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PARASITISM OF PHOTOSYNTHETIC DINOFLAGELLATES BY THREE STRAINS OF *AMOEBOPHRYA* (DINOPHYTA): PARASITE SURVIVAL, INFECTIVITY, GENERATION TIME, AND HOST SPECIFICITY[1]

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***Amoebophrya ceratii* (Koeppen) Cachon is an obligate parasite of dinoflagellates and may represent a species complex. However, little is known about the biology and host range of different strains of *Amoebophrya* Cachon. Here, we determined parasite generation time and dinospore infectivity, survival, and ability to infect nonprimary hosts for strains of *Amoebophrya* from *Akashiwo sanguinea* (Hirasaka) G. Hansen et Moestrup, *Gymnodinium instriatum* (Freudenthal et Lee) Coats comb. nov., and *Karlodinium micrum* (Leadbeater et Dodge) J. Larsen. *Akashiwo sanguinea* was readily infected, with parasite prevalence reaching 100% in dinospore:host inoculations above a 10:1 ratio. Parasitism also approached 100% in *G. instriatum*, but only when inoculations exceeded a 40:1 ratio. *Karlodinium micrum* appeared partially resistant to infection, as parasite prevalence saturated at 92%. Parasite generation time differed markedly among *Amoebophrya* strains. Survival and infectivity of dinospores decreased over time, with strains from *G. instriatum* and *A. sanguinea* unable to initiate infections after 2 and 5 days, respectively. By contrast, dinospores from *Amoebophrya* parasitizing *K. micrum* remained infective for up to 11 days. *Akashiwo sanguinea* and *G. instriatum* were not infected when exposed to dinospores from nonprimary *Amoebophrya* strains. *Karlodinium micrum*, however, was attacked by dinospores of *Amoebophrya* from the other two host species, but infections failed to reach maturity. Observed differences in host–parasite biology support the hypothesis that *Amoebophrya ceratii* represents a complex of host-specific species. Results also suggest that *Amoebophrya* strains have evolved somewhat divergent survival strategies that may encompass sexuality, heterotrophy during the "free-living" dinospore stage, and dormancy.**

***Key index words:* *Akashiwo sanguinea*; *Gymnodinium instriatum*; *Karlodinium micrum*; plankton; protist; red tide**

Dinoflagellates serve as hosts for a variety of intracellular symbionts, including viruses, bacteria, fungi, and protists (Elbrächter and Schnepf 1998, Norén et al. 1999, Tarutani et al. 2001). Bacteria that inhabit the cytoplasm and/or nucleus of dinoflagellates are generally believed to have a commensal or mutualistic relationship with their host (Silva and Franca 1985, Doucette et al. 1998); however, Kirchner et al. (1999) recently linked bacterial infections with decreased growth of *Noctiluca scintillans* (Macartney) Kofoid and Swezy, suggesting that some bacteria–dinoflagellate associations may be parasitic. Most other intracellular symbionts of dinoflagellates are clearly parasitic and have marked effects on host survival and reproduction (Elbrächter and Schnepf 1998).

Parasite-induced mortality and associated decreases in reproduction can have strong influences on dinoflagellate population dynamics. For example, fungal parasites cause major losses of cysts and vegetative cells of the freshwater dinoflagellate *Ceratium hirundinella* (O. F. Müller) and thereby contribute to short-term and the long-term reductions in host density (Canter and Heaney 1984, Sommer et al. 1984, Heaney et al. 1988). In marine environments, parasitic dinoflagellates of the genus *Amoebophrya* Cachon and the perkinsozoa *Parvilucifera infectans* Norén et Moestrup infect numerous taxa of photosynthetic and heterotrophic dinoflagellates, including several toxic species (Coats 1999, Norén et al. 1999). Outbreaks of these parasites have been linked to declines of dinoflagellate blooms in several marine systems (Cachon 1964, Taylor 1968, Nishitani and Chew 1984, Coats et al. 1996).

Amoebophrya species appear well adapted to exploit dinoflagellate blooms in enriched coastal environments (Yih and Coats 2000) where parasite prevalence (= percent hosts infected) can exceed 80% (Coats 1999). Particularly high infection levels in the toxic dinoflagellate *Alexandrium catenella* (Whedon and Kofoid) Balech led Taylor (1968) to suggest that parasites, specifically *Amoebophrya ceratii* (Koeppen) Cachon, might be intentionally used to control red tides; however, his idea was later rejected by Nishitani et al. (1985), because *A. ceratii* appeared to lack the host specificity needed to target any particular harmful bloom-forming species. Subsequently, Coats et al. (1996) demonstrated that cultures of *Amoebophrya ceratii* ex *Akashiwo sanguinea* (Hirasaka) G. Hansen et Moestrup (formally *Gymnodinium sanguineum* Hirasaka) failed to infect several other dinoflagellate species and suggested that *A. ceratii* represented a species complex composed of several host-specific taxa. Recent molecular studies support that argument by showing marked genetic diversity among strains of *Amoe-*

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bophrya from different host species (Gunderson et al. 2000). Thus, the possibility of using parasites to control harmful algal blooms is now receiving renewed attention (Anderson 1997, Norén et al. 1999, Erard-Le Denn et al. 2000).

