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Multiple Strains of the Parasitic Dinoflagellate *Amoebophrya* Exist in Chesapeake Bay

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ABSTRACT. Small subunit rRNA sequences were amplified from *Amoebophrya* strains infecting *Karlodinium micrum, Gymnodinium instriatum* and an unidentified *Scrippsiella* species in Chesapeake Bay. The alignable parts of the sequences differed from each other and from the previously reported rRNA sequence of the *Amoebophrya* strain infecting *Akashiwo sanguinea* in Chesapeake Bay by 4 to 10%. This is a greater degree of difference than sometimes found between sequences from separate genera of free-living dinoflagellates. These sequence differences indicate that the *Amoebophrya* strains parasitizing dinoflagellates in Chesapeake Bay do not all belong to the same species. In spite of their relative dissimilarity, the sequences do group together into a single clade with high bootstrap support in phylogenetic trees constructed from the sequences.

Key Words. Alveolate, biological control, blooms, host specificity, parasite, PCR, red tides, rRNA, small subunit, Syndiniophyceae.

A WIDE variety of free-living dinoflagellates can be infected with organisms identified as the parasitic dinoflagellate *Amoebophrya ceratii* (Koeppen) Cachon. Such infected organisms have been found in the Baltic and Mediterranean Seas, the Atlantic and Pacific Oceans, the Tasman Sea, and the Chesapeake Bay (Cachon 1964; Cachon and Cachon 1987; Coats and Bockstahler 1994; DWC pers. observ.; Elbrächter 1973; Fritz and Nass 1992; Nishitani et al. 1985; Taylor 1968). Host dinoflagellates are killed as the parasite grows inside them, so epidemics can strongly influence the abundance of host species. Since some of the host species are responsible for red tides (Cachon 1964; Nishitani et al. 1985), the parasites might be useful for the biological control of these dinoflagellate blooms (Taylor and Pollingher 1988).

Coats et al. (1996) raised the possibility that "*Amoebophrya ceratii*" in Chesapeake Bay actually represents a species complex composed of morphologically similar parasites having narrow host ranges rather than a single species with a broad host range. They noted that *Amoebophrya* epidemics occurred in single host species at a time, even when other host species susceptible to infection by *Amoebophrya* were abundant. They also showed that while Chesapeake Bay populations of *Gymnodinium sanguineum* (= *Akashiwo sanguinea*), *Gyrodinium uncatenum, Ceratium furca,* and *Scrippsiella trochoidea* could all be found naturally infected with *Amoebophrya*, the *Amoebophrya* strain from *Akashiwo sanguinea* was not capable of infecting the other host species in the laboratory. It was shown by Janson et al. (2000) that an "*Amoebophrya ceratii*" strain from the Baltic Sea had a small-subunit rRNA sequence distinct from that of a Chesapeake Bay strain of *Amoebophrya* (Gunderson et al. 1999), supporting the contention that morphological uniformity in these parasites does not reflect genetic uniformity.

The role of *Amoebophrya* in the ecology of free-living dinoflagellate species cannot be studied effectively without determining the host range of the parasites, and such knowledge is also required for any future development of *Amoebophrya* as a biological control agent. We therefore amplified and sequenced the small-subunit rRNA (SSU rRNA) genes of *Amoebophrya* infecting several species of dinoflagellates in Chesapeake Bay in order to determine whether or not the sequences were identical. Significant differences would indicate the existence of a species complex.

MATERIALS AND METHODS

Source and growth of cells. Stock cultures of Chesapeake Bay isolates (DWC) of the photosynthetic dinoflagellates *Gymnodinium instriatum* (Freudenthal et Lee) Coats and *Karlodinium micrum* (Leadbeater and Dodge) Larsen were grown in f/2-Si medium (Guillard and Ryther, 1962) formulated using 15‰ Chesapeake Bay water supplemented with 5% (v/v) soil-water (GR+) (Starr and Zeikus 1993). Parasitic dinoflagellates infecting these hosts and conforming in morphology and life cycle to *Amoebophrya ceratii* (Koeppen) Cachon were maintained by transferring aliquots of infected host cultures to uninfected stocks at approximately three-day intervals. All cultures were maintained at 20 °C on a 14:10 light:dark cycle, with cool white fluorescent bulbs providing ~ 100 $\mu E\ m^{-2}\ s^{-1}$. Naturally infected *Scrippsiella* sp. cells were collected directly from Chesapeake Bay.

