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The Phylogenetic Position of *Amoebophrya* sp. Infecting *Gymnodinium sanguineum*

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ABSTRACT. The small-subunit rRNA sequence of a species of *Amoebophrya* infecting *Gymnodinium sanguineum* in Chesapeake Bay was obtained and compared to the small subunit rRNA sequences of other protists. Phylogenetic trees constructed with the new sequence place *Amoebophrya* between the remaining dinoflagellates and other protists.

Supplementary key words. Dinoflagellate, rRNA, Syndiniophyceae.

THE motile stages of the parasitic syndiniophyceans have the general appearance of other dinoflagellates, and have a typical dinoflagellate transverse flagellum. However, they differ from most other dinoflagellates in having chromosomes and a mitotic pattern more typical of other eukaryotes [3]. They also lack chloroplasts, which many other dinoflagellates possess. This combination of characteristics could be explained if the lineage containing the syndiniophyceans diverged from other dinoflagellates before the nucleus had developed the aberrant characteristics so typical of the group and before chloroplasts had been acquired [16].

Ribosomal RNA sequences have been used to infer phylogeny within the dinoflagellates [1, 10, 12, 13, 15, 16, 18], but the sequences used in these analyses have not, to date, included any from the syndiniophyceans. We present here the results of phylogenetic analyses that include the small-subunit rRNA sequence from an *Amoebophrya* species infecting *Gymnodinium sanguineum* in Chesapeake Bay.

This strain has been referred to in earlier papers as *Amoebophrya ceratii* [4, 5] as it matches the original description of *A. ceratii*. However, morphological and developmental differences exist among strains of "*A. ceratii*" infecting different dinoflagellate species in Chesapeake Bay [4], something also noted by Cachon [2] in *Amoebophrya* infecting dinoflagellates in the Mediterranean Sea. It is possible that "*A. ceratii*" represents a species complex rather than a single species, and because we are not certain that the strain studied by us is identical to the organism originally given that name, we are referring to it as *Amoebophrya* sp.

MATERIALS AND METHODS

Source and growth of cells. Stock cultures of a Chesapeake Bay isolate (DWC) of the photosynthetic dinoflagellate *Gymnodinium sanguineum* Hirasaka were grown in f/2-Si medium [9] formulated using 15‰ Chesapeake Bay water supplemented with 5% (v/v) soilwater [GR+] [17]. Parasitic dinoflagellates conforming in morphology and life cycle to *Amoebophrya ceratii* (Koeppen) were established in culture by adding a single cell of *G. sanguineum* in late-stage infection to an exponentially growing culture of the same host species [5]. Stock cultures of the parasite were subsequently maintained by transferring aliquots of infected host culture to uninfected *G. sanguineum* stocks at approximately three-day intervals. All cultures were maintained at 20–23° C on a 14:10 light:dark cycle, with cool white fluorescent bulbs providing ~ 100 $\mu E\ m^{-2}\ s^{-1}$.

Isolation of DNA. Dinospores, the infective dispersal stage of the parasite [5], were separated from host cells by size fractionation using an autoclaved Sterifil holder (Millipore, Bedford, MA) fitted with a 12-μm pore Nuclepore filter. The < 12 μm fraction was transferred to sterile 15-ml conical tubes and centrifuged at 1000 *g* for 20 min to produce dinospore pellets. The supernatant overlying each pellet was removed using a sterile micropipette and pellets were immmediately resuspended in a solution of 4 M guanidium isothiocyanate and 0.1% beta-mercaptoethanol to promote cell lysis and stabilize parasite DNA. Genomic DNA was purified with a Wizard PCR Preps DNA Purification System (Promega, Madison, WI) according to the manufacturer's instructions, except that the water used for eluting DNA was at a temperature of 80° C. The high temperature permitted large fragments of DNA to be eluted.

Amplification of rRNA sequences. The small-subunit rRNA coding region was amplified with the primers described elsewhere [11]. Thirty five amplification cycles were used, each containing a 30-s denaturation step at 92° C, a 1-min annealing step at 42° C, a 3-min ramp to 72° C, and a 2-min extension at 72° C. After the final cycle, the reaction mixture was incubated at 72° C for 9 min. Each 100-μl reaction volume contained 1 unit of *Taq* DNA polymerase (Promega), 10 μl 10× buffer (Promega), 0.2 mM dNTPs (Promega), and 0.2 μM each primer. Amplified DNA was purified with a Wizard PCR Preps DNA Purification System according to the manufacturer's instructions.

