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INFECTION OF THE BLOOM-FORMING THECATE DINOFLAGELLATES *ALEXANDRIUM AFFINE* AND *GONYAULAX SPINIFERA* BY TWO STRAINS OF *AMOEBOPHRYA* (DINOPHYTA)[1]

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In autumn 2002, parasitic dinoflagellates of the genus *Amoebophrya* Koeppen infected populations of the bloom-forming thecate dinoflagellates *Alexandrium affine* (Inoue and Fukuyo) Balech and *Gonyaulax spinifera* (Claparede and Lachmann) Diesing in coastal waters of Korea. Here we present the first documentation of *Amoebophrya* infections in *A. affine* and *G. spinifera* and use host–parasite cultures to provide information on parasite development and total generation times. Parasites of the two dinoflagellate hosts differed in their site of infection, developing in the cytoplasm of *A. affine* but in the nucleus of *G. spinifera*. Developmental stages of the parasite strains were similar to the previous descriptions of *Amoebophrya* spp. infecting other dinoflagellates. A prominent feature of *Amoebophrya* infection in *A. affine* from natural field samples was the presence of abnormal "giant cells" in the long chains formed by this host species. The characteristic green autofluorescence of *Amoebophrya* infections was not evident under blue light excitation until very late in the infection cycle of the *A. affine–Amoebophrya* sp. system but was detectable throughout the infection cycle in the *G. spinifera–Amoebophrya* sp. system. Despite the relatively long duration (2–10 min) of the emergence process of *Amoebophrya* spp. from these thecate hosts, total parasite generation times were shorter (53–55 h) than those previously reported for athecate host–parasite systems. These observations provide the basis for better assessing the impact of parasitism by *Amoebophrya* sp. as a significant loss factor for thecate dinoflagellate populations in Korean waters.

***Key index words:* *Alexandrium affine*; *Amoebophrya*; dinoflagellate; *Gonyaulax spinifera*; parasite generation time; parasitism; red tide**

Dinoflagellates can act as hosts for a variety of prokaryotic and eukaryotic parasites, as parasites of protists and metazoa, and even as hyperparasites (i.e. a parasite of another parasite) of their parasitic relatives (Cachon and Cachon 1987, Elbrächter and Schnepf 1998, Coats 1999). Parasitic dinoflagellates of the genus *Amoebophrya* Koeppen received considerable attention in recent years and are of particular interest due to their potential value as biological control agents for harmful algal blooms (Taylor 1968). Of the seven species of *Amoebophrya* that have been formally described (Cachon and Cachon 1987), one, *A. ceratii* (Koeppen) Cachon, is now known to be a species complex capable of infecting many free-living photosynthetic and heterotrophic dinoflagellates. Roughly 40 different species representing more than 24 genera of dinoflagellates have been reported to serve as hosts for members of the *Amoebophrya ceratii* complex (Park 2002, Park et al. 2004). Although three-fourths of these host species are thecate taxa, our knowledge of *Amoebophrya* biology (e.g. life cycle, generation time, and impact on host physiology) is largely derived from the study of *Amoebophrya* spp. that have been cultured using athecate hosts (Coats and Bockstahler 1994, Coats and Park 2002, Park et al. 2002a,b). Recently, however, *Amoebophrya* sp. from *Prorocentrum minimum* (Pavillard) Schiller has been established in culture and used to document aspects of the parasite's developmental stages (Kim et al. 2002).

In September and October 2002, *Amoebophrya* spp. infected populations of the bloom-forming thecate dinoflagellates *Alexandrium affine* (Inoue and Fukuyo)

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Balech and *Gonyaulax spinifera* (Claparede and Lachmann) Diesing on the south and west coasts of Korea, respectively. The two host–parasite systems were successfully isolated from field samples and have been maintained as stock cultures. Here we present the first documentation of *Amoebophrya* infections and parasite developmental stages in *A. affine* and *G. spinifera*. Further, we estimated total parasite generation times (i.e. time required for infection of hosts, intracellular development, vermiform emergence, and extracellular maturation) for the two thecate host–parasite systems. These data will provide important information necessary to assess the impact of parasitism by *Amoebophrya* sp. as a significant loss factor for thecate dinoflagellate populations in Korean coastal waters.

