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Genetic Diversity of Parasitic Dinoflagellates in the Genus *Amoebophrya* and Its Relationship to Parasite Biology and Biogeography

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ABSTRACT. We determined 18S rRNA gene sequences of *Amoebophrya* strains infecting the thecate dinoflagellates *Alexandrium affine* and *Gonyaulax polygramma* from Korean coastal waters and compared those data with previously reported sequences of *Amoebophrya* from cultures, infected cells concentrated from field samples, and environmental 18S rRNA gene sequences obtained from a variety of marine environments. Further, we used these data to examine genetic diversity in *Amoebophrya* strains relative to geographic origin, host phylogeny, site of infection, and host specificity. In our analyses of known dinoflagellate taxa, the 13 available *Amoebophrya* sequences clustered together within the dinoflagellates as three groups forming a monophyletic group with high bootstrap support (maximum likelihood, ML: 100%) or a posterior probability (PP) of 1. When the *Amoebophrya* sequences were analyzed along with environmental sequences associated with Marine Alveolate Group II, nine subgroups formed a monophyletic group with high bootstrap support (ML: 100%) and PP of 1. Sequences known to be from *Amoebophrya* spp. infecting dinoflagellate hosts were distributed in seven of those subgroups. Despite differences in host species and geographic origin (Korea, United States, and Europe), *Amoebophrya* strains (Group II) from *Gymnodinium instriatum*, *A. affine*, *Ceratium tripos* (AY208892), *Prorocentrum micans*, and *Ceratium lineatum* grouped together by all of our tree construction methods, even after adding the environmental sequences. By contrast, strains within Groups I and III divided into several lineages following inclusion of environmental sequences. While *Amoebophrya* strains within Group II mostly developed within the host cytoplasm, strains in Groups I and III formed infections inside the host nucleus, a trait that appeared across several of the subgroups. Host specificity varied from moderately to extremely species-specific within groups, including Group II. Taken together, our results imply that genetic diversity in *Amoebophrya* strains does not always reflect parasite biology or biogeography.

Key Words. *Alexandrium affine*, *Amoebophrya*, *Gonyaulax polygramma*, host specificity, molecular phylogeny, parasite, 18S rRNA gene.

DINOFLAGELLATES previously assigned to the species *Amoebophrya ceratii* are endoparasites that infect several free-living dinoflagellates, including toxin-producing and harmful algal bloom species (Cachon 1964; Coats 1999; Park, Yih, and Coats 2004; Taylor 1968). Cachon (1964) reported the first detailed description of the morphology and life cycle of the parasite while studying *Amoebophrya* infections in a number of dinoflagellate hosts in the Mediterranean Sea. He also recognized the possibility that organisms classified as *Amoebophrya ceratii* might represent more than one species, noting conspicuous developmental differences in parasites among host species, different sites of infection inside hosts, and considerable variation in the morphology of the infective, dispersal ''dinospore'' stage. From field observations and laboratory experiments, Coats et al. (1996) argued that *A. ceratii* was a species complex composed of several host-specific parasites. In addition, Coats and Park (2002) reported that *Amoebophrya* strains from *Akashiwo sanguinea*, *Gymnodinium instriatum*, and *Karlodinium veneficum* had a high degree of host specificity, along with marked biological differences (e.g. parasite generation time, dinospore survival, and infectivity) among the strains. More recently, Kim et al. (2004a) showed that *Amoebophrya* strains from the thecate dinoflagellates *Alexandrium affine* and *Gonyaulax polygramma* parasitized different sites within their hosts (i.e. cytoplasm and nucleus, respectively) and had faster generation times than previously reported for athecate hosts. Subsequently, Kim (2006) showed that the two strains of *Amoebophrya* infecting *A. affine* and *G. polygramma* lacked host specificity, thereby revealing that the *A. ceratii* complex includes non-host-specific to extremely host-specific species. She also noted that host specificity was more pronounced in *Amoebophrya* infecting athecate dinoflagellates. These observations, along with molecular studies showing considerable genetic divergence in 18S rRNA gene sequences among several *Amoebophrya* strains (Gunderson, Goss, and Coats 1999, 2000; Gunderson et al. 2002; Janson et al. 2000; Salomon, Janson, and Granéli 2003), support the species complex hypothesis of Coats et al. (1996).

In this study, we sequenced the 18S rRNA genes of *Amoebophrya* strains infecting *A. affine* and *G. polygramma* from Korean coastal waters and compared those data with previously reported sequences for *Amoebophrya* strains from other dinoflagellate hosts. We then incorporated into our analysis environmental 18S rRNA gene sequences associated with the *Amoebophrya* group reported from a variety of marine environments. We used these comparisons to assess whether genetic diversity in *Amoebophrya* strains reflects geographic origin, host phylogeny, site of infection, and host specificity of parasite strains.

MATERIALS AND METHODS

Source and culture of cells. Stock cultures of the thecate dinoflagellates *A. affine* and *G. polygramma* were grown in f/2-Si medium (Guillard and Ryther 1962) formulated using 30 psu (practical salinity unit) Korean coastal seawater. Two strains of *Amoebophrya*, one each in the host species *A. affine* and *G. polygramma*, were established in cultures by adding a single infected host cell from field samples to cultures of complementary host species (Kim et al. 2004a). Parasites were subsequently propagated by transferring aliquots of infected host cultures to uninfected host stocks at approximately 2–3 day intervals. All cultures were maintained at 20 °C on a 14:10 h light:dark cycle under cool white fluorescent light at 50 μmol photons/m^2/s.