DNA sequencing. A SequiTherm Cycle Sequencing Kit (Epicentre Technologies, Madison, WI) was used for direct sequencing of the PCR product. Primers listed by Elwood et al. [6] were labelled with ^{33}P (ICN, Costa Mesa, CA) according to the kit manufacturer's instructions and used in sequencing reactions with 25 ng of amplified DNA. Twenty cycles were used in each sequencing reaction, each containing a 30-s denaturation step at 95° C, a 30-s annealing step at 42° C, a 3-min ramp to 72° C, and a 2-min extension at 72° C. Sequencing reactions were electrophoresed on 5% or 8% polyacrylamide gels, and sequencing ladders detected by exposure to Biomax MR film (Eastman Kodak, Rochester, NY).

Sequence analysis. The *Amoebophrya* sequence was aligned with the other protistan sequences listed in Table 1. The number of aligned positions used in constructing trees varied between 1669 and 1725, according to which sequences were used in building trees. Trees were built using the parsimony programs DNAPARS or DNAPENNY, the neighbor-joining program NEIGHBOR in which distances were produced using the Kimura 2-parameter model in DNADIST, and the maximum-likelihood program DNAML. Data sets for bootstrapping were produced by SEQBOOT, and majority-rule consensus trees produced with CONSENSE. All of these programs are from PHYLIP 3.57c [7]. Bootstrapped DNAML and DNAPENNY trees

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Table 1. List of species used in constructing phylogenetic trees. GenBank accession numbers are listed to the right of each species.

Species	Accession number
Alexandrium fundyense	U09048
Amphidinium belauense	L13719
Amphidinium carterae	(Sogin lab, unpublished)
Blepharisma americanum	M97909
BBSR 323	USU52356
Ceratium tenue	AF022192
Collozoum caudatum symbiont	USU52354
Crypthecodinium cohnii	M34847
Dictyostelium discoideum	K02641
Gloeodinium viscum	L13716
Gymnodinium catenatum	AF022193
Gymnodinium fuscum	AF022194
Gymnodinium mikimotoi	AF022195
Gymnodinium sanguineum	GSU41085
Gymnodinium sp.	AF022196
Gyrodinium impudicum	AF022197
Heterocapsa triquetra	AF022198
Lepidodinium viride	AF022199
Noctiluca scintillans	AF022200
Pentapharsodinium tyrrhenicum	AF022201
Peridinium sp.	AF022202
Perkinsus sp.	L07375
Prorocentrum micans	M14649
Scrippsiella nutricula	SNU52357
Spongostaurus symbiont	USU52355
Symbiodinium microadriaticum	M88521
Tetrahymena thermophila	M10932
Toxoplasma gondii	U03070

were limited to 9 organisms in order to keep the computation time reasonable. Sixteen sequence sets were used with each of these two types of algorithms. All contained *Toxoplasma gondii, Perkinsus* sp., *Amoebophrya* sp., *Noctiluca scintillans,* and five other randomly chosen dinoflagellate species. Each consensus tree was produced from 200 bootstrap replicates. Trees were drawn with TREEVIEW 1.5.0 [14].

RESULTS

Amplification of *Amoebophrya* genomic DNA with primers complementary to the ends of eukaryotic small-subunit rRNA coding regions yielded a product 1826 nucleotides long (GenBank Accession No. AF069516), 72 of these positions being complementary to the primers. Nucleotide usage was unambiguous; there was no evidence for the existence of multiple slightly different rRNA gene copies in the genome.

Initial parsimony trees constructed with DNAPARS and containing *Dictyostelium discoideum,* the ciliates, apicomplexans, and dinoflagellates always placed *Amoebophrya* with the apicomplexans and dinoflagellates, as would be expected. *D. discoideum* and the ciliates were then removed from subsequent analyses so that more nearly complete sequences could be used for tree construction.