MATERIALS AND METHODS

Sample collection. A bucket was used to gently collect surface seawater samples from coastal waters off Kunsan (36°2′N, 126°25′E) during September 2002 and from Jinhae Bay (35°N, 128°40′E) during October 2002. Aliquots of samples were preserved with acid Lugol's fixative (2% final concentration) and/or modified Bouin's solution (5% final concentration; Coats and Heinbokel 1982). Live field samples were transported to the laboratory to establish host–parasite cultures.

Laboratory cultures. Isolates of *A. affine* and *G. spinifera* were maintained as stock cultures in f/2-Si medium (Guillard and Ryther 1962) formulated using 30 psu seawater collected near Kunsan or from Jinhae Bay. Two strains of parasitic dinoflagellates belonging to the genus *Amoebophrya*, one each in the host species *A. affine* and *G. spinifera*, were established in culture using a glass micropipette to transfer individual infected dinoflagellates from field samples to cultures of complementary host species. Parasites were propagated by sequentially transferring aliquots of infected host culture into uninfected host stock at roughly 2-day intervals. Stock cultures were maintained at 20° C under a 14:10-h light:dark cycle with cool white fluorescent light at 50 and 15 μmol photons $\cdot$ m^{-2} $\cdot$ s^{-1} for *A. affine* and *G. spinifera*, respectively.

Parasite morphology and development. Bright field and/or epifluorescence microscopy of living and protargol-stained specimens was used to document parasite morphology and development inside host cells. For *Amoebophrya* sp. infecting *A. affine*, pictures were taken of live and Lugol-fixed specimens using a microscope (Olympus) equipped with differential interference contrast. Epifluorescence micrographs (Fluorescence Cube U-MWB2, 450- to 480-nm excitation, 500-nm emission) of *Amoebophrya* sp. from *G. spinifera* were obtained for live specimens from culture using a digital camera (PowerShot G5, Canon) coupled to the microscope and set to the automatic exposure mode. Bouin's-preserved field and culture samples were stained using the quantitative protargol technique (Montagnes and Lynn 1993) to characterize the site of infection and developmental stages of *Amoebophrya* spp. within host cells.

Q1 Q2 Q3

Total parasite generation time. For each host species, duplicate 200-mL cultures at initial densities of approximately 1×10^3 cells $\cdot$ mL^{-1} were inoculated with recently formed ($\leq$6 h) dinospores to give a dinospore-to-host ratio of 1:1, incubated under growth conditions described above, and sampled over time to determine the abundance of host cells and parasite dinospores. A relatively low dinospore-to-host ratio was selected for these experiments to avoid possible alteration of parasite generation time resulting from multiple infections that can occur at high dinospore-to-host ratios (Coats and Park 2002). To obtain recently formed dinospores, stock parasite cultures were gravity filtered through polycarbonate filters of 12 μm pore size (Isopore™, Millipore, Bedford, MA, USA) to produce filtrates containing dinospores of unknown age. These dinospores were added to uninfected host cultures, with resulting infections and subsequent generation of dinospores followed over time to enable harvesting of young ($\leq$6 h old) dinospores using filtration procedures described above.

Host abundance was determined from acid Lugol (for *A. affine*) or Bouin's-fixed (for *G. spinifera*) samples using triplicate Sedgwick-Rafter chambers. In experiments using *G. spinifera*, the abundance of empty host theca that exhibited two-thirds or more of the host cell profile was also determined. Dinospore abundance was determined from samples preserved with $CaCO_3$ buffered formaldehyde (2% final concentration) using a hemacytometer and an epifluorescence microscope (Olympus microscope, 200 ×; Fluorescence Cube U-MWB2, 450- to 480-nm excitation, 500-nm emission). For each sample, duplicate counts for dinospores were made within 30 min after fixation to avoid fading of the parasite's green autofluorescence (Coats and Bockstahler 1994). Total parasite generation time (i.e. time required for infection of host cells, intracellular development, vermiform emergence, and extracellular maturation) was estimated from the temporal occurrence of new dinospores after infection of host cells, according to the method of Coats and Park (2002). Plot area from the appearance of new dinospores to peak dinospores concentrations was integrated, with elapsed time corresponding to half of the integrated area considered as total parasite generation time. In experiments using *G. spinifera*, dinospore success (i.e. the percent of dinospores that infected host cells) was estimated from parasite prevalence (= percent host cells infected) and dinospore abundance following the method of Coats and Park (2002). Data are reported as means ± SE.