DNA extraction, polymerase chain reaction (PCR) amplification, cloning, and sequencing. *Alexandrium affine* and *G. polygramma* host cells were harvested from exponentially

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Table 1. Primers used for PCR amplification and sequencing in this study.

Primer[a]	Sequence (5′ → 3′)
18S-0009f[b]	GATCCTGCCAGTAGTCATAT
18S-1797r[b]	GATCCTTCYGCAGGTTCACCTAC
18S-0302f[c]	AGTTTCTGACCTATCAG
18S-0437r[c]	GCGCCTGCTGCCTTCCTTA
18S-0613f[c]	GCGGTTAAAAAGCTCGTAGT
18S-0897f[c]	AGAGGTGAAATTCTTGGAT
18S-1179f[c]	CTTAATTTGACTCAACACG
18S-1435f[c]	AACAGGTCTGTGATGCCCTT

[a]Primer nomenclature corresponds to *Prorocentrum micans* SSU rDNA position (Herzog and Maroteaux, 1986); ''f'' and ''r'' represent forward and reverse primers, respectively.
[b]Primers for PCR amplification and sequencing.
[c]Primers for sequencing.
PCR, polymerase chain reaction.

Table 2. Information of isolates sequenced in this study and the GenBank accession numbers for their 18S rRNA gene sequences.

Species	Sampling site	Sampling date	Accession number
Alexandrium affine	Jinhae Bay, Korea	October 2002	AY775286
Gonyaulax polygramma	Coastal water near Gunsan, Korea	September 2002	AY775287
Amoebophrya sp.[a]	Jinhae Bay, Korea	October 2002	AY775284
Amoebophrya sp.[b]	Coastal water near Gunsan, Korea	September 2002	AY775285

[a]The parasite *Amoebophrya* sp. infecting *Alexandrium affine*.
[b]The parasite *Amoebophrya* sp. infecting *Gonyaulax polygramma*.

growing cultures, and their genomic DNAs were extracted using a slightly modified LiCl method (Hong et al. 1995). For PCR amplification of the two *Amoebophrya* strains, vermiforms (i.e. the parasite stage immediately after emergence from the host) were individually captured using a glass micropipette and transferred directly to a PCR tube.

Amplification of 18S rRNA genes was performed using PCR protocols with eukaryote-specific primers 18S-0009f and 18S-1797r (Kim et al. 2004b; Table 1). The 50 µl reactions contained 1 × *Ex Taq*™ buffer, 250 µM of each dNTP, 1 µl of genomic DNA (10 ng/µl) or single cells, each primer at a concentration of 0.2 µM, and 1.25 unit of *TaKaRa Ex Taq*™ (TaKaRa, Shiga, Japan). Polymerase chain reaction amplification of eukaryotic 18S rDNA was conducted according to the following cycle parameters: an initial denaturation step (3 min, 94 °C) was followed by 35 cycles consisting of denaturation (30 s, 94 °C), annealing (1 min, 55 °C), and extension (1 min, at 72 °C) with a final 7-min extension step at 72 °C. The size of PCR products was approximately 1.8 kb for *A. affine*, *G. polygramma*, and the two *Amoebophrya* strains. The appropriate PCR bands were excised and purified using a QIAquick™ Gel Extraction Kit (Qiagen, Hilden, Germany).

Polymerase chain reaction products of *A. affine* and *G. polygramma* were directly sequenced using various eukaryotic sequencing primers. The PCR products of each *Amoebophrya* strain were ligated into pCR®2.1 vector, which was used to transform *Escherichia coli* (INVαF′) with an Original TA Cloning® Kit (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. After the color-based selection of transformants using X-gal, four positive clones were cultured, and their plasmid DNAs were extracted and purified using a Quantum Prep® Plasmid Miniprep Kit (Bio-Rad, Hercules, CA). When using cloned fragments, the four clones were sequenced using the various sequencing primers (Table 1) to detect and clarify possible ambiguities. Cloned fragments generated an identical 18S rRNA sequence from each parasite strain. A cycle-sequencing reaction was performed with the PCR primer set and internal sequencing primers (Table 1) using an ABI Prism BigDye™ Terminator v3.0 Cycle Sequencing Kit (Perkin-Elmer, Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The sequencing reaction was run on an ABI 3100 Sequencer (Perkin-Elmer). Sequence data were deposited in GenBank (AY775284 to AY775287, Table 2).

