoid-attached micronemes [*sic*]' of Perkins (1976) and not tubules. We were unable to discover any indication of a pair of pre-conoidal rings or of the pair of intraconoidal microtubules that are integral to the conoid of apicomplexans (Fig. 1A). Moreover, an apical complex is found in all aflagellate apicomplexan and not in the flagellated microgamete stage. The putative conoid in *Perkinsus* is found only in the flagellated zoospore stage and not in any aflagellate stage in the life-cycle. Apicomplexan conoids are never found with attached vesicular structures typical of *Perkinsus* species, and are never enclosed by an alveolar membrane (Fig. 1B). Structures resembling the apicomplexan conoid can be found in dinoflagellates such as the apical microtubular basket (Fig. 1C) of the feeding peduncle in *Amphidinium poecilochroum* (see Larsen, 1988). We are not suggesting morphological homology of this structure with a true conoid, but wish to underscore the arbitrary nature of such homology statements between the microtubular ribbon of *Perkinsus* species and the conoid of apicomplexans. We conclude that *Perkinsus* does not have a conoid and does not have an apical complex.

Other 'apicomplexan' structures

Micropores (Fig. 2A) have been found in *Perkinsus* species (see Perkins, 1969, 1976). In apicomplexans

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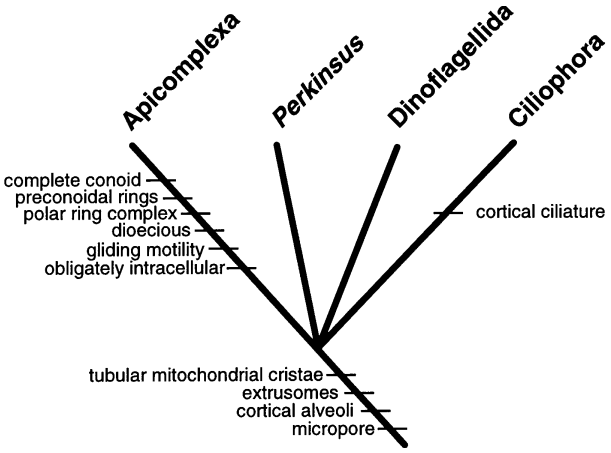

Fig. 3. Cladogram based solely on morphological characteristics indicating the lack of resolution resulting from these data being interpretable only as symplesiomorphies or as autapomorphies in the Alveolata.

these pinocytic structures appear as small invaginations of the plasmalemma inserting through discontinuities of the inner cortical alveolar membranes and are surrounded by an electron-dense cytoplasmic matrix (Fig. 2B). The micropores of *Perkinsus* species are indistinguishable from those in apicomplexans. Identical structures called 'collared pits' (Fig. 2C) are common in dinoflagellates (e.g. Fig. 14 in Dodge & Crawford (1971), Fig. 5 in Roberts & Roberts (1991) and Fig. 12 in Roberts, Heimann & Wetherbee (1995). Moreover, ultrastructurally identical features have been described from the ciliates (Fig. 2D) under a variety of terms such as 'pit-like invaginations' (e.g. Figs 3, 6, 8 and 19 in Hascall & Rudzinska (1970)), Figs 6, 7 and 9 in Millecchia & Rudzinska, (1970)), 'pellicular pores' (e.g. Fig. 12 in Lom & Corliss (1971)) or 'parasomal sacs' when found in association with cilia (e.g. Figs 13 and 14 in Lom & Corliss (1971) and Figs 4 and 7 in Paulin (1973)). We conclude that all alveolate phyla have micropores.

Perkins (1976) referred to 2 distinct groups of organelles as 'micronemes' in *Perkinsus* zoospores. One of these, the 'conoid [*sic*] attached micronemes (CAM)', is composed of elongate and electron-lucent vesicular structures that terminate on the right edge of the apical ribbon of tubules (Fig. 1B). Unlike apicomplexan micronemes which occur in all asexual stages, these structures are found only in the zoospore of *Perkinsus* species. Because the CAM are not electron dense and are firmly attached to the apical ribbon there is nothing to suggest homology with apicomplexan micronemes. Unlike the hundreds of small scattered sac-like apicomplexan micronemes, the 'rectilinear microneme' (RM) of *Perkinsus* species (Perkins, 1969) is found in bundles of relatively rigid structures that extend almost the full length of the zoospore. In cross-section, they have an electron-dense core surrounded by a more lucent matrix inside the limiting membrane of the organelle. This morphology is typical of toxicysts and other extrusomes in dinoflagellates and ciliates. In fact, the toxicysts of the ciliate *Didinium nasutum* (Fig. 5 in Wessenberg & Antipa (1970)) more closely resemble the RM structures of *Perkinsus marinus* than do apicomplexan micronemes. Although we concur that the RM structures of *Perkinsus* species are likely homologues of the micronemes of apicomplexans, we conclude that they are no more so than any other extrusomes common to all alveolates.

The other ultrastructural features on which Perkins (1976, 1996) premised his hypothesis of an apicomplexan affinity were the presence of a trilaminar pellicle (= cortical alveoli) and the subpellicular microtubules. As with the preceding features, these are common to ciliates, dinoflagellates and apicomplexans and serve to identify their clade, the Alveolata.

Morphological equivocation

The morphological features Perkins (1976, 1996) used to show an apicomplexan affinity for *Perkinsus* are tenuous, and there are some features which might indicate a closer affinity with the dinoflagellates. *Perkinsus* species and many dinoflagellates have 2 unequal and orthogonally arranged flagella in the asexual free-swimming trophozoite stages which frequently exhibit hair-like structures (e.g. Möestrup, 1982; Larsen, 1988) called 'mastigonemes' by Perkins (1976). Although apicomplexans do have flagella, these are found only in the short-lived microgamete stage, are equal in length, are arranged at an acute angle and never exhibit hair-like structures. It is likely that the orthogonal-hairy morphology represents another plesiomorphy for the alveolates because they resemble the flagella of various algal taxa, particularly the closely related chrysophytes like *Ochromonas* species (Pitelka, 1970; Hill & Outka, 1974; Möestrup, 1982).