Q4

RESULTS

Parasite morphology, development, and emergence. A prominent feature of *Amoebophrya* infections in *A. affine* from natural field samples was the presence of abnormal "giant cells" in the long cell chains formed by this host species (Fig. 1, A and B). Up to four giant cells (on average 0.6 ± 0.08 cells per chain, $n = 100$) occurred within the long chains of *A. affine* from natural field samples, with the largest cells reaching 60 μm in width. In culture, however, the significant difference in cell size was not often evident between infected and uninfected host cells. A closer examination of these abnormally large cells revealed that each contained a single fully developed trophont (beehive stage) of *Amoebophrya* sp. (Fig. 1C). These beehive stage trophonts were always oriented perpendicular to the axis of the cell chain, resulting in the apical aperture of the mastigocoel being pressed against the cingulum of the host (Fig. 1D). The green autofluorescence of *Amoebophrya* sp. in *A. affine* was not easily detected until the parasite had developed into a mature trophont. Infections occurred in the cytoplasm of *A. affine*, where they grew in size to eventually produce a multinucleated and multiflagellated trophont with the characteristic "beehive" appearance (Fig. 1, E–G). At maturity, the trophont ruptured through the cingulum of the host and transformed into a strongly motile vermiform stage (Fig. 1 H).

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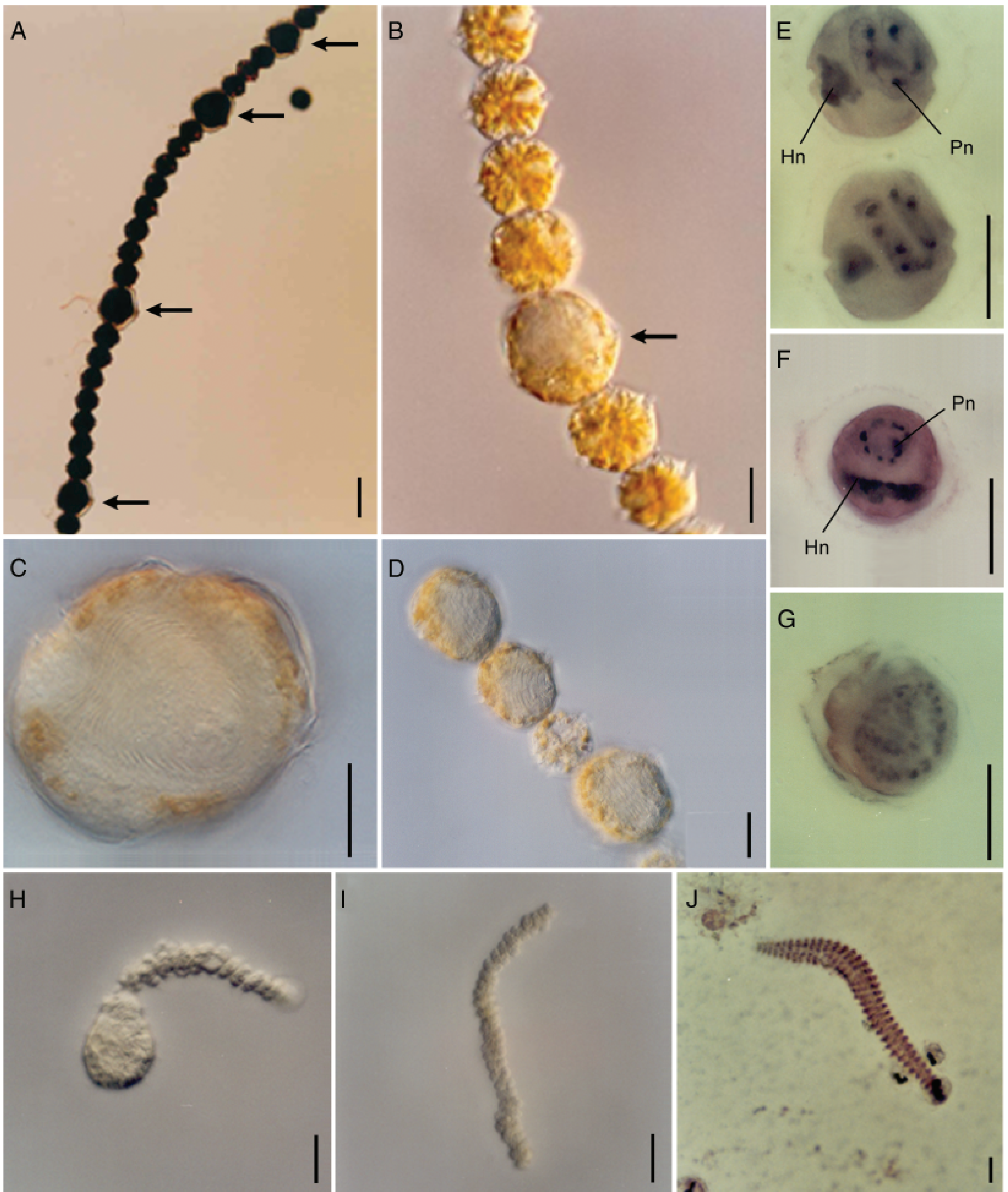