Phylogenetic analyses. The 18S rRNA gene sequences obtained from the cultures were compared to the sequences of related taxa obtained from the GenBank database using a BLASTN search. The sequences were manually aligned using the 18S rRNA secondary structure (Van de Peer et al. 2000). A total of 1,521 unambiguously aligned sites were retained for phylogenetic analysis of dinoflagellates. Only homologous positions in the 18S rRNA gene sequences were used for all the phylogenetic analyses. Apicomplexa (*Eimeria nieschulzi* and *Toxoplasma gondii*) were used as an outgroup. We also constructed a dataset including several taxa and phylotypes in the order Syndiniales with a significantly larger portion of the 18S rRNA gene sequences (1,625 sites). These alignments are available on request. Phylogenetic trees were inferred by the maximum likelihood (ML) (Felsenstein 1985) method using PAUP*4b10 (Swofford 2002) and by Bayesian analysis using MrBAYES 3.0 (Huelsenbeck and Ronquist 2001). Modeltest version 3.04 (Posada and Crandall 1998) was used to select the GTR (i.e. general-time reversible)+gamma+I model for analyses of 52 alveolate taxa representing the Apicomplexa, Syndiniales, and Dinoflagellata and the TIM (i.e. transition model)+gamma+I model for in-group analyses of taxa belonging to the alveolate order Syndiniales. Parameter values for the likelihood analysis were estimated from a test tree obtained using PAUP*. For each ML analysis, the best tree was found using 20 random additions and tree bisection-reconstruction (TBR) branch-swapping, and a 200-replicate bootstrap analysis was performed (neighbor-joining starting trees, then TBR). To estimate Bayesian posterior probabilities, four simultaneous Markov Chain Monte Carlo (MCMC) chains were run for 1,000,000 generations and sampled every 500 generations (burn-in 200,000 generations).

GenBank accession numbers. GenBank sequences included in the analyses of shown in Fig. 1 were as follows: AY831408, AJ535375, AF033869, AF022156, AJ276699, AF022154, AF022155, AJ833631, AB036837, AF225965, AF099183, AY421786, L13716, AF080098, AF080097, AF080096, AF022200, M64245, AF022199, AB073119, AB073117, AJ506974, AF239261, AF033865, AF022198, Y16238, M14649, AF172712, AF231805, U41085, U52357, L13719, AF022201, AF172714, AF172713, AF472553, AY208894, AY260469, AY775285, AF069516, AF239260, AF472554, AY775284, AY208892, AY208893, AY260467, AF472555, AY260468, AF286023, AF126013, U40263 and X65508 (outgroup taxa).

Prior reports show that some members of *Duboscquella* belong to the Marine Alveolate Group I, while *Amoeophyra*, *Hematodinium*, and *Syndinium* belong to the Marine Alveolate Group II (Harada, Ohtsuka, and Horiguchi 2007; Skovgaard et al. 2005). In the present study, we aimed to examine the phylogenetic relationship between *Amoebophyra* strains and *Amoebophyra*-like sequences from environmental samples within Marine Alveolate Group II. To do this, the following Genbank sequences were included in the analyses of the *Amoebophyra* group (i.e. in Fig. 7): DQ145107, EF173016, AY208894, AY129043, AJ402338,