Isolation of DNA. Plankton samples containing infected *Scrippsiella* cells were collected from the Rhode River subestuary of Chesapeake Bay (38° 52′ N, 76° 32′ W) in August 1997. The cells were suspended in a solution of 4 M guanidium isothiocyanate and 0.1% beta mercaptoethanol to promote cell lysis and stabilize parasite DNA. Infected *Karlodinium micrum* cells were treated in the same way. Dinospores of the *Amoebophrya* strain infecting *Gymnodinium instriatum* were separated from host cells by size fractionation using an autoclaved Sterifil holder (Millipore, Bedford, MA) fitted with an 8 μm-pore Nuclepore filter. The < 8 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 then immmediately resuspended in the guanidium isothiocyanate/beta mercaptoethanol solution. Genomic DNA from all three samples 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. KARLAMO1R and SCRIPAMO1R are primers complementary to the sequence of the *Amoebophrya* strain parasitizing *Akashiwo sanguinea* which were successfully used to amplify DNA from the parasites of *K. micrum* and *Scrippsiella* sp., respectively. KARLAMO2F is complementary to the sequence of the *Amoebophrya* strain from *K. micrum*. SCRIPAMO2F and SCRIPAMO3F are complementary to the sequence of the *Amoebophrya* strain from *Scrippsiella* sp. EUK AA is a primer complementary to a region at the 5′ end of most SSU rRNA genes, 3FCL is a primer

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The GenBank accession numbers for the sequences reported here are AF472553 (*Amoebophrya* ex *Karlodinium micrum*), AF472554 (*Amoebophrya* ex *Gymnodinium instriatum*), and AF472555 (*Amoebophrya* ex *Scrippsiella* sp.).

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Table 1. Sequencing and amplification primers used to obtain small subunit rRNA sequences from *Amoebophrya* strains. The positions complementary to the *Amoebophrya*-specific primers in the full-length amplification products obtained from either the *Karlodinium micrum* or *Scrippsiella* sp. parasites are given in parentheses after the sequence.

	Amplification primers
EUK AA	GAATTCGTCGACAACCYGGTYGATCCTGCCA
EUK BSE	AGTTACTCTTCAGTGGCAGGTTCACCTAC
3FCL	TTAGGGAATTCGTCGACTCYGKTTGATCCYG
1400R	ACGGGCGGTGTGTRC
SCRIPAMO1R	AGCTTCATTCGATTATTAT (260–278)
SCRIPAMO2F	CAGAACCAATACCCTCGG (227–244)
SCRIPAMO3F	ACCTTACTTCGGTAAAGTAG (1360–1379)
KARLAMO1R	CAAGGCGAAAGCCTGCCGTG (784–803)
KARLAMO2F	CAGAACCAATGCTCCTTG (230–247)
	Sequencing primers
82F	GAAACTGCGAATGGCTC
AMOEB690F	AGAGGTGGAATTCT
1055F	GGTGGTGCATGGCCG
1200F	CAGGTCTGTGATGCTC
536R	GWATTACCGCGCGGKGCTG
570R	TTAAYATACGCTATTGG
1200R	GGGCATCACAGACCTG

having approximately the same target site, EUK BSE is complementary to a region at the 3′ end of most eukaryotic SSU rRNA genes, and 1400R is complementary to a conserved region found in all SSU rRNA genes. The sequences and positions of the amplification primers are given in Table 1.

The SSU rRNA coding region of the *Scrippsiella* parasite was obtained as three overlapping pieces. EUK AA and SCRIPAMO1R were used together to obtain the 5′ end of the gene, SCRIPAMO2F and 1400R were used together to obtain the middle segment, and SCRIPAMO3F was used with EUK BSE to obtain the 3′ end.