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FIG. 1. Bright field micrographs of *Amoebophrya* sp. in *Alexandrium affine*. Scale bars: (A) 40 µm, (B–J) 20 µm. (A) Chain of *A. affine* containing four "giant cells" (arrows) resulting from development of mature trophonts of *Amoebophrya* sp. (Lugol-preserved field material). (B) Live chain of *A. affine* containing giant cell (arrow) viewed with differential interference contrast (DIC) microscopy. (C and D) Live *A. affine* cells showing the mature *Amoebophrya* sp. trophonts. In D the mastigocoeles were all orientated in the same direction within the host cells in the chain (DIC). (E–G) Protargol-stained specimens showing early to mid-, mid-, and late (beehive stage) infections, respectively. Pn, parasite nuclei; Hn, host nucleus. (H) The emergence process of *Amoebophrya* sp. vermiform from an *A. affine* cell (DIC). (I and J) Live and protargol-stained vermiforms of *Amoebophrya* sp. from *A. affine*, respectively (DIC).

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Emergence of *Amoebophrya* sp. from *A. affine* typically required 3 to 10 min and left behind a distorted empty host theca that could not be easily identified by shape. Emergence of a vermiform from an individual host cells within a long chain of *A. affine* fragmented the chain into two or more shorter pieces. Vermiforms were approximately 250 μm long (Fig. 1, I and J) and over a period of about 1 h gradually produced fragments of variable sizes, with each fragment releasing numerous new dinospores.

Amoebophrya sp. infecting *G. spinifera* exhibited green autofluorescence throughout its life cycle (Fig. 2). Fluorescence microscopy was useful in detecting dinospores attached to the surface of host cells (Fig. 2A) and developing inside the nucleus of *G. spinifera* (Fig. 2B). With continued growth (Fig. 2, C and D), the parasite eventually occupied most of the host cell and assumed the characteristic "beehive" appearance of the mature trophont. Emergence of the vermiform stage from *G. spinifera* typically required 2 to 3 min and occurred through an opening produced by dislodging one of the hypothecal plates, leaving behind a relatively intact but empty host theca. Vermiforms of *Amoebophrya* sp. from *G. spinifera* were approximately 200 μm long and persisted for about 30 min before suddenly breaking apart to release numerous dinospores (Fig. 2, E and F).

Total parasite generation time. Densities of *A. affine* and *G. spinifera* in cultures inoculated with dinospores at a 1:1 ratio, respectively, showed a gradual increase or remained relatively constant for the first 40 h, probably due to low growth rates (Fig. 3). Abundance of *A. affine* decreased slightly from 44 to 52 h and then increased toward the end of the experiment (Fig. 3A). By comparison, densities of *G. spinifera* declined gradually to the end of the experiment, accompanied by the accumulation of empty host thecae and the formation of new dinospores (Fig. 3B). Dinospores densities exhibited distinct peaks in both *A. affine* (at 61 h) and *G. spinifera* (at 56 h) cultures, thus permitting calculation of total parasite generation time. Estimates for total parasite generation time averaged 55 ± 1.7 h and 53 ± 1.1 h for *A. affine* and *G. spinifera*, respectively; the two means were not significantly different ($P = 0.409$, *t*-test). In experiments using *G. spinifera*, parasite prevalence averaged 7.5% ± 2.50%. The percent of dinospores from *G. spinifera* that successfully established infections (i.e. dinospore success) was 6.9% ± 1.81%.