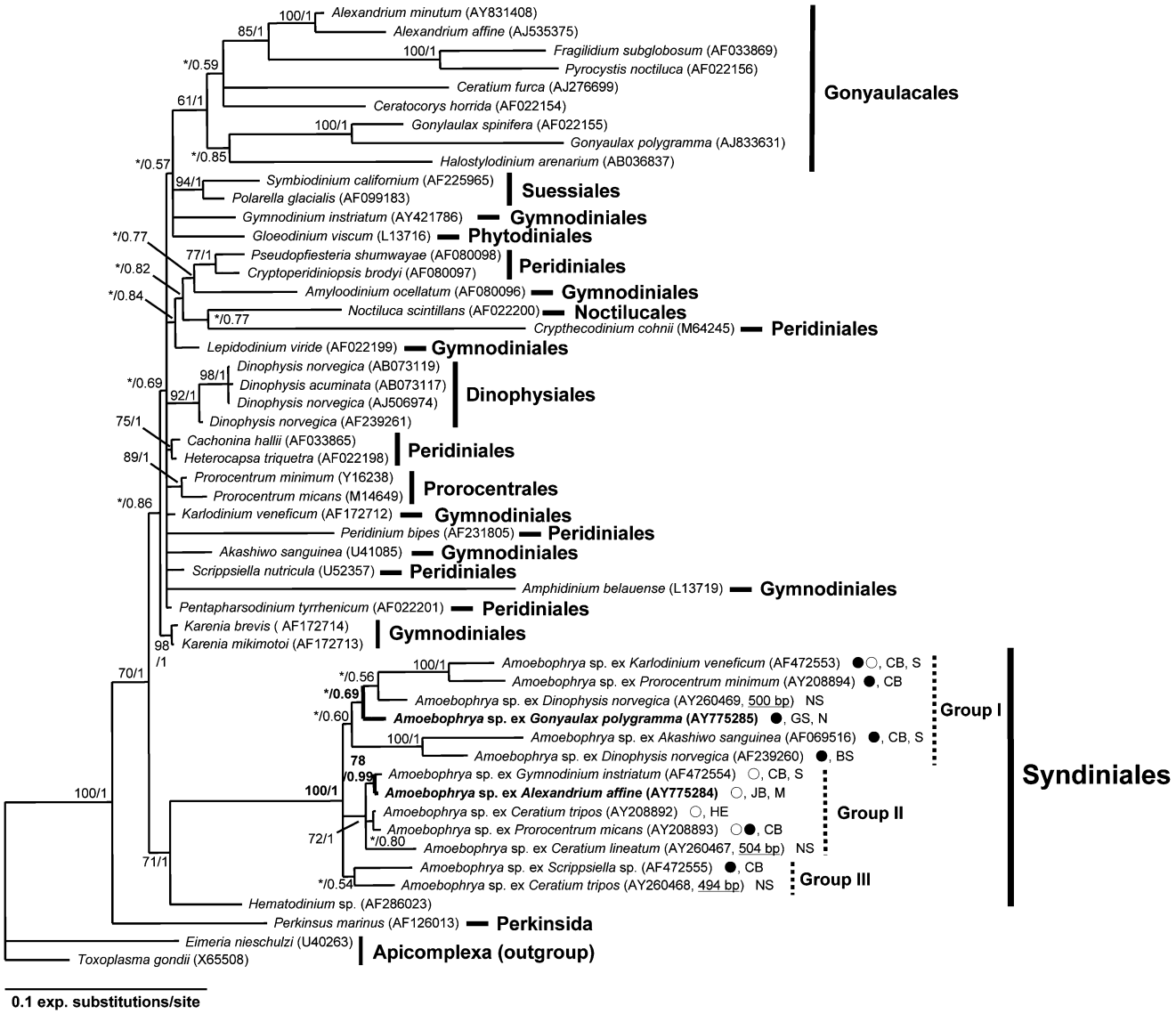

Fig. **1.** An 18S rRNA gene tree showing the phylogenetic position of *Amoebophrya* strains and other representative dinoflagellates using GTR (i.e. general-time reversible)+gamma+I model. The apicomplexa *Eimeria nieschulzi* and *Toxoplasma gondii* were used as outgroup taxa. Bootstrap values from maximum likelihood (ML; 200 replicates) and Bayesian posterior probability (PP) are indicated at nodes (presented in order ML/PP). Accession numbers of each taxon are presented in parenthesis. *Bootstrap value of $<50\%$ in ML analysis. The two strains analyzed in this study are in bold. Origins of the *Amoebophrya* strains are indicated in abbreviation as follows: CB, Chesapeake Bay; NS, North Sea; KS, Gunsan of Korea; JB, Jinhae Bay of Korea; BS, Baltic Sea; HE, Helsingor of Denmark. Site of infection: ○, cytoplasmic infections; ●, nuclear infections. Information of site of infection was derived from Park et al. (2004) and this study. S, M, and N represent extremely host species-specific, moderately host species-specific, and non-specific in host specificity, respectively. Data on host specificity of *Amoebophrya* strains were derived from Coats and Park (2002) and Kim (2006).

EF172965, AY295692, AY295731, DQ186526, AY129031, AF472553, AF290077, AY260468, DQ186527, AY129051, AY260467, AY129046, AY129054, AY208892, AY208893, AF472554, AY775284, DQ145110, AY295588, AJ402326, AY129038, AF239260, EF172968, AY295500, AY295714, AF069516, DQ186528, DQ186531, AY295373, AY937888, EF173006, AF472555, AY129040, AY260469, AY295690, AY775285, AJ402330, AF290068, AF286023 (outgroup taxon).

Protargol staining. Bouin's-preserved field samples that were collected from Chesapeake Bay and the Kattegat near Helsingør, Denmark, simultaneously with material used for *Amoebophrya* gene sequences, available as Genbank accession numbers AY208892 (*Ceratium tripos*), AF472555 (*Scrippsiella* sp.), AY208894 (*P. minimum*), and AY208893 (*Prorocentrum micans*), were stained using the quantitative protargol technique (Montagnes and Lynn 1993) to characterize the site of infection within host cells.

RESULTS

Molecular sequencing. The 18S rRNA gene sequences of *Amoebophrya* strains from *A. affine* and *G. polygramma* clustered together with the eleven previously reported sequences for *Amoebophrya* strains (Fig. 1). All phylogenetic trees clearly showed that our two strains of *Amoebophrya* are members of the order Syndiniales with high bootstrap value (ML: 100%) or posterior

probability (PP) 1. While the Gymnodiniales and Peridiniales formed paraphyletic or polyphyletic groups, the order Syndiniales with *Hematodinium* sp. placed at the base of *Amoebophrya* group, formed a monophyletic group with high bootstrap support (ML: 71%) or a PP of 1.

The *Amoebophrya* strains appeared to sort into three groups. Group I contained *Amoebophrya* strains infecting *K. veneficum*, *P. minimum*, *D. norvegica*, *G. polygramma*, and *A. sanguinea*. Group II included *Amoebophrya* spp. from *G. instriatum*, *A. affine*, *C. tripos* (AY208892), *P. micans*, and *Ceratium lineatum* and Group III included *Amoebophrya* sp. ex *Scrippsiella* sp. and *Amoebophrya* sp. ex *C. tripos* (AY260468). Group II was supported by high bootstrap support (ML: 72%) or PP 1, but nodes for the other two groups had relatively low bootstrap values and PP. Group II had relatively short branches compared to the other groups.

Biogeography, site of infection, and host specificity. The phylogeny of *Amoebophrya* strains did not appear to be linked with either the phylogeny of their dinoflagellate hosts or geographic origin (Fig. 1). For example, the *Amoebophrya* strain infecting *P. minimum* belonging to Group I was more strongly related to the *Amoebophrya* strain infecting *K. veneficum* (Group I) than the *Amoebophrya* strain infecting *P. micans* (Group II). Further, *Amoebophrya* strains infecting the same host species (i.e. *C. tripos*) grouped to Groups II and III. In addition, the five *Amoebophrya* strains forming the well-supported Group II originated from distant geographic regions: *Amoebophrya* spp. ex *G. instriatum* and ex *P. micans* originated from Chesapeake Bay, USA, *Amoebophrya* sp. ex *A. affine* from Jinhae Bay, Korea, *Amoebophrya* sp. ex *C. tripos* from Helsingør, Denmark, and *Amoebophrya* sp. ex *C. lineatum* from North Sea.