In the majority of trees produced with either DNAPENNY or DNAML, a branch leading only to *Amoebophrya* diverged between *Perkinsus* sp. and the remaining dinoflagellates. However, many of the most parsimonious trees produced with DNAPENNY united *Noctiluca* and *Amoebophrya* in the most deeply diverging dinoflagellate clade, while few of the trees produced with DNAML displayed this topology. The results of bootstrapping show that this apparent difference in trees produced by the two methods is an illusion. Neither method strongly supports the divergence of a lineage at the base of the dinoflagellate tree which contains only *Amoebophrya,* and the bootstrap values for branches produced in DNAPENNY and DNAML trees were usually very similar when produced from the same data set. The bootstrap value for a dinoflagellate clade separated from *T. gondii* and *Perkinsus* sp. ranged from 60–89%, with the average for the 32 trees being 76%. Alternative topologies placed *Noctiluca, Amoebophrya* or *Crypthecodinium* between *T. gondii* and *Perkinsus.* The support for a dinoflagellate clade that excluded *Amoebophrya,* that is, for a tree topology in which *Amoebophrya* was separated from *Noctiluca* and emerged by itself from the base of the dinoflagellates, was much lower. Bootstrap value for such a dinoflagellate clade ranged from 37–62%, with the average value being 48%. Although this arrangement occurred less than 50% of the time, the alternative topologies occurred even less frequently. The usual alternative topology was one in which *Noctiluca* and *Amoebophrya* emerged together on a common branch diverging from the other dinoflagellates.

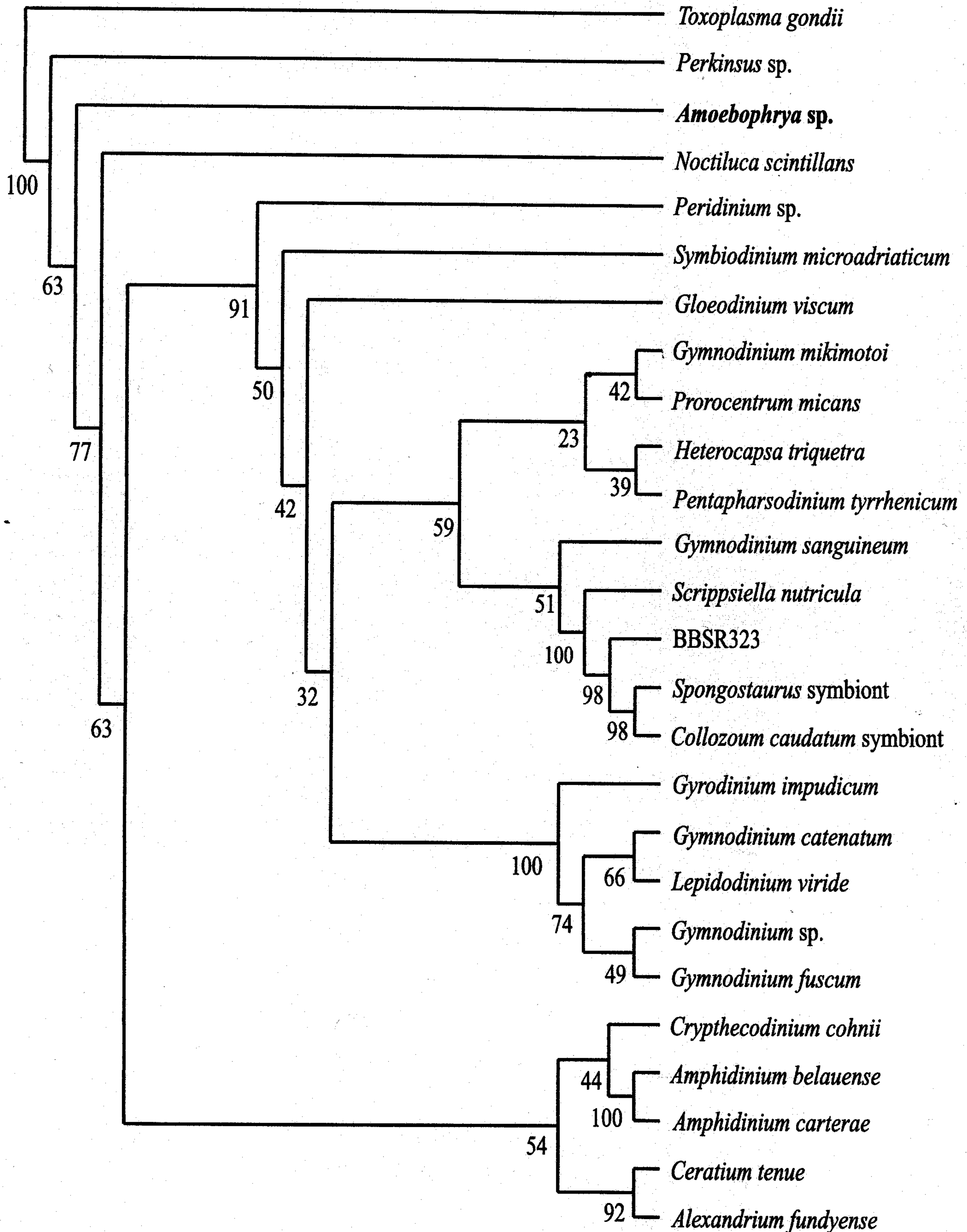
Figure 1 shows a bootstrapped neighbor-joining tree. The speed of the algorithm allows many more species to be included in a tree, but possibly at the cost of accuracy. Its topology is in general agreement with the relationships revealed by the smaller trees (although the bootstrap figures are different), and is therefore a useful summary figure.