DISCUSSION

Several different methods have been used to detect infection of planktonic dinoflagellates by *Amoebophrya* spp. Most of these techniques have relied on examination of preserved samples using cytological staining techniques, DNA-specific fluorochromes, or fluorescent *in situ* hybridization probes (Park et al. 2004). Only two approaches, epifluorescence microscopy to reveal green autofluorescence of the parasite (Coats and Bockstahler 1994, this study) and detection of "giant" host cells as reported here, have been used to recognize *Amoebophrya* infections in living dinoflagellates. The characteristic green autofluorescence previously noted for *Amoebophrya* spp. from a number of dinoflagellate host species (Coats and Bockstahler 1994, Coats and Park 2002, Salomon et al. 2003) was not evident in infected *A. affine* until very late in the infection cycle. Delayed appearance of green fluorescence has also been reported for *Amoebophrya* spp. that infect *Ceratium furca* (Ehrenberg) Claparede et Lachmann and *Ceratium tripos* (Mueller) Schiller (Park et al. 2004). Whether this condition reflects some attribute of the host cytoplasmic environment or an inherited trait shared by *Amoebophrya* strains that infect *A. affine* and the two *Ceratium* species is uncertain. The development of abnormally giant cells as a consequence of *Amoebophrya* infection may be an uncommon phenomenon. For example, cultures of *Amoebophrya* spp. ex. *Akashiwo sanguinea* (Hirasaka) G. Hansen et Moestrup and *Gymnodinium instriatum* (Freudenthal et Lee) Coats showed no noticeable increase in host size during the infection cycle (Coats and Park 2002). Indeed, the only prior report of giant cells being produced by *Amoebophrya* infections comes from Taylor's (1968) examination of formalin-preserved samples of the chain-forming dinoflagellate *Alexandrium catenella* (Whedon and Kofoid) Balech from Sequim Bay, USA. However, significant differences in cell size can be seen in the genus *Alexandrium* depending on the life cycle; for example, planozygotes resulting from the fusion of gametes and planomeiocytes from a newly germinated cyst can be much larger than vegetative cells (Anderson and Wall 1978). Thus, although providing simple and rapid means of detecting and isolating some species of *Amoebophrya*, parasite autofluorescence and production of giant cells are not characteristic of all parasite strains and should not be used as the primary means to estimate parasite prevalence.

Although *Amoebophrya* spp. are known to infect both thecate and athecate host species, our understanding of host–parasite biology is largely based on the study of athecate species (Coats and Park 2002, Park et al. 2002a,b). Because completion of the *Amoebophrya* life cycle is dependent on penetration of infective dinospores into the host cell and rupture of the reproductive vermiform through the host pellicle, difference in the surface architecture of thecate and athecate dinoflagellates may pose different constraints on the timing of parasite developmental processes. Cultures of *Amoebophrya* spp. established here for *A. affine* and *G. spinifera* provide the first opportunity to explore that possibility. Estimates of parasite generation time for our two thecate hosts (53–55 h) are shorter than those reported for athecate hosts, including *A. sanguinea*, *G. instriatum*, and *Karlodinium micrum* (Leadbeater and Dodge) J. Larsen (59–71 h; Coats and Park 2002). These results suggest that the plates of thecate dinoflagellate are not a major obstacle to infection by *Amoepophrya* spp. Further, the percent (6.9% ± 1.81%) of dinospores from *G. spinifera* that successfully