Cytological stains of field samples obtained from Chesapeake Bay and Helsingør, Denmark revealed that *Amoebophrya* infections developed inside the nucleus of *Scrippsiella* sp. and *P. minimum* (Fig. 2, 3), but within the cytoplasm of *C. tripos* (Fig. 4). In *P. micans* (Fig. 5, 6), most infections occurred in the nucleus, but cytoplasmic infections were observed in a few instances. Overall, *Amoebophrya* strains within Group II tend to develop within the host cytoplasm, whereas those within the other groups usually form infections inside the host nucleus (Fig. 1).

There is at present insufficient data on host specificity of *Amoebophrya* strains to carefully evaluate its relationship to parasite diversity. Nonetheless, two of the three *Amoebophrya* groups include strains with high host specificity (ex *K. veneficum* and ex *A. sanguinea* Group I; ex *G. instriatum* Group II), as well as moderately, or non-host-specific strains (ex *G. polygramma* Group I; ex *A. affine* Group II) (Fig. 1). Thus, host specificity, or lack thereof, appears not to sort across *Amoebophrya* groups.

Phylogeny of in-group including environmental sequences. Many environmental sequences obtained from a variety of marine ecosystems clustered together with *Amoebophrya* strains forming a monophyletic group with high bootstrap support (ML: 100%) or high PP of 1 (Fig. 7). This group was composed of nine-assemblage (subgroups), although some subgroups (7–9) had weak bootstrap support and low PP. Sequences from *Amoebophrya* spp. known to infect dinoflagellate hosts were present in seven subgroups. Subgroup 3 contained the largest number of *Amoebophrya* sequences from cultures and infected cells concentrated from field samples (five of 13 sequences; i.e. *Amoebophrya* strains infecting *C. tripos* (AY208892), *P. micans*, *C. lineatum*, *G. instriatum*, and *A. affine*), whereas Subgroup 5 contained only environmental sequences. Even after adding environmental sequences, *Amoebophrya* strains that grouped together within Group II in Fig. 1 still clustered together, whereas strains within Groups I and III were divided into several lineages.

When we included either additional parasite taxa (i.e. *Duboscquella* and *Syndinium*) and 20 environmental sequences within the Marine Alveolate Group I, as in previous report (Dolven et al. 2007; Harada, Ohtsuka, and Horiguchi 2007), or used a variety of outgroup taxa from the other dinoflagellate groups (i.e. *Alexandrium minutum* (AY831408), *Ceratium furca* (AJ276699), *Noctiluca scintillans* (AF022200), *Scrippsiella nutricula* (U52357), and *Symbiodinium californium* (AF225965)) in our phylogenetic analysis, the topological position of *Amoebophrya* sequences studied here did not substantially change in the nine-assemblage clade (data not shown).

DISCUSSION

Parasitic dinoflagellates of the genus *Amoebophrya* have been reported from over 40 different free-living dinoflagellates inhabiting coastal waters of the world (Park et al. 2004). All but one of these parasites were historically classified as *Amoebophrya ceratii*, the single exception being *A. leptodisci* from the heterotrophic dinoflagellate *Pratjetella medusoides* (Cachon 1964). Recently, however, ecological, physiological, and molecular investigations of *Amoebophrya*–host associations have revealed that *A. ceratii* represents a species complex (Coats and Park 2002; Coats et al. 1996; Gunderson et al. 2000, 2002; Janson et al. 2000; Salomon et al. 2003). Within the same time frame, pioneering studies by Díez, Pedros-Alió, and Massana (2001), López-García et al. (2001), and Moon-van der Staay, De Wachter, and Vaulot (2001), along with other reports (e.g. Groisillier et al. 2006; Not et al. 2007; Worden 2006), have revealed tremendous eukaryotic molecular diversity for environmental samples of the pico- and nanoplankton size fractions from coastal to oceanic environments and from surface waters to the deep ocean. Phylogenetic analyses of environmental 18S rRNA gene sequences have also demonstrated that ''*Amoebophrya*-like'' organisms are abundant in coastal and oceanic ecosystems (Groisillier et al. 2006; Not et al. 2007). Our molecular analysis of *Amoebophrya* from *A. affine* and *G. polygramma* brings the number of SSU rDNA sequences available for *Amoebophrya* strains from cultures and known host species concentrated from field samples to 13, providing a sufficient database for examining genetic diversity among parasite strains, as well as their relationships to parasite biology, biogeography, and environmental sequences.

Phylogenetic trees based on host and parasite 18S rRNA gene sequences placed all 13 *Amoebophrya* sequences as a monophyletic group having 100% bootstrap support within the order Syndiniales. By comparison, members of other orders (e.g. Gymnodiniales and Peridiniales) formed paraphyletic or polyphyletic groups, suggesting that *Amoebophrya* species have congener sequences that are not shared with other dinoflagellates. More importantly, some *Amoebophrya* strains, here designated as Group II, always grouped together by all of our tree construction methods, even after adding the environmental sequences, despite differences in host species (i.e. from *G. instriatum*, *A. affine*, *C. tripos* [AY208892], *P. micans*, and *C. lineatum*) and geographic origin (Korea, United States, and Europe).