All of the morphological characteristics considered in Fig. 3 can be interpreted as symplesiomorphies for the Alveolata or as autapomorphies for individual phyla. They do not indicate a phylogenetic affinity for *Perkinsus* species except to place them with the alveolates (Fig. 3). Similarly, Simpson & Patterson's (1996) decision to place the genus *Colpodella* with the Apicomplexa would seem to be premature because these flagellates are indistinguishable from *Perkinsus* zoospores (Brugerolle & Mignot, 1979).

Partitioned molecular phylogeny

Early suggestions that molecular approaches to phylogenetics should be more reliable than mor-

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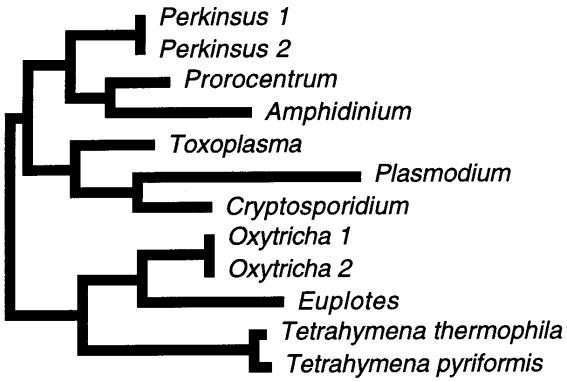
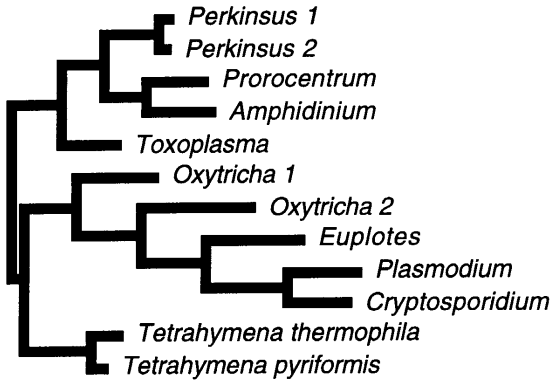


A



B

Fig. 4. Phylogenetic hypotheses based on single-gene analyses of (A) 18S-like rDNA data, and of (B) actin nucleotide data. Branch lengths are proportional to amount of change.

phology, being 'closer to the gene', have now been refined in light of findings that the molecular approach is not necessarily a panacea (Hillis & Dixon, 1991; Graybeal, 1994; Wheeler, 1995). Nonetheless, nucleotide sequences have proved useful, in part because the tentative homology statements of sequence alignments are not as open to subjective interpretation as are morphological characters. In fact, it was the use of 18S-like rDNA sequences in phylogenetic analyses which solidified the notion that the ciliates, dinoflagellates and apicomplexans represent a monophyletic group, the Alveolata (Preparata *et al*. 1988, Barta, Jenkins & Danforth, 1991, Gajadhar *et al*. 1991). The use of this gene in phylogenetic analyses repeatedly has supported a closer relationship for *Perkinsus* species with dinoflagellates than with apicomplexans (Fong *et al*. 1993; Goggin & Barker, 1993; Siddall *et al*. 1995; Flores *et al*. 1996).

The alignment of 18S-like rDNA sequences obtained by Malign, when cladistically analysed, yielded 1 most parsimonious tree for the alveolates (Fig. 4A) with a length of 1491 steps and a consistency index (CI) of 0·66. This tree supports monophyly of each of the phyla Apicomplexa, Ciliophora and Dinoflagellida. As with previous analyses for this gene, it supports a sister-group relationship between *Perkinsus* species and the 2 included dinoflagellate genera. Inspection of branch-lengths on this tree, however, suggest an extreme rate of divergence for the genus *Plasmodium*. This particular taxon has proved problematic in other analyses using ribosomal sequences (Johnston *et al*. 1990; Barta *et al*. 1991) and might even be expected to confound the placement of unrelated taxa. Moreover, because the 18S-like rDNA genes can have variable GC content, it could be claimed that the errors this introduces may be responsible for the grouping of *Perkinsus* with the dinoflagellates instead of with the Apicomplexa.

An advantage to using the actin gene over the use of non-coding 18S-like rDNA genes is that it can be aligned by matching inferred amino acid sequences. With the exception of 2 single amino acid deletions for the *Amphidinium* gene and 1 insertion for the *Oxytricha*1 sequence, this was a simple process. Phylogenetic analysis of the actin nucleotide data set, however, was less trivial. Although it yielded a single most parsimonious tree (length = 1217; CI = 0·60), this tree hypothesizes some awkward relationships (Fig. 4B). In it, the 2 *Oxytricha nova* isolates are not closest relatives of each other and the apicomplexan genera *Plasmodium* and *Cryptosporidium* are hypothesized to belong to the ciliate clade. Nonetheless, the 2 *Perkinsus* isolates form a clade and these are hypothesized to be more closely related to the dinoflagellates than to any of the apicomplexans. Although translated genes are less likely to suffer from overall base-composition biases, they can be influenced by the vagaries of third-position flexibility. For many codons, any nucleotide in the third position will result in the same amino acid, yet for some taxa there can be non-random representations of certain bases in the third position collectively referred to as 'codon usage bias'. The use of rare codons for certain apicomplexan species (Ellis *et al*. 1993; Char *et al*. 1996) could explain the erroneous placement of these taxa in Fig. 4B. As reasonable as this may seem, it puts us in the awkward position of postulating that the tree is misleading regarding the apicomplexans while simultaneously asserting that the sistergroup relationship of *Perkinsus* with the dinoflagellates is correct.