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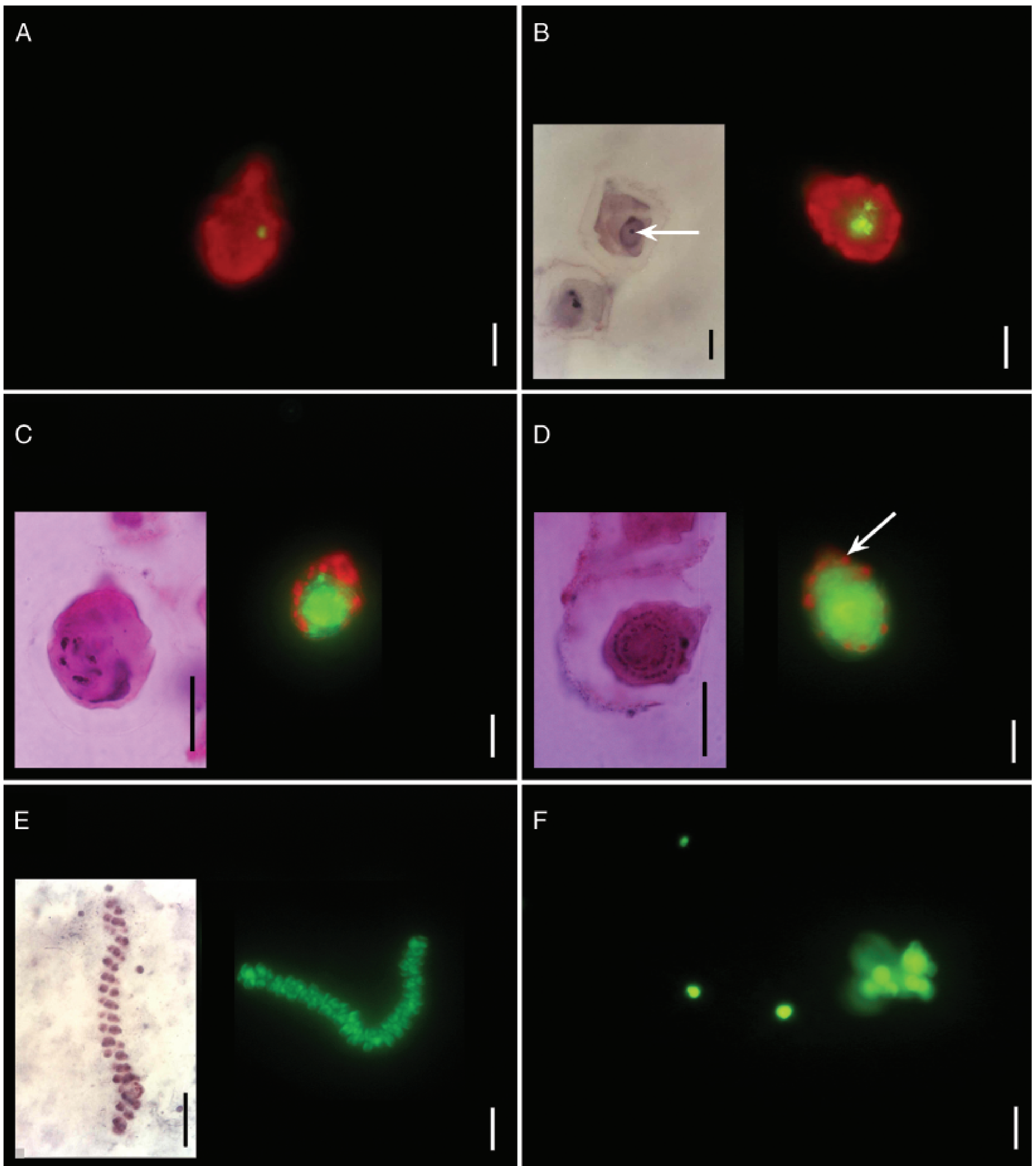

FIG. 2. Epifluorescence and bright field micrographs of *Amoebophrya* sp. in *Gonyaulax spinifera*. Scale bars, 20 μm. (A) Attachment of dinospore (green fluorescence) of *Amoebophrya* sp. to the surface of *G. spinifera* (red fluorescence) observed under epifluorescence. (B and C) Early and mid-infections where the green autofluorescing parasite is surrounded by red fluorescing chloroplasts of *G. spinifera*. (B, inset) Protargol-stained specimen showing young trophont visible as a lightly staining circle inside the host's nucleoplasm; arrow indicates parasite nucleus. (C, inset) Protargol-stained specimen showing mid-infection with several irregularly arranged parasite nuclei. (D) Epifluorescence of a mature trophont; the remnants of the host's chloroplasts (arrow) are also shown. (Inset) Protargol-stained specimen showing late (beehive stage) infection. (E and F) Vermiform of *Amoebophrya* sp. under epifluorescence and snapshot of the process releasing new dinodpores, respectively. (E, inset) Protargol-stained vermiform of *Amoebophrya* sp. from *G. spinifera*.