Thirty-five amplification cycles were used, each containing a 30 s denaturation step at 95 °C, a 1-min annealing step at 45 °C, and a 3-min extension at 73 °C. After the final cycle, the reaction mixture was incubated at 73 °C for 10 min. Each 100 μl reaction vol. contained 5 units of *Taq* DNA polymerase (Promega), 10 μl 10× buffer (Promega), 0.2 mM dNTPs (Promega), and 0.2 μM each primer.

The SSU rRNA coding region of the *Karlodinium micrum* parasite was obtained as two overlapping pieces. 3FCL and KARLAMO1R were used together to obtain the 5′ end of the gene. KARLAMO2F and EUK BSE were used together to obtain the 3′ end. The amplification conditions used were the same as those for the *Scrippsiella* parasite gene fragments, except that only one unit of *Taq* DNA polymerase was used in each reaction. The SSU rRNA coding region of the *Gymnodinium instriatum* parasite was obtained from purified dinospore DNA by using primers EUK AA and EUK BSE together in amplification reactions otherwise identical to those used with *Karlodinium micrum* parasite DNA. These reaction conditions were also used to obtain the dominant SSU rRNA gene sequences present in DNA extracted from the *Scrippsiella* sp. and *Karlodinium micrum* samples, except that EUK AA and 536R (targeting a sequence generally found in eukaryotic rRNA) were used.

PCR products were electrophoresed in 1% agarose gels and visualized by ethidium bromide staining. Amplified DNA was purified with a Wizard PCR Preps DNA Purification System according to the manufacturer's instructions.

DNA sequencing. A ThermoSequenase Radiolabeled Terminator Cycle Sequencing Kit (USB, Cleveland, OH) was used for direct sequencing of the PCR products following the manufacturer's instructions. The unlabelled nucleotide mix contained dITP. Primers listed by Elwood et al. (1985) and others given in Table 1 were used in sequencing reactions with 25 ng of amplified DNA. Thirty-five cycles were used in each sequencing reaction, with each cycle containing a 30 s denaturation step at 95 °C, a 30 s annealing step at 42 °C, a 1 min ramp to 60 °C, and a 7 min extension at 60 °C. Sequencing reactions were electrophoresed on 5% and 8% polyacrylamide gels, and sequencing ladders detected by exposure to Biomax MR film (Eastman Kodak, Rochester, NY).

Sequence analysis. The new *Amoebophrya* sequences were aligned with each other, with the *Amoebophrya* sequence obtained from *Dinophysis norvegica* (Janson et al. GenBank AF239260), and with sequences listed in Gunderson et al. (1999), where GenBank accession numbers are provided. We are here referring to the sequence deposited as *Gymnodinium sanguineum* (GenBank AF276818) as *Akashiwo sanguinea*, and *Gyrodinium impudicum* (GenBank AF022197) as *Gymnodinium impudicum*, in accordance with the taxonomic revision of Daugbjerg et al. (2000).

Similarity values were determined by comparing alignable positions in the sequences. Phylogenetic trees were constructed using PAUP* 4.0b10 (Swofford, 2002). Trees were built using maximum likelihood and parsimony algorithms. Parameters used for maximum likelihood trees were determined by using Modeltest 3.06 (Posada and Crandall 1998) and varied according to which sequences were used for building trees. Trees were drawn with TREEVIEW 1.6.5 (Page 1996).

RESULTS AND DISCUSSION

We attempted to amplify *Amoebophrya* sequences from *Scrippsiella* sp. and *Karlodinium micrum* host cells at a time when only a single *Amoebophrya* sequence was available (Gunderson et al. 1999). A series of primers complementary to regions that differed between the single *Amoebophrya* sequence and other dinoflagellate sequences was synthesized and used in these amplification reactions. Of these primers, only SCRIPAMO1R and KARLAMO1R yielded amplification products containing rRNA sequences.