Combined molecular phylogeny

Much attention recently has been given to the issue of whether or not to combine data sets in phylogenetic analyses. The argument against combining data into single analyses stems from concerns regarding the swamping of phylogenetic signal with excessively biased or noisy data (Miyamoto & Fitch,

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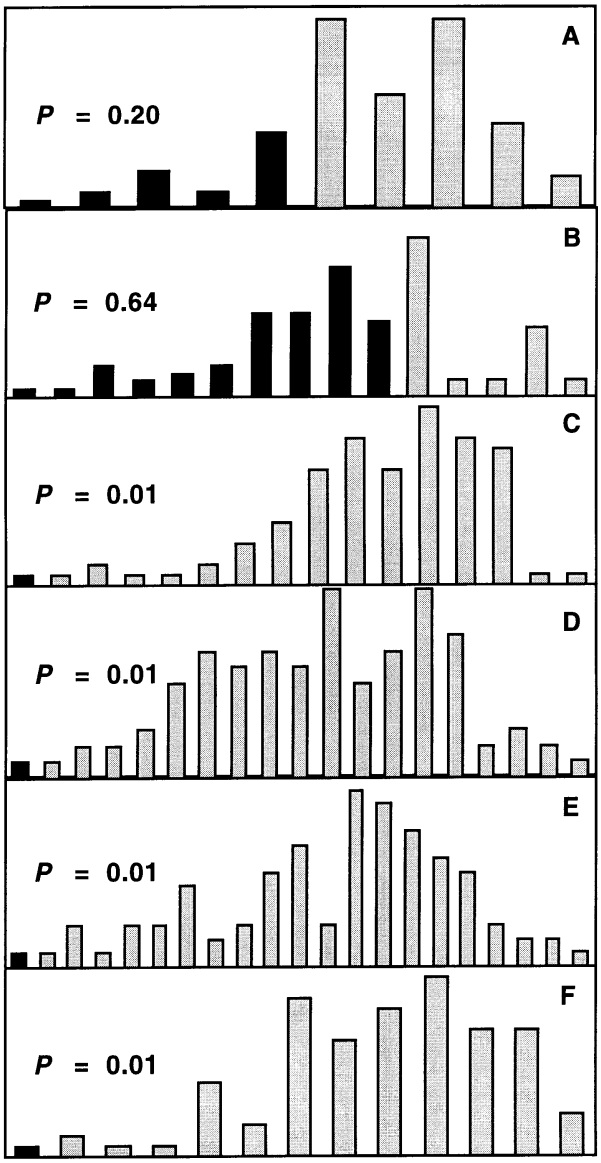

Fig. 5. Incongruence length difference test histograms of combined tree lengths and corresponding levels of significance of incongruence for (A) 18S-like rDNA versus translated amino acid sequences, (B) 18S-like rDNA versus first and second codon positions of actin nucleotides, (C) 18S-like rDNA versus all actin nucleotides, (D) 18S-like rDNA versus first position versus second position versus third position actin nucleotides, (E) 18S-like rDNA plus first and second position actin nucleotides versus third position actin nucleotides and (F) first and second position actin nucleotides versus third position actin nucleotides. The dark bars represent the tail-distribution in which random data partitions were at least as incongruent as the proposed partition.

1995). Those in favour of combining data sets, also called the 'principle of total evidence', hold that the hypothesis which explains more data is to be preferred over that which explains less (Mickevitch & Farris, 1981; Kluge, 1989; Kluge & Wolf, 1993). Paraphrasing Wheeler *et al.* (1993), another expression of the latter point of view is: although 1 data set, such as the 18S-like rDNA, might be intrinsically misleading due to the potential for saturating substitution rates and nucleotide composition, and another data set, such as the actin nucleotides, might be intrinsically misleading because of the confounding effects of codon usage bias, it is considerably less likely that *both* data sets will be misleading in *precisely* the same way. Thus, where the combination of the 2 data sets better corroborates monophyletic groups, this must be due to some extrinsic phenomenon, i.e. history. This is not to suggest that phylogeneticists should be unconcerned about incongruent data sets. The incongruence length difference (ILD) test (Farris *et al.* 1994) offers a mechanism for assessing the relative combinability of data set partitions. Any data partition has an associated number of extra steps required for the most parsimonious hypothesis. It is impossible for a combined data set to have less homoplasy than the sum of its parts, but it can have more. The difference between the length of the combined analysis and the sum of its parts is the incongruence introduced by combining the data sets. If this incongruence is greater than that found for 95 % of random partitions of the same data sets, then they are considered to be significantly incongruent. This feature allows the test to investigate hypotheses about which data set might be problematic. That is, using this technique, one can directly address the conundrum regarding third position nucleotide biases causing erroneous placement of the genera *Plasmodium* and *Cryptosporidium* in Fig. 4B.

Of the 6 ILD tests conducted, only 2 partitionings were found to be congruent, namely 18S-like rDNA versus first and second actin codon positions (Fig. 5B) and 18S-like rDNA versus translated actin amino acid sequences (Fig. 5A). The patterns found in the other 4 partitionings is revealing. Although 18S-like rDNA versus all actin nucleotides indicated significant incongruence (Fig. 5C), Fig. 5B suggests that this is not due to the first and second positions. The common feature of the remaining 3 partitions (Fig. 5D–F) is that the third position of the actin data set is set apart as 1 of the partitions. This suggests that the cause of incongruence between 18S-like rDNA and actin sequence data sets is due solely to the third position nucleotide biases as postulated above. Eliminating the third position, or ameliorating third position bias by using the translated amino acids, eliminates this problem.

Parsimony analysis of the combined 18S-like rDNA and actin data sets yielded the single most parsimonious tree shown in Fig. 6 irrespective of using translated amino acid sequences or first and second positions or even all nucleotide positions in phylogeny reconstruction. Unlike the use of the 18S-like rDNA data set alone, long branch length heterogeneities are not evident. As well, the 2 *Oxytricha nova* isolates are closest relatives in

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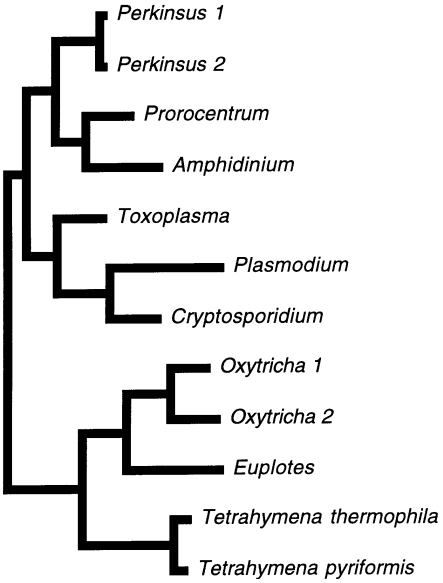

Fig. 6. The most parsimonious tree resulting from the combined analysis of both 18S-like rDNA data and actin sequence data irrespective of whether the latter consisted of all actin nucleotides, only the first and second position actin nucleotides or the translated actin amino acid sequences.

contrast to results from the use of actin nucleotides alone. This tree indicates monophyly of the ciliates, monophyly of the Apicomplexa, monophyly of the dinoflagellates and a sister group relationship for *Perkinsus marinus* and the dinoflagellates.