Amoebophrya infections typically develop either within the host cytoplasm, or inside the host nucleus, depending on the particular dinoflagellate species being parasitized. Exceptions to this pattern are *C. tripos*, *K. veneficum*, and *P. micans* for which both cytoplasmic and nuclear infections have been reported (Coats and Park 2002; Koeppen 1899; this study). Nuclear infections are uncommon in *Amoebophrya* ex *K. veneficum* and usually accompany multiple infection of host cells (M.G.P. & D.W.C., pers. observ.). In that parasite–host system, differences in site of infection presumably reflect within species variation, as observations were derived from a host-parasite culture initiated using a single infected

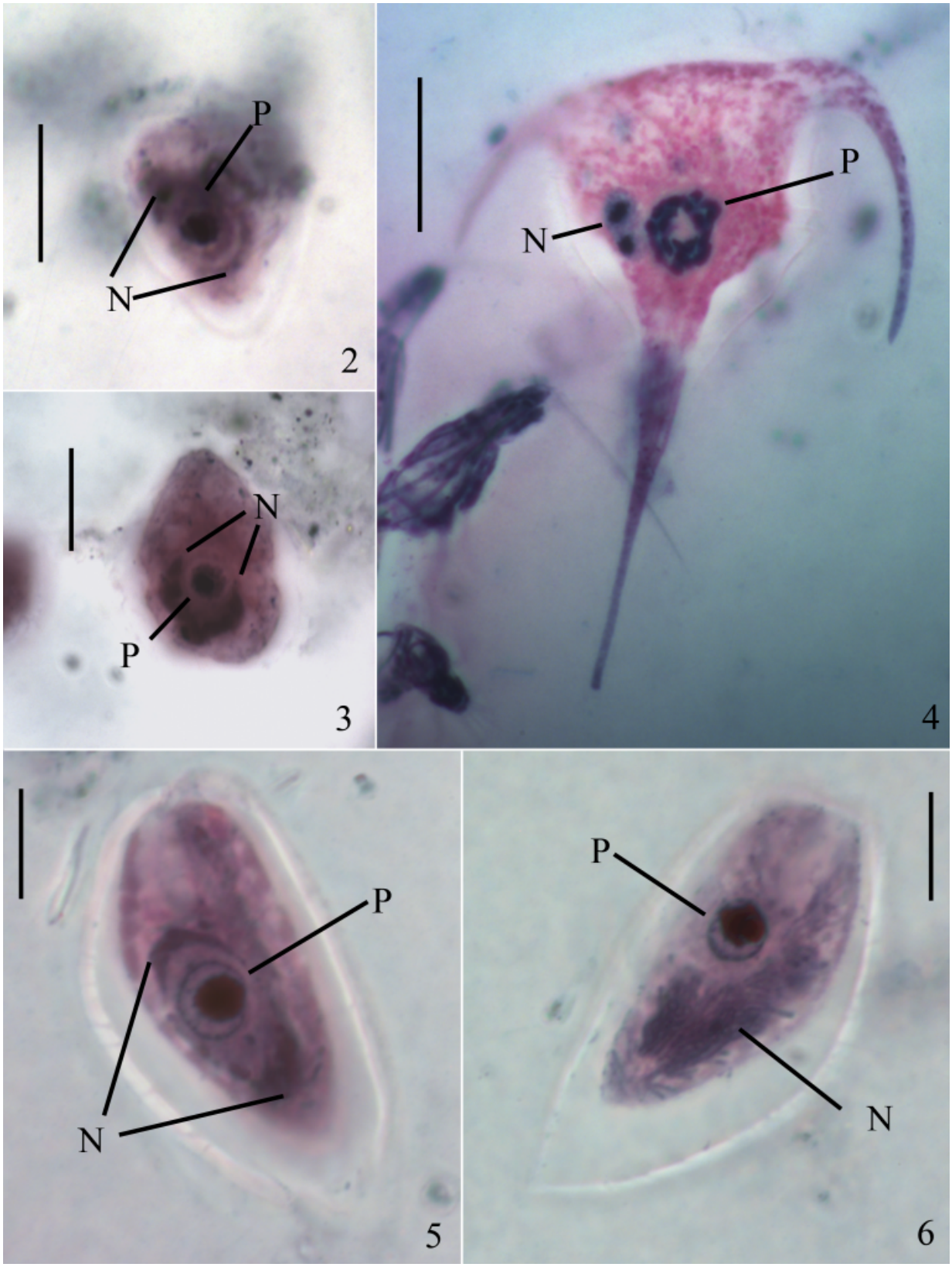

Fig. **2–6.** Photomicrographs of protargol-stained specimens showing site of infection by *Amoebophrya* strains in *Prorocentrum minimum* (**2**), *Scrippsiella* sp. (**3**), *Ceratium tripos* (**4**), and *Prorocentrum micans* (**5**, **6**). P = parasite; N = host nucleus. Scale bars for Fig. 2, 3, 5, 6 represent 10 μm, and that for Fig. 4 represents 50 μm.