After obtaining the 5′ end of the molecule, it was then possible to synthesize primers specific for the two *Amoebophrya* sequences (SCRIPAMO2F, SCRIPAMO3F, and KARLAMO2F) that could be used in conjunction with general eukaryotic amplification primers to amplify the remainder of the se-

quence. KARLAMO2F was used successfully with EUK BSE to generate the remainder of the sequence from the *K. micrum* parasite. SCRIPAMOF2 did not yield an amplification product with EUK BSE, but did produce one when used with 1400R. Another forward primer (SCRIPAMOF3) complementary to a section in the amplification product produced with SCRIPAMOF2 and 1400R was used with EUK BSE to generate the missing 3′ end. The reason for the failure of SCRIPAMO2F to work with EUK BSE is not known. The *Scrippsiella* amplification reactions did not work as well as the others, requiring a higher concentration of *Taq* DNA polymerase and yielding smaller quantites of DNA. The concentration of parasite DNA might have been much lower, it might have been degraded, or the DNA might be modified in a way that interferes with the activity of *Taq* DNA polymerase.

The three overlapping fragments of the SSU rRNA coding region together yielded a sequence 1,812 nucleotides long. EUK AA and SCRIPAMO1R produced a fragment extending from position 1 through 278 of the reconstructed gene. SCRIPAMO2F and 1400R produced a fragment extending from position 227 through position 1654. SCRIPAMO3F and EUK BSE produced a fragment extending from position 1360 through 1812. There was no indication from the sequencing ladders that more than one sequence was present in any of these three amplifications.

Sequences of the different amplification products matched exactly in the regions of overlap. Although the overlap in the 227–278 region is very short, it passes through a region where all the known *Amoebophrya* sequences differ from each other. Identity of the nucleotides in this region suggests that the two overlapping fragments are from the same gene. Secondary structure evidence supports this contention. The 5′ and middle fragments fall out in a secondary structure diagram into hybrid helical regions in regions in which they don't overlap. That is, one side of helices 3, 4, 5, 7, 8 and the base of E10_1 (following the numbering system of Wuyts et al. 2000) are contained in the 5′ fragment while the complementary side of these helices is contained in the middle fragment. They base-pair in a way that preserves the same secondary structure as seen in other *Amoebophrya* species. A comparison with other *Amoebophrya* sequences reveals that at positions in which these two fragments differ from other *Amebophrya* sequences, they have simultaneously changed in such a way as to maintain base-pairing.

The two overlapping fragments of the SSU rRNA coding region obtained from the *Amoebophrya* strain infecting *K. micrum* yielded a complete sequence 1,817 nucleotides long. 3FCL and KARLAMO1R produced a fragment extending from position 1 through 803 of the reconstructed gene. KARLAMO2F and EUK BSE produced a fragment extending from position 230 through 1817. The sequences matched each other exactly through the region of overlap.

A comparison of the primers SCRIPAMO1R and KARLAMO1R with their targets in the completed rRNA sequences revealed that neither matched their targets exactly. There were four mismatches between each of these primers and their target sequences. However, none of the mismatches was near the 3′ end of the primers and this allowed them to be successfully used in the amplifications.

Neither of the completed sequences obtained with the putative *Amoebophrya*-specific primers matched the sequences obtained by amplifying the same DNA samples with the general eukaryotic primers EUK AA and 536R. The two short sequences obtained using these primer pairs appeared among free-living dinoflagellate sequences in phylogenetic trees constructed using PAUP* (results not shown) and represent the host sequences.

Table 2. Similarity between the SSU rRNA sequences of a series of dinflagellates. Similarity values are given in percentages. They were obtained through comparison of the alignable part of the sequences (1525 nucleotides). The *Amoebophrya* sequences are less similar to each other than are some sequences from dinoflagellates assigned to separate genera.