Tests of stability

The bootstrap (Felsenstein, 1985) can be considered either an assessment of the sensitivity of clades to random resampling of the aligned characters, or as a determination of sensitivity to the combined effects of random inclusion and randomly re-weighting included characters (Trueman, 1993). Although the character bootstrap is the most commonly used metric of clade stability, there are concerns regarding its limitations (Sanderson, 1989; Zarkickh & Li, 1992; Hillis & Bull, 1993; Wilkinson, 1996). An alternative is character jackknifing (Farris *et al*. 1996) which examines only the effects of randomly including characters on clade stability. As with the bootstrap, characters are included in pseudoreplicates according to a resampling scheme, though a character cannot be included more than once. The Jackknife Monophyly Index (Siddall, 1995), in contrast, examines the effects of including and excluding taxa. Figs 7–9 illustrate stability found for each of these techniques as applied to the 18S-like rDNA partition, the actin nucleotide partition, and the combined data set of all nucleotides from both data sets. Any nodes receiving less than 0·50 bootstrap values are collapsed in Fig. 7. Except for the *Oxytricha*/*Euplotes* clade which remained unchanged, all clades found in partitioned analyses and in the combined analysis were more stable in the latter. In accordance with the specified sampling theory (Farris *et al*. 1996), any nodes receiving less than 63% support in parsimony jackknifing of characters are collapsed in Fig. 8. Only the dinoflagellate/apicomplexan clade was marginally less stable (0·930 *vs*. 0·948) in the combined analysis. All other clades were found to be as or more robust to varying character information. Siddall (1995) made no recommendations regarding collapsing clades relative to JMI values, therefore all clades are shown with their sensitivities to taxonomic sampling in Fig. 9. Here again, the combined analysis outperforms the partitioned analyses and there is only 1 clade with less that 100% support in the tree resulting from using all of the data.

Phylogenetic and related implications

Our findings indicate that there is little remaining support for the inclusion of the genus *Perkinsus* in the phylum Apicomplexa. None of the morphological criteria postulated by Perkins (1976) and Levine (1978) withstands rigorous scrutiny. As Vivier (1982) has already asserted, *Perkinsus* does not have a conoid and all the other putatively apicomplexan characteristics are general to the Alveolata. Recently, Perkins (1996) has acknowledged the growing body of evidence in favour of a dinoflagellate relationship for these parasites but concluded that *Perkinsus* species represent 'the first apicomplexan to diverge from the evolutionary line which gave rise to the rest of the Apicomplexa. Future findings will probably support [this] suggestion.' We disagree. Findings subsequent to Perkins' (1976) initial suggestion of a relationship with the Apicomplexa have indicated a more recent common ancestry with the dinoflagellates, subsequent and unrelated to the divergence of the apicomplexan lineage (Vivier, 1982; Fong *et al*. 1993; Goggin & Barker, 1993; Siddall *et al*. 1995; Flores *et al*. 1996; Reece *et al*. 1997).

Analyses of data from a non-coding structural gene suggests that *Perkinsus* is (or is more closely related to) a dinoflagellate. Analyses of data from an unrelated, genetically unlinked, translated protein-coding gene supports precisely the same hypothesis both at the nucleotide and amino acid levels of analysis. The combination of these data provides a single fully resolved tree, which is robust when investigated by the most contemporary methods. Therefore, we suggest that the removal of the genus *Perkinsus* from Apicomplexan classifications is overdue and, to avoid any persisting confusion, that its ultrastructural and developmental features no longer be characterized in an apicomplexan framework. To facilitate this, and in keeping with the dinoflagellate affinity, we concur with Perkins' (1996) suggestions regarding terminology, that is: *palintomy* (instead of