DISCUSSION

No single placement of *Amoebophrya* in our trees is so strongly supported by bootstrapping as to exclude the likelihood of a different arrangement. However, all of the alternatives place *Amoebophrya* in a lineage at or near the base of the dinoflagellates. The two most strongly supported alternatives (which taken together probably do make other arrangements unlikely) are one in which a branch containing only *Amoebophrya* emerges at the base of the dinoflagellates (as seen in Fig. 1) and one in which the most deeply diverging lineage among the dinoflagellates contains both *Amoebophrya* and *Noctiluca.* The syndiniophycean nuclear state is comparable to that of other eukaryotes, and the phylogenetic position of *Amoebophrya* in relation to other dinoflagellates is one more indication that the ancestral dinoflagellates had a typical eukaryotic nucleus.

A possible association with *Noctiluca* isn't entirely surprising. *Noctiluca* has a dinokaryotic nucleus only during the haploid stage of the life cycle. The morphological and life cycle characteristics of *Noctiluca* place it outside of the typical dinophyceans [8], and it was the most divergent dinoflagellate in trees constructed from a set of small-subunit rRNA sequences which, however, did not include sequences from *Oxyrrhis* or syndiniophyceans [16]. The *Amoebophrya-Noctiluca* relationship might be better resolved once more sequences are obtained from more deeply diverging dinoflagellate lineages. It would be desirable to have sequences from *Oxyrrhis,* representatives of the Blastodiniophyceae, and a few more syndiniophyceans.

The remaining sections of our trees are in general agreement with trees produced from both SSU and LSU rRNA sequences by other authors [12, 13, 16]. All indicate that *Crypthecodinium* and *Noctiluca* are in lineages that diverged early in dinoflagellate evolution rather than being derived forms, and that the lineage containing *Prorocentrum* appeared more recently in dinoflagellate evolution, so the *Prorocentrum* morphotype cannot represent the form of primitive dinoflagellates. A lineage that diverged early in dinoflagellate evolution contains *Alexandrium, Amphidinium, Crypthecodinium,* and some other genera not appearing in our tree. A large number of organisms belong to a group that appeared more recently. This group includes the Gymnodiniales, Peridiniales, and the Prorocentrales. Relationships within this group are very poorly resolved by the available sequence information.


Toxoplasma gondii
Perkinsus sp.
Amoebophrya sp.
Noctiluca scintillans
Peridinium sp.
Symbiodinium microadriaticum
Gloeodinium viscum
Gymnodinium mikimotoi
Prorocentrum micans
Heterocapsa triquetra
Pentapharsodinium tyrrhenicum
Gymnodinium sanguineum
Scrippsiella nutricula
BBSR323
Spongostaurus symbiont
Collozoum caudatum symbiont
Gyrodinium impudicum
Gymnodinium catenatum
Lepidodinium viride
Gymnodinium sp.
Gymnodinium fuscum
Crypthecodinium cohnii
Amphidinium belauense
Amphidinium carterae
Ceratium tenue
Alexandrium fundyense
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LITERATURE CITED

1. Adachi, M., Sako, Y. & Ishida, Y. 1996. Analysis of *Alexandrium* (Dinophyceae) species using sequences of the 5.8S ribosomal DNA and internal transcribed spacer regions. *J. Phycol.*, **32**:424–432.

2. Cachon, J. 1964. Contribution a l'étude des péridiniens parasites. Cytologie, cycles évolutifs. *Ann. Sci. Nat. Zool.*, **6**:1–158.

3. Cachon, J. & Cachon, M. 1987. Parasitic dinoflagellates. *In:* Taylor, F. J. R. (ed.), The Biology of Dinoflagellates. Botanical Monographs, Blackwell Scientific, Oxford. **21**:571–610.