Amoebophrya ex *K. micrum*: *Amoebophrya* ex *Scrippsiella* sp.	93
Amoebophrya ex *K. micrum*: *Amoebophrya* ex *G. instriatum*	94
Amoebophrya ex *K. micrum*: *Amoebophrya* ex *A. sanguinea*	90
Amoebophrya ex *K. micrum*: *Amoebophrya* ex *D. norvegica*	92
Amoebophrya ex *Scrippsiella* sp.: *Amoebophrya* ex *G. instriatum*	96
Amoebophrya ex *Scrippsiella* sp.: *Amoebophrya* ex *A. sanguinea*	91
Amoebophrya ex *Scrippsiella* sp.: *Amoebophrya* ex *D. norvegica*	93
Amoebophrya ex *G. instriatum*: *Amoebophrya* ex *A. sanguinea*	92
Amoebophrya ex *G. instriatum*: *Amoebophrya* ex *D. norvegica*	94
Amoebophrya ex *A. sanguinea*: *Amoebophrya* ex *D. norvegica*	94
Scrippsiella nutricula: *Gymnodinium catenatum*	97
Scrippsiella nutricula: *Gymnodinium mikimotoi*	98
Scrippsiella nutricula: *Heterocapsa triquetra*	98
Scrippsiella nutricula: *Lepidodinium viride*	98

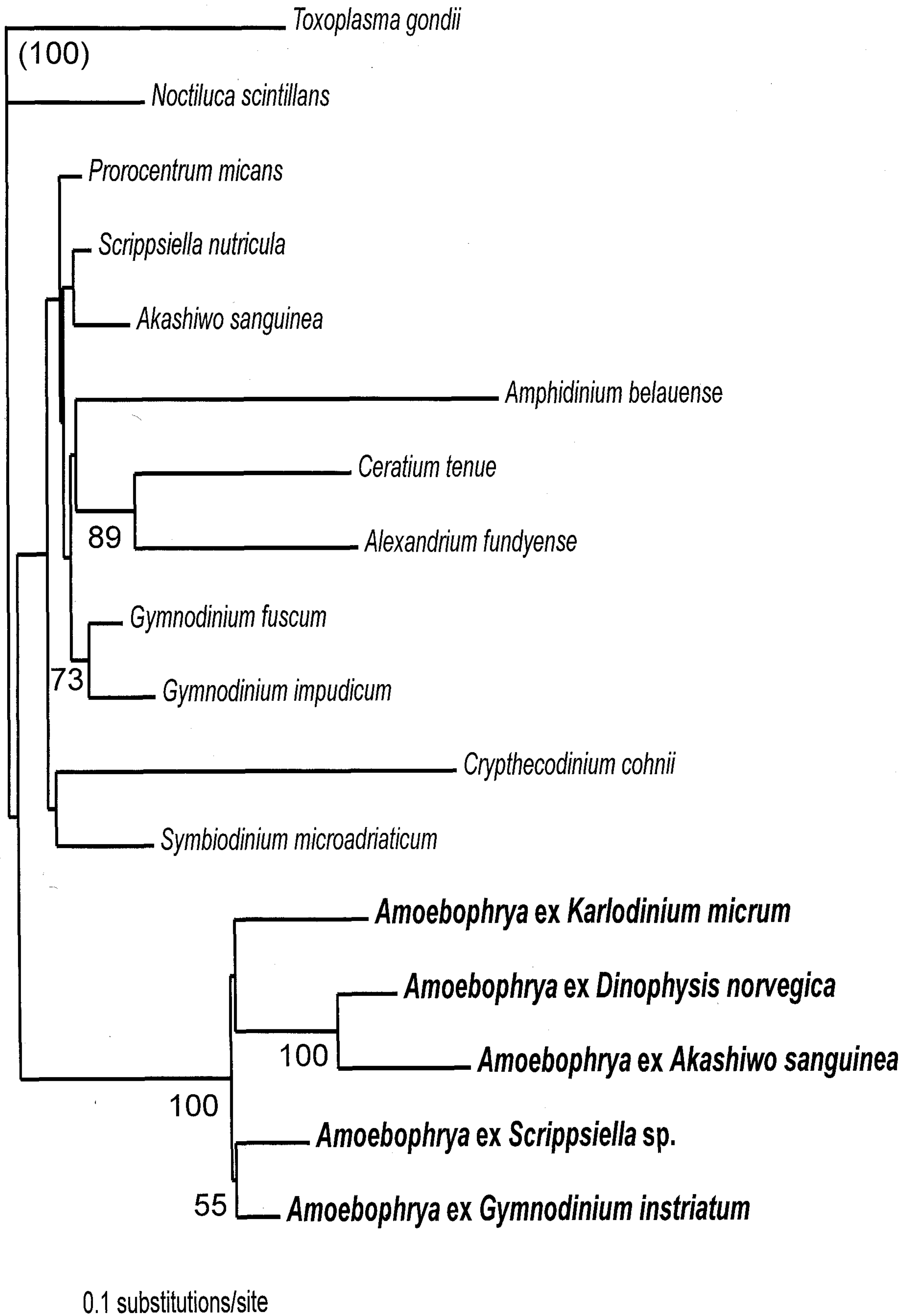
This provides further evidence that the *Amoebophrya* primers were amplifying parasite rather than host DNA.