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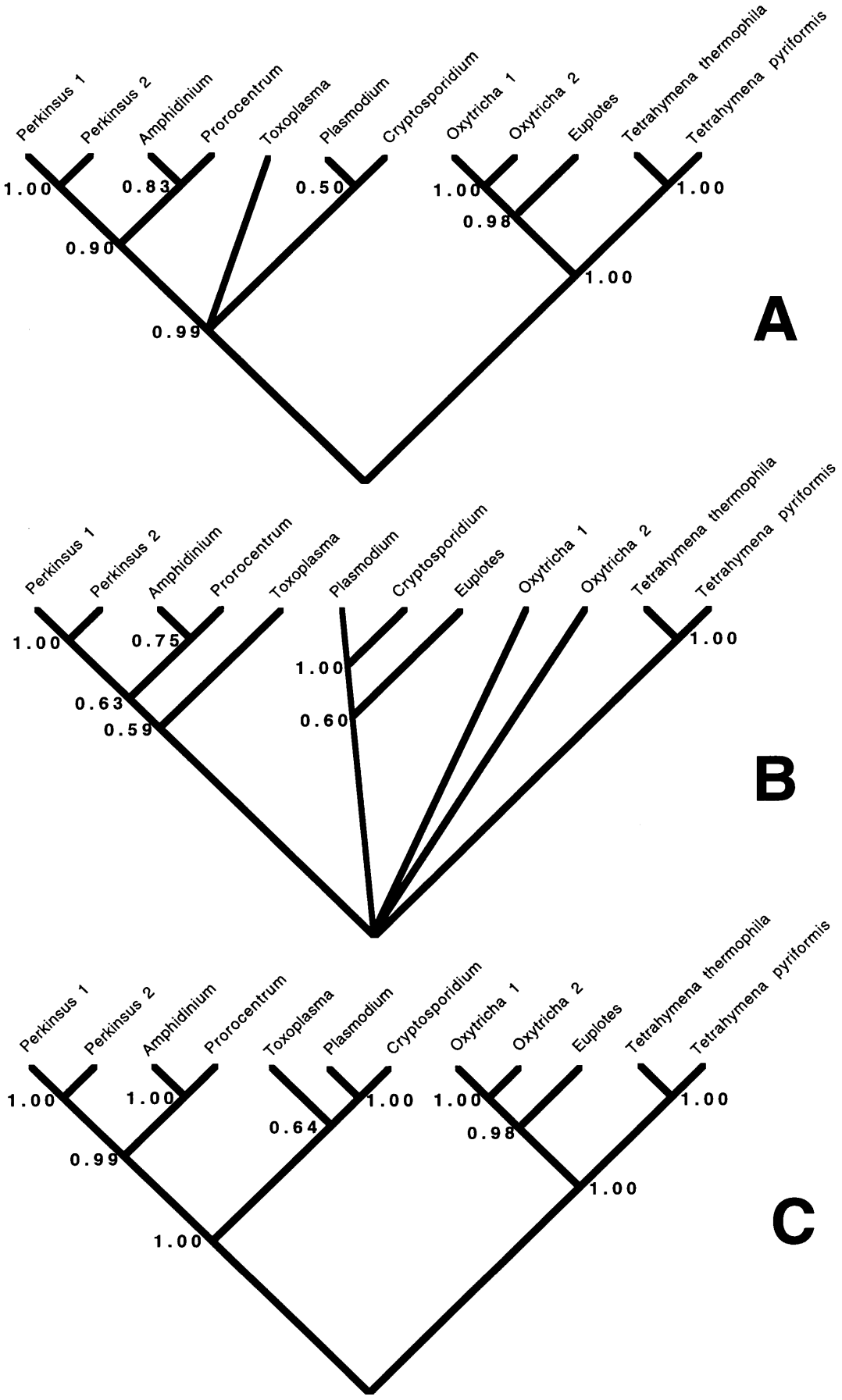

Fig. 7. Clade support values obtained from bootstrap analysis of (A) the 18S-like rDNA data alone, (B) the actin nucleotide data alone and (C) the combined dataset of all nucleotides from both genes. Clades with less than 0·50 bootstrap values are not shown.

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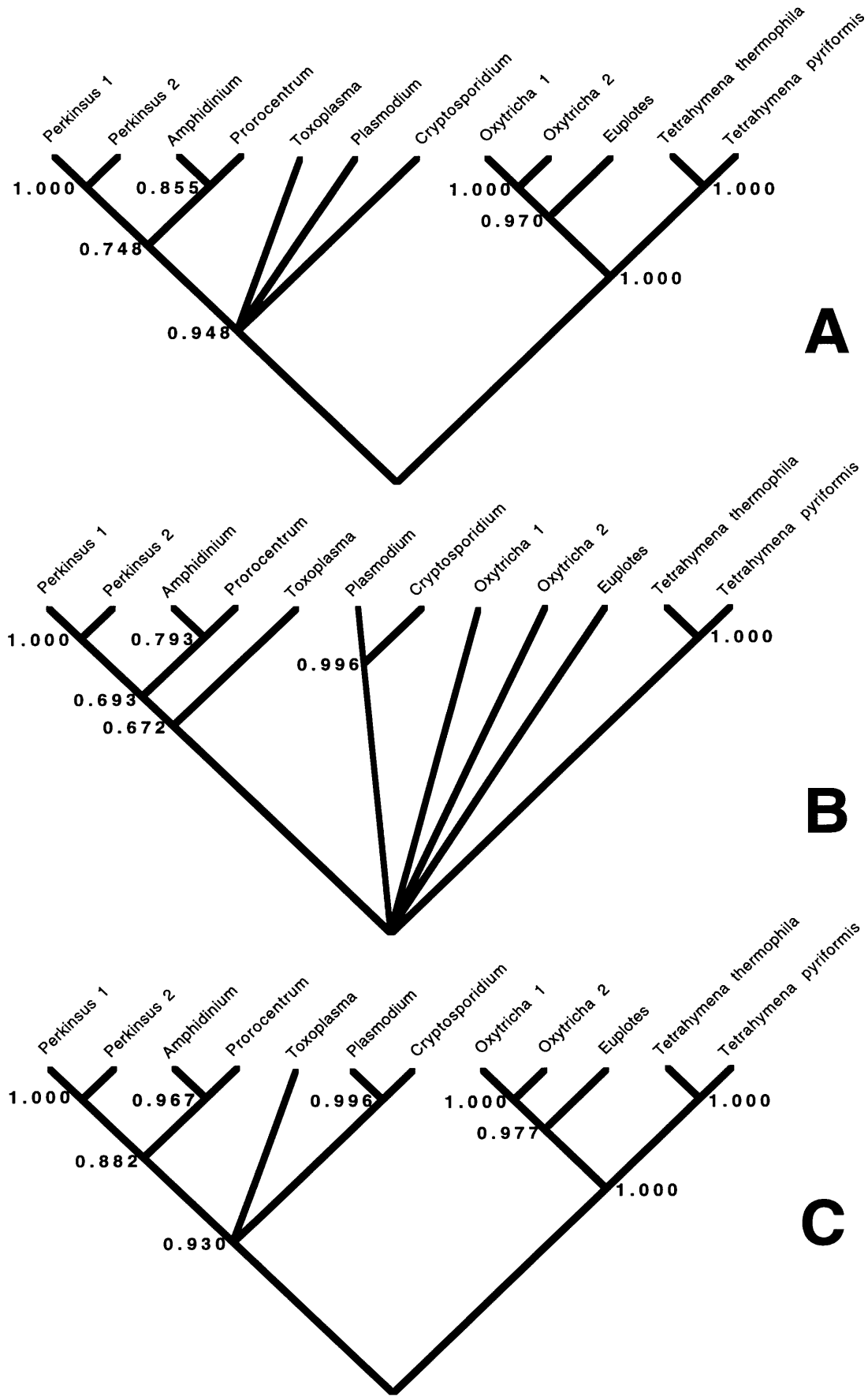

Fig. 8. Clade support values obtained from parsimony character jackknifing analysis of (A) the 18S-like rDNA data alone, (B) the actin nucleotide data alone and (C) the combined dataset of all nucleotides from both genes. Clades with less than 0·63 jackknife values are not shown.