4. Coats, D. W., Adam, E. J., Gallegos, C. L. & Hedrick, S. 1996. Parasitism of photosynthetic dinoflagellates in a shallow subestuary of Chesapeake Bay, USA. *Aquat. Microb. Ecol.*, **11**:1–9.

5. Coats, D. W. & Bockstahler, K. R. 1994. Occurrence of the parasitic dinoflagellate *Amoebophrya ceratii* in Chesapeake Bay populations of *Gymnodinium sanguineum*. *J. Euk. Microbiol.*, **41**:586–593.

6. Elwood, H. J., Olsen, G. J. & Sogin, M. L. 1985. The small subunit RNA gene sequences from the hypotrichous ciliates *Oxytricha nova* and *Stylonychia pustulata*. *Mol. Biol. Evol.*, **2**:399–410.

7. Felsenstein, J. 1993. PHYLIP: phylogeny inference package, version 3.5c. University of Washington, Seattle, WA.

8. Fensome, R. A., Taylor, F. J. R., Norris, G., Sarjeant, W. A. S., Wharton, D. I. & Willliams, G. L. 1993. A classification of living and fossil dinoflagellates. *Micropaleontol. Spec. Publ.* **7**.

9. Guillard, R. R. L. & Ryther, J. H. 1962. Studies on marine planktonic diatoms. I. *Cyclotella nana* Hustedt and *Detonula confervacea* (Cleve) Gran. *Can. J. Microbiol.*, **8**:229–239.

10. Gast, R. J. & Caron, D. A. 1996. Molecular phylogeny of symbiotic dinoflagellates from planktonic foraminifera and radiolaria. *Mol. Biol. Evol.*, **13**:1192–1197.

11. Gunderson, J., Hinkle, G., Leipe, D., Morrison, H. G., Stickel, S. K., Odelson, D. A., Breznak, J. A., Nerad, T. A., Muller, M. & Sogin, M. L. 1995. Phylogeny of trichomonads inferred from small-subunit rRNA sequences. *J. Euk. Microbiol.*, **42**:411–415.

12. Lenaers, G., Maroteaux, L., Michot, B. & Herzog, M. 1989. Dinoflagellates in evolution. A molecular phylogenetic analysis of large-subunit ribosomal RNA. *J. Molec. Evol.*, **29**:40–51.

13. McNally, K. L., Govind, N. S., Thomé, P. E. & Trench, R. K. 1994. Small-subunit ribosomal DNA sequence analyses and a reconstruction of the inferred phylogeny among symbiotic dinoflagellates (Pyrrophyta). *J. Phycol.*, **30**:316–329.

14. Page, R. D. M. 1996. TREEVIEW: An application to display phylogenetic trees on personal computers. *CABIOS*, **12**:357–358.

15. Rowan, R. 1992. Ribosomal RNA sequences and the diversity of symbiotic dinoflagellates (zooxanthellae). *Proc. Natl. Acad. Sci. USA*, **89**:3639–3643.

16. Saunders, G. W., Hill, D. R. A., Sexton, J. P. & Andersen, R. A. 1997. Small-subunit ribosomal RNA sequences from selected dinoflagellates: testing classical evolutionary hypotheses with molecular systematic methods. *Pl. Syst. Evol.* (Suppl.), **11**:237–259.

17. Starr, R. C. & Zeikus, J. A. 1993. UTEX—The culture collection of algae at the University of Texas at Austin. *J. Phycol.*, **29**(Suppl.).

18. Zardoya, R., Costas, E., López-Rodas, V., Garrido-Pertierra, A. & Bautista, J. M. 1995. Revised dinoflagellate phylogeny inferred from molecular analysis of large-subunit ribosomal RNA gene sequences. *J. Molec. Evol.*, **41**:637–645.

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Fig. 1. Consensus neighbor-joining tree illustrating relationships among the dinoflagellates. The topology is in general agreement with the arrangement of species seen in smaller maximum likelihood and branch-and-bound parsimony trees; all trees indicate that the lineage containing *Amoebophrya* diverges at or near the base of the dinoflagellates. Bootstrap values are given at the nodes, and represent percentages of 200 replicates. The bootstrap value for a dinoflagellate clade (63%) is lower than was normally seen in parsimony and maximum likelihood trees. CONSENSE produces trees in which branch lengths are proportional to the frequency of a clade in the input trees rather than the number of substitutions per site, so the tree is drawn as a rectangular cladogram without branch lengths.