The purified dinospore DNA from the *Amoebophrya* strain parasitizing *Gymnodinium instriatum* yielded an amplification product 1,816 nucleotides long. Indels in hypervariable regions of SSU rRNA accounted for the slight length differences among the three *Amoebophrya* sequences.

The four Chesapeake Bay *Amoebophrya* sequences differ from each other to a degree incompatible with their assignation to a single species. The sequences differ by 4–10% over those positions that can reliably be aligned (Table 2). It is easy to find examples of sequences coming from representatives of different genera that differ by less, and a few of these are listed in Table 2. The Chesapeake Bay strains of *Amoebophrya* are not more similar to each other (with the exception of the parasites from *Scrippsiella* and *G. instriatum*) than they are to the strain infecting *D. norvegica* in the Baltic Sea.

In spite of the great degree of sequence difference between them, trees constructed with parsimony or maximum likelihood programs in PAUP* placed these *Amoebophrya* sequences into a monophyletic group with a high level of bootstrap support. Figure 1 is an example of such a maximum likelihood tree. It represents the best tree found (score = 7592.14014) in ten heuristic searches through the same aligned sequence set (1,525 positions, alignment available on request) using random addition of sequences and TBR (tree-bisection-reconnection) branch-swapping. *Toxoplasma gondii* was used as an outgroup. Modeltest selected a GTR + Γ + I model as the best one for the data set. T to C and C to T changes were assigned a rate of 6.2143, A to G and G to A changes were assigned a rate of 3.3286 and all other changes were assigned a rate of 1 in the rate matrix used. The shape parameter for the gamma distribution was 0.7803. There were four rate categories, with each category rate represented by the mean in that category. The proportion of invariable sites was 0.3915, and the nucleotide frequencies were: A = 0.27730, C = 0.18010, G = 0.24910 and T = 0.29350.

After obtaining the tree in Fig. 1, 500 bootstrap replications were performed on the data set and maximum likelihood trees constructed with the same parameter settings as before. The percentage of times a group in the optimum tree appeared in



Toxoplasma gondii
(100)
Noctiluca scintillans
Prorocentrum micans
Scrippsiella nutricula
Akashiwo sanguinea
Amphidinium belauense
Ceratium tenue
89
Alexandrium fundyense
Gymnodinium fuscum
73
Gymnodinium impudicum
Crypthecodinium cohnii
Symbiodinium microadriaticum
Amoebophrya ex Karlodinium micrum
Amoebophrya ex Dinophysis norvegica
100
Amoebophrya ex Akashiwo sanguinea
100
Amoebophrya ex Scrippsiella sp.
55
Amoebophrya ex Gymnodinium instriatum
0.1 substitutions/site

Table **3**. Conserved sequence positions at which *Amoebophrya* sequences carry a different nucleotide than sequences from other dinoflagellates. The sequence given is that of the *Ameobophrya* strain infecting *Akashiwo sanguinea* (Gunderson et al. 1999). The base unique to all *Amoebophrya* strains is shown in bold and underlined. The base used by other dinoflagellates at that position is given in parentheses. In some cases more than one base is used by other dinoflagellates; then the base given in parentheses is that found in the majority of dinoflagellates. Since the sequences were determined from amplified DNA products, they are presented as DNA rather than RNA sequences. The locations are given in accordance with the secondary structure model of Wuyts et al. (2000).