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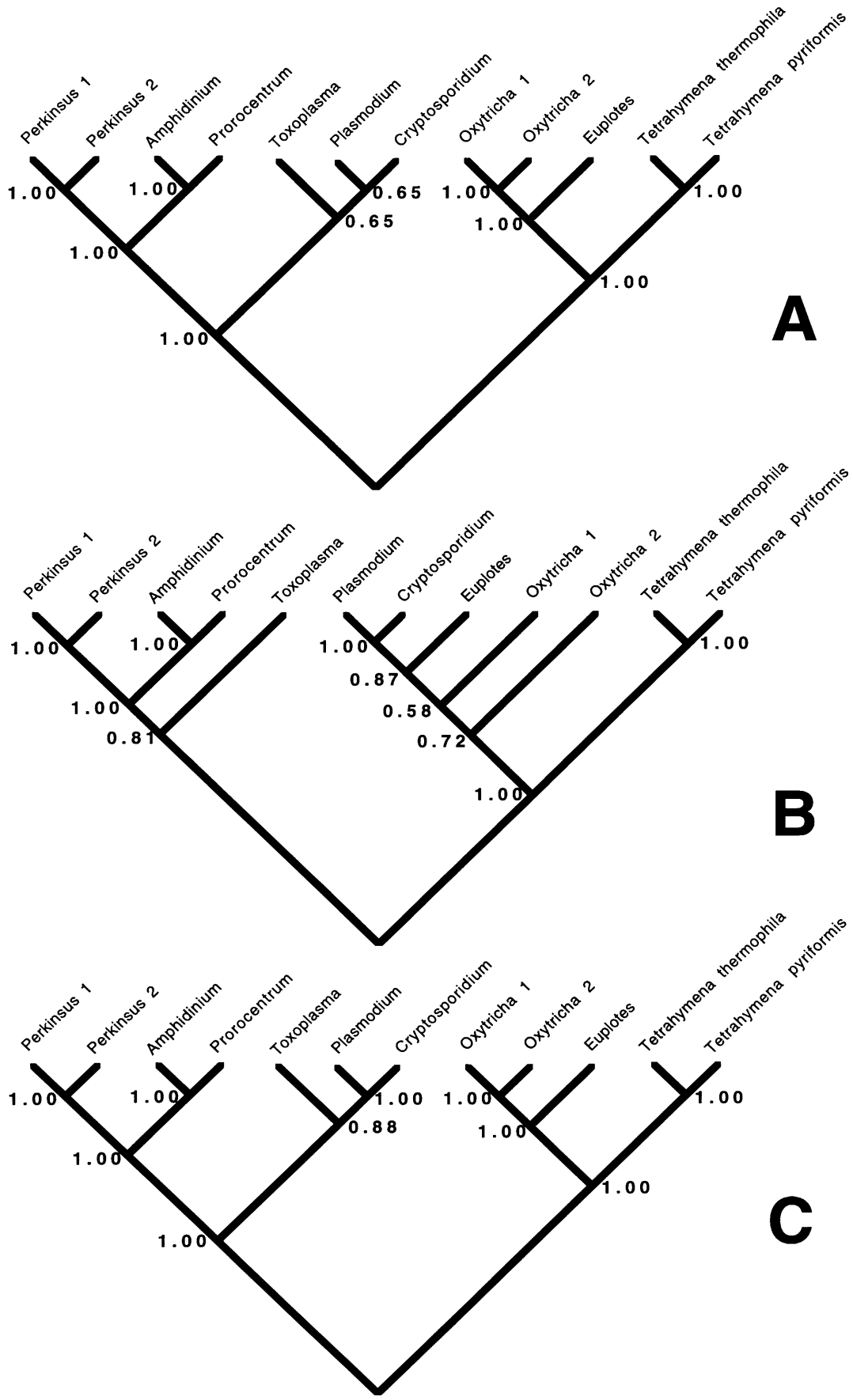

Fig. 9. Clade support values obtained from taxonomic jackknife analysis of (A) the 18S-like rDNA data alone, (B) the actin nucleotide data alone and (C) the combined dataset of all nucleotides from both genes.

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merogony), *tomont* (instead of meront), *trophozoite* (instead of merozoite) and *zoosporangium* (instead of sporocyst). Furthermore, we propose expanding on this, and in keeping with the suggestions of Roberts & Roberts (1991), we urge the following: *apical ribbon* (instead of conoid), *ribbon associated vesicles* (instead of conoid attached micronemes), and *toxicysts* (instead of rectilinear micronemes). The terms *micropore* and *cortical alveoli* probably should be applied to all alveolate phyla equally.

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REFERENCES

AZEVEDO, C. (1989). Fine structure of *Perkinsus atlanticus* n. sp. (Apicomplexa, Perkinsea) parasite of the clam *Ruditapes decussatus* from Portugal. *Journal of Parasitology* **75**, 627–635.

AZEVEDO, C., CORRAL, L. & CACHOLA, R. (1990). Fine structure of zoosporulation in *Perkinsus atlanticus* (Apicomplexa: Perkinsea). *Parasitology* **100**, 351–358.

BARTA, J. R., JENKINS, M. C. & DANFORTH, H. D. (1991). Evolutionary relationships of avian *Eimeria* species among other Apicomplexan protozoa: monophyly of the Apicomplexa is supported. *Molecular Biology and Evolution* **8**, 345–355.

BRUGEROLLE, G. & MIGNOT, J. P. (1979). Observations sur le cycle l'ultrastructure et la position systématique de *Spiromonas perforans* (*Bodo perforans* Hollande 1938), flagellé parasite de *Chilomonas paramecium*: ses relations avec les dinoflagellés et sporozoaires. *Protistologica* **XV**, 183–196.