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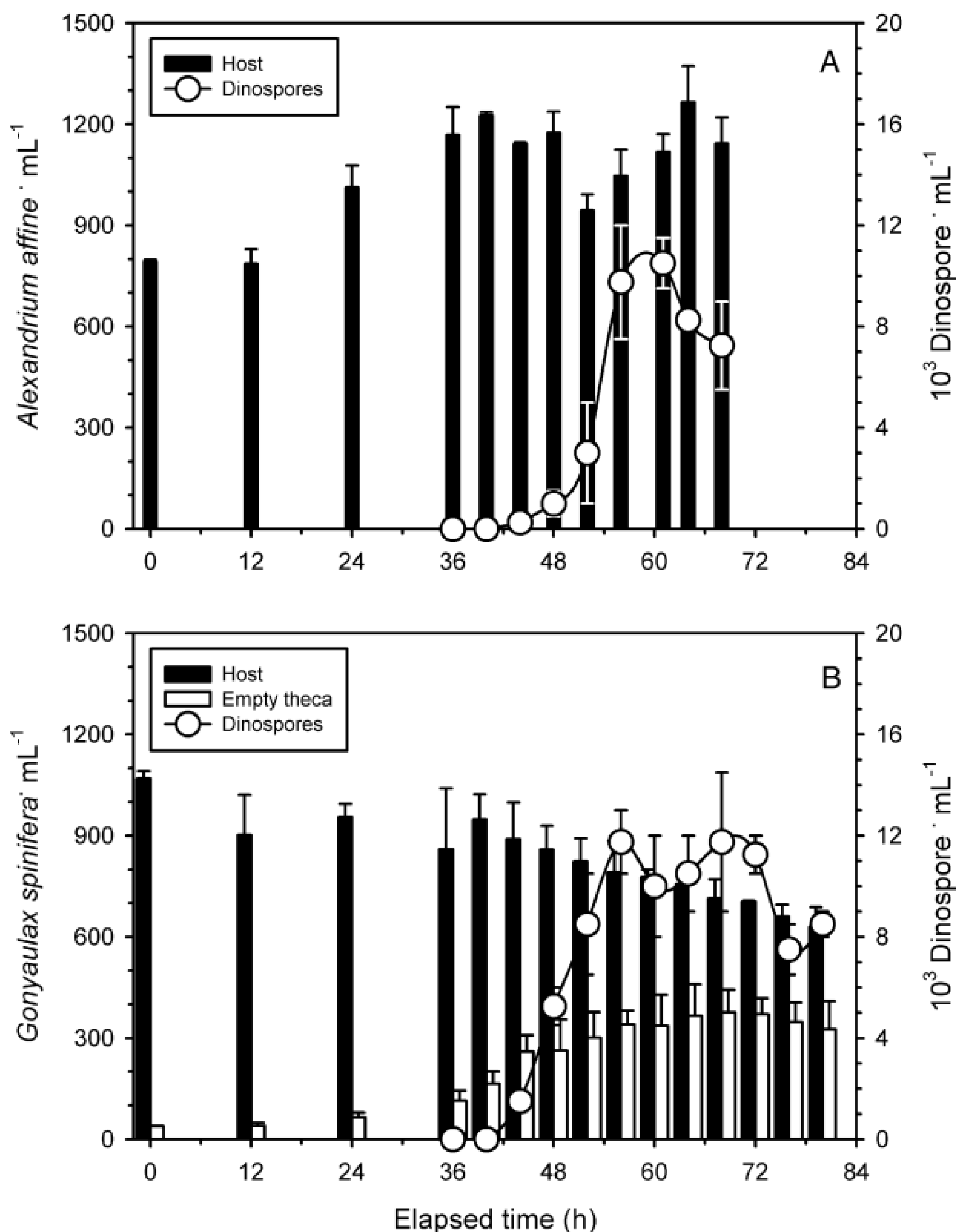

Fig. 3. Time-course studies of *Amoebophrya* spp. infecting (A) *Alexandrium affine* and (B) *Gonyaulax spinifera* inoculated at dinospore-to-host ratios of 1:1. Black bars represent host abundance, and white bars represent the empty theca. Open circles are dinospore abundance. Errors bars indicate SE.