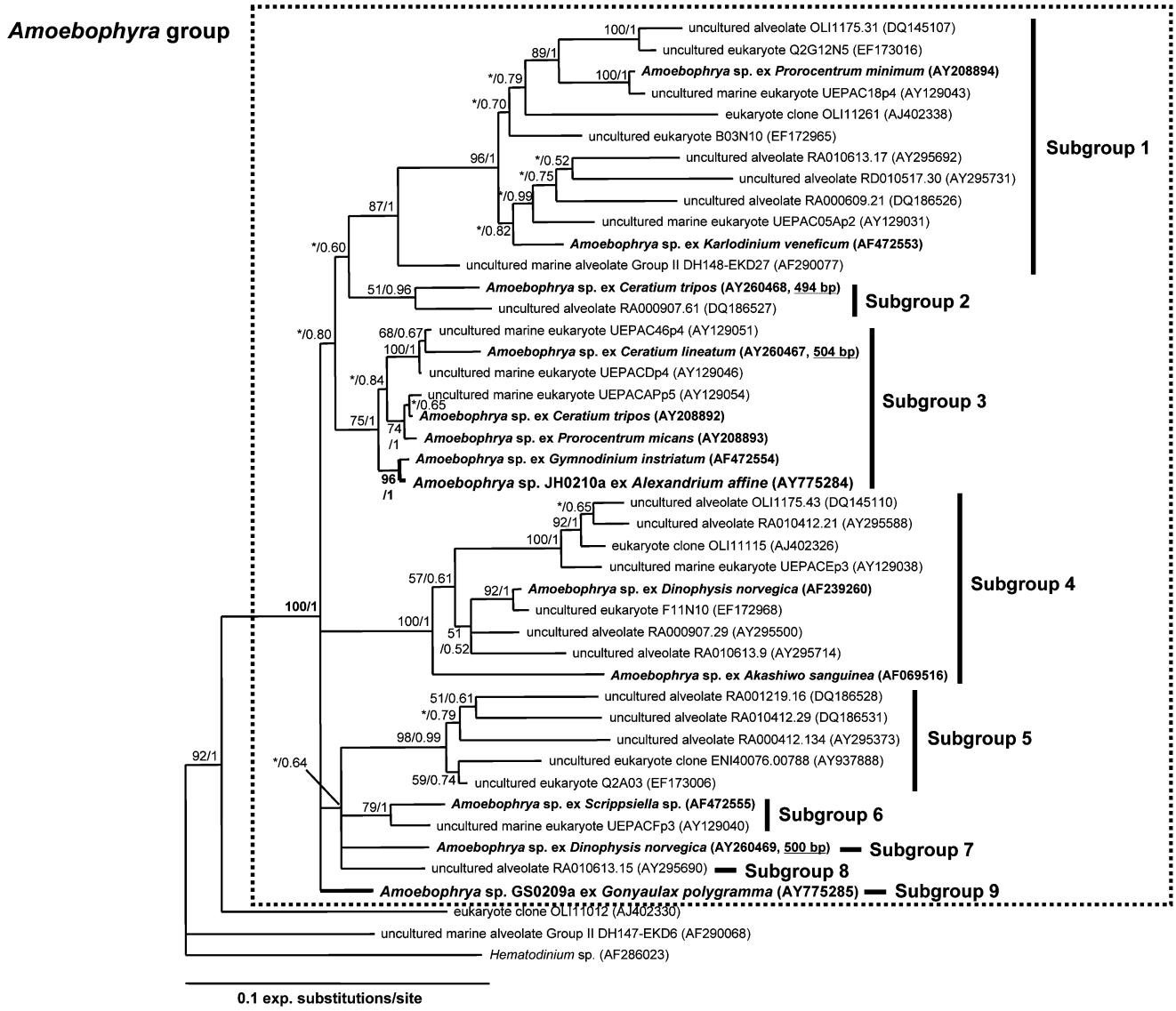

Fig. **7.** An 18S rRNA gene tree showing the phylogenetic position of *Amoebophrya* strains from cultures and infected cells concentrated from field samples as well as environmental 18S rRNA gene sequences using TIM (i.e. transition model)+gamma+I model. *Hematodinium* sp. was used as outgroup taxon. Bootstrap values from maximum likelihood (ML; 200 replicates) and Bayesian posterior probability (PP) are indicated at nodes (presented in order ML/PP). Accession numbers of each taxon are presented in parentheses. *Bootstrap value of $<50\%$ in ML analysis. *Amoebophrya* taxa used in Fig. 1 were indicated in bold in this figure.

host cell (Coats and Park 2002) and yielding only one parasite 18S rRNA gene sequence (Gunderson et al. 2002). That may not, however, be the case for *C. tripos* and *P. micans*, as observations have been based on field samples and may contain more than one parasite species. For example, Elbrächter (1971, 1973) considered nuclear and cytoplasmic infections in *C. tripos* to be different parasites and classified only those that developed in the host nucleus as *A. ceratii*. However, cytological stains of field samples used by Gunderson et al. (2002) to obtain 18S rRNA gene sequence data for *Amoebophrya* ex *C. tripos* (AY208892) revealed only the presence of cytoplasmic infections (Fig. 4). Thus, differences in site of infection in that host species may be attributable to different parasite species. This also may be the case for *P. micans*. While Cachon (1964) noted that *Amoebophrya* sp. formed cytoplasmic infection in *P. micans*, cytological stains of field samples used by Gunderson et al. (2002) to obtain 18S rRNA gene sequence data for *Amoebophrya* ex *P. micans* (AY208893) revealed the presence of predominately intranuclear infections, with rare occurrence of cytoplasmic infections.