CHAR, S., KELLY, P., NAEEM, A. & FARTHING, M. J. G. (1996). Codon usage in *Cryptosporidium parvum* differs from that in other Eimeriorina. *Parasitology* **66**, 357–362.

DE QUEIROZ, A., DONOGHUE, M. J. & KIM, J. (1995). Separate versus combined analysis of phylogenetic data. *Annual Review of Ecology and Systematics* **26**, 657–681.

DODGE, J. D. & CRAWFORD, R. M. (1971). Fine structure of the dinoflagellate *Oxyrrhis marina*. II. The flagellar system. *Protistologica* **VII**, 399–423.

ELLIS, J., GRIFFIN, H., MORRISON, D. & JOHNSON, A. M. (1993). Analysis of dinucleotide frequency and codon usage in the phylum Apicomplexa. *Gene* **126**, 163–170.

FARRIS, J. S. (1988). *Hennig86, version 1.5*. Software distributed by the author, Port Jefferson Station, N.Y.

FARRIS, J. S. (1995). *MM.EXE. Software distributed by the author*. Molecular Systematics Laboratory, Box 50007, Swedish Museum of Natural History, Stockholm, S-10405, Sweden.

FARRIS, J. S., ALBERT, V., KÄLLERSJÖ, M., LIPSCOMB, D. & KLUGE, A. G. (1996). Parsimony jackknifing outperforms neighbor-joining. *Cladistics* **12**, 99–124.

FARRIS, J. S., KÄLLERSJÖ, M., KLUGE, A. G. & BULT, C. (1994). Testing significance of incongruence. *Cladistics* **10**, 315–320.

FELSENSTEIN, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.

FLORES, B. S., SIDDALL, M. E. & BURRESON, E. M. (1996). Phylogeny of the Haplosporidia (Eukaryota: Alveolata) based on small subunit ribosomal RNA gene sequence. *Journal of Parasitology* **82**, 616–623.

FONG, D., RODRIGUEZ, R., KOO, K., SUN, J., SOGIN, M. L., BUSHEK, D., LITTLEWOOD, D. T. L. & FORD, S. E. (1993). Small subunit ribosomal RNA gene sequence of the oyster parasite *Perkinsus marinus*. *Molecular Marine Biology and Biotechnology* **2**, 346–350.

GAJADHAR, A. A., MARQUARDT, W. C., HALL, R., GUNDERSON, J., ARIZTIA-CARMONA, E. V. & SOGIN, M. L. (1991). Ribosomal RNA sequences of *Sarcocystis muris*, *Theileria annulata* and *Crypthecodinium cohnii* reveal evolutionary relationships among apicomplexans, dinoflagellates, and ciliates. *Molecular and Biochemical Parasitology* **45**, 147–154.

GOGGIN, C. L. & BARKER, S. C. (1993). Phylogenetic position of the genus *Perkinsus* (Protista, Apicomplexa) based on small subunit ribosomal RNA. *Molecular and Biochemical Parasitology* **60**, 65–70.

GRAYBEAL, A. (1994). Evaluating the phylogenetic utility of genes: a search for genes informative about deep divergences among vertebrates. *Systematic Biology* **43**, 174–193.

HARPER, D. S. & JAHN, C. L. (1989). Differential use of termination codons in ciliated protozoa. *Proceedings of the National Academy of Sciences, USA* **86**, 3252–3256.

HASCALL, G. K. & RUDZINSKA, M. A. (1970). Metamorphosis in *Tokophyra infusionum*; an electron-microscope study. *Journal of Protozoology* **17**, 311–323.

HILL, F. G. & OUTKA, D. E. (1974). The structure and origin of mastigonemes in *Ochromonas minuta* and *Monas* sp. *Journal of Protozoology* **21**, 299–312.

HILLIS, D. M. & DIXON, M. T. (1991). Ribosomal DNA: molecular evolution and phylogenetic inference. *Quarterly Review of Biology* **66**, 411–453.

HILLIS, D. M. & BULL, J. J. (1993). An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. *Systematic Biology* **42**, 182–192.

HUELSENBECK, J. P., BULL, J. J. & CUNNINGHAM, C. W. (1996). Combining data in phylogenetic analysis. *Trends in Ecology and Evolution* **11**, 152–158.

JOHNSTON, A. M., FIELKE, R., LUMB, R. & BAVERSTOCK, P. R. (1990). Phylogenetic relationships of *Cryptosporidium* determined by ribosomal RNA sequence comparison. *International Journal for Parasitology* **20**, 141–147.

KLUGE, A. (1989). A concern for evidence and a phylogenetic hypothesis for relationships among *Epicrates* (Boidae, Serpentes). *Systematic Biology* **38**, 1–25.

KLUGE, A. G. & WOLF, A. J. (1993). Cladistics: what's in a word? *Cladistics* **9**, 183–199.

LARSEN, J. (1988). An ultrastructural study of *Amphidinium poecilochroum* (Dinophyceae), a phagotrophic dinoflagellate feeding on small species of cryptophytes. *Phycologia* **27**, 366–377.

LEVINE, N. D. (1978). *Perkinsus* gen. n. and other new taxa in the protozoan Phylum Apicomplexa. *Journal of Parasitology* **64**, 549.

LOM, J. & CORLISS, J. O. (1971). Morphogenesis and cortical ultrastructure of *Brooklynella hostilis*, a dysteriid ciliate ectoparasite on marine fishes. *Journal of Protozoology* **18**, 261–281.

MACKIN, J. G. (1951). Histopathology of infection of *Crassostrea virginica* (Gmelin) by *Dermocystidium marinum* Mackin, Owen and Collier. *Bulletin of Marine Science of the Gulf and Caribbean* **1**, 72–87.

MACKIN, J. G. & BOSWELL, J. L. (1956). The life cycle and relationships of *Dermocystidium marinum*. *Proceedings of the National Shellfisheries Association* **46**, 112–115.

MACKIN, J. G., OWEN, H. M. & COLLIER, A. (1950). Preliminary note on the occurrence of a new protistan parasite, *Dermocystidium marinum* n. sp. in *Crassostrea virginica* (Gmelin). Science **111**, 328–329.