established infections (i.e. dinospore success) was within the range (4.7%–18.0%) reported for athecate hosts (Coats and Park 2002), again suggesting that thecal plates do not act as a barrier to successful penetration of dinospores into host cells and thereby do not reduce parasite success. Although the time required for dinospores to penetrate into host cells is unknown, the duration of vermiform emergence from *A. affine* and *G. spinifera* (2–10 min) is only slightly longer than that recorded for the athecate host *A. sanguinea* (1–2 min; Coats and Bockstahler 1994). Salomon et al. (2003) also reported a similar emergence time (2–8 min) for *Amoebophrya* sp. from thecate dinoflagelate *Dinophysis norvegica* Claparede and Lachmann. Thus, the emergence process is rapid in both thecate and athecate hosts and represents a relatively short portion of the parasite's life cycle. Whether the timing of dinospore penetration into the host differs among thecate and athecate hosts and is sufficiently long to influence parasite generation time remains an open question.

Parasite generation time may be influenced by a number of factors, including 1) parasite load, 2) host cell size or biovolume, 3) growth conditions (e.g. temperature, salinity, and light intensity, etc.), and 4)

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intrinsic host-specific differences in development times among *Amoebophrya* spp. For example, inoculation of *G. instriatum* at a high dinospore-to-host ratio (20:1) increased parasite load and shortened parasite generation times, apparently due to more rapid utilization of host resources with growth of multiple parasites (Coats and Park 2002). The short generation times observed here for *Amoebophrya* from *A. affine* and *G. spinifera*, however, are not likely the result of multiple infections, because cultures were inoculated at a low dinospore-to-host ratio (i.e. 1:1). Generation times of *Amoebophrya* spp. that infect athecate dinoflagellates appear positively related to host size ranging from 59 h for *K. micrum* to 71 h for *G. instriatum* (Coats and Park 2002). Based on host size, *Amoebophrya* from *A. affine* and *G. spinifera* would be expected to have generation times within this range, yet our estimates (55 h and 53 h for parasites of the two host species, respectively) are less than the shortest parasite generation time reported by Coats and Park (2002). Thus, host cell size (and/or biovolume) may be not the primary factor governing parasite generation time. Our experiments were conducted at the same temperature (20° C) used in the study by Coats and Park (2002), but our cultures were grown at a higher salinity (30 vs. 15 psu) and at lower irradiance (15–50 μmol photons $\cdot m^{-2} \cdot s^{-1}$ vs. 95–175 μmol photons $\cdot m^{-2} \cdot s^{-1}$). Thus, we cannot exclude the possibility that differences in growth conditions account for the faster generation time observed for *Amoebophrya* for our thecate hosts relative to those of strains infecting athecate dinoflagellates (Coats and Bockstahler 1994, Coats and Park 2002). Nonetheless, intrinsic host-specific differences in intracellular and extracellular development times among *Amoebophrya* species appear the most plausible explanation for observed differences in parasite generation times.

Parasite intracellular development time, representing 60%–80% of total parasite generation time (Coats and Park 2002), has been used along with estimates of parasite prevalence to assess the impact of parasitism by *Amoebophrya* sp. on dinoflagellate populations in the field (Coats and Bockstahler 1994). In some instances, generation times obtained for *Amoebophrya* spp. from athecate hosts have been used to calculate the impact of parasitism on thecate host species (Gisselson et al. 2002, Salomon et al. 2003). Because *Amoebophrya* spp. likely have intrinsic host-specific differences in generation times, applying data for one parasite–host system to estimate the influence of parasitism on a different host species may produce unpredictable biases as recognized by Salomon et al. (2003). Thus, data for intracellular development times and/or total parasite generation times for a variety of athecate and thecate host–parasite systems are needed to better define the significance of parasitism as a loss factor for dinoflagellate populations in nature.

Amoebophrya infections in chain-forming dinoflagellates can facilitate the fragmentation of long cell chains into multiple short pieces as observed here for *A. affine*. Because swimming speed of chain-forming dinoflagellates is a function of chain length (Fraga et al. 1989), parasitism may reduce host's swimming speed by reducing the length of cell chains. The resulting decrease in host swimming speed may increase the chance of infection by facilitating attachment of parasites to host cells.

Our observations and data for parasite generation times in *A. affine* and *G. spinifera* increase knowledge of thecate dinoflagellate host–parasite systems. Further, our results provide the basis for better assessing the impact of parasitism by *Amoebophrya* sp. as a significant loss factor for thecate dinoflagellate populations in nature.

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