Amoebophrya strains that typically form cytoplasmic infections grouped exclusively within Group II, while those that usually produce nuclear infections occurred in the other two groups and across several subgroups when environmental sequences were considered. In addition to showing similarity in site of infection, *Amoebophrya* strains within Group II had relatively short branches, raising the probability that *Amoebophrya* strains with cytoplasmic infections may have a slower rate of molecular evolution than those with intranuclear infections.

Coats et al. (1996) and later Coats and Park (2002) used host specificity of *Amoebophrya* strains in culture to reinforce the suggestion that *A. ceratii* is a species complex. Unlike the site of infection, however, host specificity appears not to appropriately

reflect variation in genetic diversity of *Amoebophrya* strains. Recently, Kim (2006) reported that host specificity of *Amoebophrya* strains varies from extremely species-specific to rather unspecific. As an example, *Amoebophrya* spp. infecting *G. instriatum* and *A. affine* show different patterns in host specificity even within the same group (Group II): *Amoebophrya* sp. ex *G. polygramma*-infected species covering five genera (*Alexandrium*, *Gonyaulax*, *Prorocentrum*, *Heterocapsa*, and *Scripsiella*), while the parasite from *A. affine* was capable of infecting only species of the genus *Alexandrium* (*A. affine*, *A. catenella*, and *A. tamarense*) (Kim 2006).

Our phylogenetic analysis revealed that the environmental sequences used in our study formed a monophyletic group with *Amoebophrya* strains, having high strong bootstrap support or high PP, as in previous studies (e.g. Groisillier et al. 2006; Not et al. 2007). Thus, all these environmental sequences likely belong to endoparasitic ''*Amoebophrya*-like'' organisms within the order Syndiniales. If these ubiquitous environmental sequences really come from ''*Amoebophrya*-like'' organisms, then they probably represent the infective, ''free-living'' dispersal stage (i.e. dinospores) in the asexual cycle of marine parasitic dinoflagellates, as the sequences were obtained from pico- and nanoplanktonic size-fractions that would exclude species known to host these syndinian parasites.

Species of *Amoebophrya* have been reported from a variety of planktonic marine organisms including ciliates, radiolarians, chaetognaths, siphonophores, and other dinoflagellates (Cachon 1964; Cachon and Cachon 1987). Dinospores of *Amoebophrya* spp. infecting dinoflagellate hosts are ca 8 μm long and slightly narrower when free-swimming (Cachon 1964). Coats and Park (2002) have used Nucleopore filters of 5-, 8-, and 12-μm pore size to harvest recently formed dinospores of *Amoebophrya* spp. from infected *K. veneficum*, *G. instriatum*, and *A. sanguinea* cultures, respectively. Further, dinospores decrease considerably in size with increasing age outside the host (M.G.P., pers. observ.). Interestingly, Groisillier et al. (2006) reported that 18S rRNA gene sequences of the *Amoebophrya* group were not detected in fractions below 1.6-μm pore-sized filter. Thus, the environmental sequences likely correspond to the dinospores ranging from 1.6–5 μm in size. An alternative explanation is that the environmental sequences may result from *Amoebophrya* cysts, as dinospores appear unable to survive for more than 2 wk without their hosts (Coats and Park 2002). While cysts have not been reported for *Amoebophrya* spp., very small (1.4 by 6.0 μm) cyst-like cells are produced by *Duboscquella cachoni*, a syndinian parasite of ciliates (Coats 1988).

That ''*Amoebophrya*-like'' organisms are widespread and genetically diverse in coastal and oceanic ecosystems raises questions about what generates and maintains such high genetic diversity within the group. One plausible explanation is that the high genetic diversity might originate from the methodological problems commonly used in this and previous studies (e.g. Díez et al. 2001; Groisillier et al. 2006; López-García et al. 2001; Moon-van der Staay et al. 2001; Not et al. 2007; Worden 2006). All these studies have analyzed a common gene, 18S rRNA gene sequences, to demonstrate genetic diversity in picoeukaryotes from environmental samples. Other conserved eukaryotic genes like heat-shock protein 90 (HSP90), tubulin, actin, and mitochondrial barcode (i.e. *cox*1) genes (e.g. Leander and Keeling 2004; Lynn and Strüder-Kypke 2006; Saldarriaga et al. 2002; Shalchian-Tabrizi et al. 2006) need to be analyzed to better elucidate the ''real'' genetic diversity of these organisms.

As mentioned above, however, some of *Amoebophrya* strains show a high degree of host specificity, whereas others have a relatively broad host range (Coats and Park 2002; Kim 2006; Park et al. 2004; Sengco et al. 2003). If each of environmental sequences comes from parasites with strong host specificity, then host specificity may greatly increase the genetic diversity of ''*Amoebophrya*-like'' organisms, and vice versa. Therefore, future work to resolve genetic diversity in the ''*Amoebophrya*-like'' organisms should explore host specificity for additional strains and relate those results to variation in 18S rRNA gene sequences. Finally, the diversity of ''*Amoebophrya*-like'' organisms may result from the presence of multiple 18S rRNA genes within individual eukaryotic organism although that issue remains more or less controversial (Graur and Li 2000; Rooney 2004; Wintzingerode, Göbel, and Stackebrandt 1997), and no evidence for multiple sequences from single *Amoebophrya* cells has yet emerged from studies done on laboratory strains or parasites from natural samples.

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