MACKIN, J. G. & RAY, S. M. (1966). The taxonomic relationships of *Dermocystidium marinum* Mackin, Owen and Collier. *Journal of Invertebrate Pathology* **8**, 544–545.

MADDISON, W. P. & MADDISON, D. R. (1992). *MacClade : Analysis of Phylogeny and Character Evolution, version 3.0*. Software distributed by Sinauer Associates, Sunderland, Mass.

MICKEVITCH, M. F. & FARRIS, J. S. (1981). The implications of congruence in *Menidia*. *Systematic Zoology* **30**, 351–370.

MILLECCHIA, L. L. & RUDZINSKA, M. A. (1970). The ultrastructure of brood pouch formation in *Tokophyra infusionum*. *Journal of Protozoology* **17**, 574–583.

MIYAMOTO, M. M. & FITCH, W. M. (1995). Testing species phylogenies and phylogenetic methods with congruence. *Systematic Biology* **44**, 64–76.

MÖESTRUP, O. (1982). Flagellar structure in algae: a review, with new observations particularly on the Chrysophyceae, Phaeophyceae (Fucophyceae), Euglenophyceae, and *Reckertia*. *Phycologia* **21**, 427–528.

PAULIN, J. J. (1973). The resorption of cilia in *Cyathodinium piriforme*. *Journal of Protozoology* **20**, 281–285.

PERKINS, F. O. (1969). Ultrastructure of vegetative stages in *Labyrinthomyxa marina* (= *Dermocystidium marinum*), a commercially significant oyster pathogen. *Journal of Invertebrate Pathology* **13**, 199–222.

PERKINS, F. O. (1976). Zoospores of the oyster pathogen, *Dermocystidium marinum*. I. Fine structure of the conoid and other sporozoan-like organelles. *Journal of Parasitology* **62**, 959–974.

PERKINS, F. O. (1996). The structure of *Perkinsus marinus* (Mackin, Owen and Collier, 1950) Levine, 1978 with comments on taxonomy and phylogeny of *Perkinsus* spp. *Journal of Shellfish Research* **15**, 67–87.

PERKINS, F. O. & MENZEL, R. W. (1967). Ultrastructure of sporulation in the oyster pathogen *Dermocystidium marinum*. *Journal of Invertebrate Pathology* **9**, 205–229.

PITELKA, D. R. (1970). Ciliate ultrastructure: some problems in cell biology. *Journal of Protozoology* **17**, 1–10.

PREPARATA, R. M., MEYER, E. B., PREPARATA, F. P., SIMON, E. M., VOSSBRINCK, C. R. & NANNEY, D. L. (1988). Ciliate evolution: the ribosomal phylogeny of tetrahymenine ciliates. *Journal of Molecular Evolution* **28**, 447–451.

RAY, S. M. (1954). Experimental studies on the transmission and pathogenicity of *Dermocystidium marinum*, a fungus disease of oysters. *Journal of Parasitology* **40**, 235.

REECE, K. S., SIDDALL, M. E., BURRESON, E. M. & GRAVES, J. E. (1997). Phylogenetic analysis of *Perkinsus* based on actin gene sequences. *Journal of Parasitology* (in the Press).

ROBERTS, K. R. & ROBERTS, J. E. (1991). The flagellar apparatus and cytoskeleton of the dinoflagellates. A comparative overview. *Protoplasma* **164**, 105–122.

ROBERTS, K. R., HEIMANN, K. & WETHERBEE, R. (1995). The flagellar apparatus and canal structure in *Prorocentrum micans* (Dinophyceae). *Phycologia* **34**, 313–322.

SANDERSON, M. J. (1989). Confidence limits on phylogenies: the bootstrap revisited. *Cladistics* **5**, 113–129.

SIDDALL, M. E. (1994). '*Random Cladistics*'. *vers. 4.0*. Available by anonymous ftp to zoo.toronto.edu.

SIDDALL, M. E. (1995). Another monophyly index: revisiting the jackknife. *Cladistics* **11**, 33–56.

SIDDALL, M. E., STOKES, N. A. & BURRESON, E. M. (1995). Molecular phylogenetic evidence that the phylum Haplosporidia has an alveolate ancestry. *Molecular Biology and Evolution* **12**, 573–581.

SIMPSON, A. G. B. & PATTERSON, D. J. (1996). Ultrastructure and identification of the predatory flagellate *Colpodella pugnax* Cienkowski (Apicomplexa) with a description of *Colpodella turpis* n. sp. and a review of the genus. *Systematic Parasitology* **5**, 1–12.

SWOFFORD, D. L. (1993). *PAUP – Phylogenetic analysis using parsimony, vers. 3.1.1*. Software distributed by the Illinois Natural History Survey, Champaign, IL.

TRUEMAN, J. W. H. (1993). Randomization confounded: a response to Carpenter. *Cladistics* **9**, 101–109.

VIVIER, E. (1982). Réflexions et suggestions à propos de la systématique des sporozoaires: création d'une classe des Hematozoa. *Protistologica* **XVIII**, 449–457.

WESSENBERG, H. & ANTIPA, G. (1970). Capture and ingestion of *Paramecium* by *Didinium nasutum*. *Journal of Protozoology* **17**, 250–270.

WHEELER, W. C. (1995). Sequence alignment, parameter sensitivity, and phylogenetic analysis of molecular data. *Systematic Biology* **44**, 321–331.

WHEELER, W. C., CARTWRIGHT, P & HAYASHI, C. Y. (1993). Arthropod phylogeny: a combined approach. *Cladistics* **9**, 1–39.

WHEELER, W. C., & GLADSTEIN, D. (1993). *Malign. Vers. 1.85*. Department of Invertebrates, American Museum of Natural History, Central Park W. at 79th Street, New York, NY.

WILKINSON, M. (1996). Majority-rule reduced consensus trees and their use in bootstrapping. *Molecular Biology and Evolution* **13**, 437–444.

ZARKICKH, A. & LI, W.-H. (1992). Statistical properties of bootstrap estimation of phylogenetic variability from nucleotide sequences. I. Four taxa with a molecular clock. *Molecular Biology and Evolution* **9**, 1119–1147.