The 3′ half of helix 11:	
CCGGGG**C**T	(A)
The loop at the end of helix 14:	
C**C**GAGAAACG	(T)
The 5′ half of helix 17:	
CAA**A**AGAGGTCC	(T)
The region between helices 18 and 19:	
GA**C**TATCAAT	(G)
Part of helix E23-11 and the region between helices E23-10 and E23-11:	
TT**CACG**GCAGGC	(TCAA; *Noctiluca scintillans* has TCAG)
The 3′ half of helix E23-14:	
AGGGCTAGG**A**	(G)
The loop at the end of helix 25:	
GTG**G**AA	(A)
The 5′ half of helix 27:	
GT**A**AGGGGATCGAAGACG	(T)
The loop at the end of helix 27:	
AT**T**AGATAC	(C)
The 3′ half of helix 27:	
CGTC**G**TAGTCTTTAC	(C, except G in *Noctiluca scintillans*)
The 3′ half of helix 29:	
GACTCGCTT**C**G	(A)
Helix 41 and its terminal loop:	
CTG**C**TTAATT**G**CG	(G, C)
Helix 48 and its terminal loop:	
G**TG**GCGCATCATCAGTGCGT**CG**C	(GC, GC)
The 5′ side of helix 49, proximal end:	
TCCTACCGATTGAG**CA**TT	(TG)

the set of bootstrapped trees is indicated by a number next to the branches, if the value is greater than 50%.

Figure 1 shows that an *Amoebophrya* clade was present in all the bootstrapped trees. The bootstrapped trees often contained a clade including *Noctiluca* as a sister taxon of the *Amoebophrya* strains, although this was not true of the optimal tree. Dinoflagellate relationships are not well resolved by SSU rRNA sequences (Gunderson et al. 1999; Litaker et al. 1999; Saldarriaga et al. 2001; Saunders et al. 1997), and our purpose in presenting a tree is not to provide a foundation for discussing these relationships. We only wish to show that in spite of the differences between the *Amoebophrya* sequences, treeing algorithms consistently place them all into a single group. The tree also demonstrates that the putative *Scrippsiella* parasite sequence, whose identity was not established through in situ hybridization, is an *Amoebophrya* sequence (the *Scrippsiella* cells in the original sample were known to contain *Amoebophrya* through microscopical observation).

It is possible to find a number of sequence elements that all known *Amoebophrya* sequences share which are not present in sequences from other dinoflagellates (Table 3). Those signature elements that are flanked by conserved regions might prove useful in creating primers for the specific amplification of *Amoebophrya* sequences from mixtures of host and parasite DNA. Such primers are necessary since most *Amoebophrya* strains cannot yet be maintained in the laboratory. Their sequences have to be obtained from plankton samples containing the host species harboring them and perhaps other dinoflagellate species as well. These unique regions may also be useful for screening plankton samples for *Amoebophrya* using in situ hybridization.

The discovery that a Baltic Sea "*Amoebophrya ceratii*" strain had a very different sequence than a Chesapeake Bay strain (Janson 2000) raised the question of whether the difference was due to geographic separation or host difference. It is possible that one or a few closely related parasite strains exist

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Fig. **1**. Maximum likelihood tree illustrating the relationship between *Amoebophrya* species and other dinoflagellates. The *Amoebophrya* sequences always group together near the base of the tree. Details of tree construction are given in the text. Bootstrap values are given in percentages at the nodes, if they are above 50%. The bar at the bottom indicates substitutions/site.

in a particular geographical area, infecting all the susceptible dinoflagellates there. This is apparently not the case. The Chesapeake Bay strains are quite different from each other and, in general, no more similar to each other than they are to the Baltic Sea strain. The greatest sequence difference seen in pairwise comparisons was the difference found between two of the Chesapeake Bay strains. The rRNA sequence evidence indicates that "*Amoebophrya ceratii*", even within the confines of Chesapeake Bay, is a collection of species.

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