Species of *Amoebophrya* that infect dinoflagellates have a relatively simple life cycle, consisting of an infective dispersal stage, the dinospore, an intracellular growth stage, the trophont, and an extracellular reproductive stage, the vermiform (Cachon 1964, Cachon and Cachon 1987). Dinospores are small (8–10 μm long) biflagellate cells that attach to the surface of host organisms, penetrate through the host pellicle, and develop into trophonts either in the cytoplasm or in the nucleus of the host, depending on the species of dinoflagellate being infected (Cachon 1964, Park et al. 2002). The trophont stage grows inside the host for 2–3 days (20–23° C; Coats and Bockstahler 1994, Yih and Coats 2000), during which time it undergoes nuclear and flagellar replication to produce a large multinucleate and multiflagellate cell known to as a "beehive." At maturity, the beehive metamorphoses into a vermiform that ruptures through the host cell membrane. Vermiforms persist for only a short time (~20 min at 23° C), with completion of cytokinesis giving rise to hundreds to thousands of new dinospores (Coats and Bockstahler 1994). Survival time of dinospores outside the host is unknown; however, grazing by ciliates may influence their ability to transmit infections to new host cells (Maranda 2001, Johansson and Coats, unpublished data).

Here we compare aspects of host–parasite biology for strains of *Amoebophrya* isolated from bloom-forming dinoflagellates of Chesapeake Bay. The host–parasite systems we examined were *Amoebophrya* sp. ex *A. sanguinea*, *Amoebophrya* sp. ex *Gymnodinium instriatum* (Freudenthal et Lee) Coats comb. nov. (formerly *Gyrodinium instriatum* Freudenthal et Lee), and *Amoebophrya* sp. ex *Karlodinium micrum* (Leadbeater and Dodge) J. Larsen (formerly *Gyrodinium galatheanum* [Braarud] Taylor). Our goal was to better define the biological diversity of *Amoebophrya* strains that infect different dinoflagellate species. For each host–parasite system, we examined the influence of inoculum size (i.e. dinospore:host ratio) on parasite prevalence, determined duration of the infection cycle, estimated total parasite generation time, and assessed survival and infectivity of dinospores with age. We also evaluated the influence of inoculum size on parasite development and assessed the ability of each parasite strain to infect the other host species.

MATERIALS AND METHODS

Laboratory cultures. Chesapeake Bay isolates of *A. sanguinea*, *G. instriatum*, and *K. micrum* were maintained as stock cultures in f/2-Si medium (Guillard and Ryther 1962). The medium was formulated using 15 ppt Chesapeake Bay water supplemented with 5% (v/v) GR+ soilwater (Starr and Zeikus 1993).

Three strains of parasitic dinoflagellates belonging to the genus *Amoebophrya*, one each in the host species *A. sanguinea*, *G. instriatum*, and *K. micrum*, were established in culture by transferring infected dinoflagellates from Chesapeake Bay plankton to cultures of the complementary host species. Parasites were propagated by sequentially transferring aliquots of infected host culture into uninfected host stock at roughly 2-day intervals for *A. sanguinea* and *G. instriatum* and twice weekly for *K. micrum*. Stock cultures were maintained at 20° C under a 14:10 light:dark cycle of cool-white fluorescent light at 175 μmol photons·m^{-2}·s^{-1} for *A. sanguinea* and 95 μmol photons·m^{-2}·s^{-1} for *G. instriatum* and *K. micrum*.

All experiments were conducted using host cultures in exponential growth and recently formed (≤6 h) dinospores of *Amoebophrya* spp. To obtain recently formed dinospores, stock parasite cultures were gravity filtered through Nucleopore filters (12-μm pore size for *A. sanguinea*, 8 μm for *G. instriatum*, 5 μm for *K. micrum*) (Costar-Nucleopore, Cambridge, MA, USA) to yield filtrates containing dinospores of unknown age. Although dinospores of our three *Amoebophrya* strains were of similar size, host size differed greatly across species. Thus, filters of different pore sizes were used in harvesting dinospores to ensure efficient removal of remaining host cells. Dinospores were inoculated into uninfected host culture in sufficient quantities (dinospore:host ratio of 20:1 to 30:1) to produce infection levels ≥80%. Dinospores produced from the subsequent parasite generation were harvested within 6 h (= recently formed dinospores) using filtration procedures described above.

Parasite prevalence versus inoculum size. Parasite prevalence was determined as a function of inoculum size in four separate experiments, one for *A. sanguinea*, one for *G. instriatum*, and two for *K. micrum*. For each experiment, sets of triplicate scintillation vials containing 10 or 20 mL of host cells at 1×10^3 mL^{-1} were inoculated with dinospores and incubated for 24–40 h under growth conditions described above. Incubation time differed for the three host species (36, 40, and 35 or 36 h for *A. sanguinea*, *G. instriatum*, and *K. micrum*, respectively) but was always shorter than the time needed for the parasite to reach maturity. Inoculum size for each set of triplicate vials was adjusted to give dinospore:host ratios of 1:2, 1:1, 5:1, 10:1, 20:1, and 40:1 for *A. sanguinea*, 1:1, 2:1, 5:1, 10:1, 20:1, 30:1 40:1 and 100:1 for *K. micrum*, and 1:1, 2:1, 5:1, 10:1, 20:1, 40:1, 80:1, and 120:1 for *G. instriatum*. After incubation, samples were preserved with $CaCO_3$ buffered formaldehyde (2% final concentration) and examined by inverted epifluorescence microscopy (Leitz Diavert, 450- to 490-nm excitation, 520 nm barrier filter, ×400) to visualize the distinctive green autofluorescence of the parasite (Coats and Bockstahler 1994). Parasite prevalence (= percent cells infected) was determined by scoring at least 100 hosts cells per sample as infected or uninfected.

Data for each host species were fitted to a single two-parameter exponential rise to maximum. The equation for the curve fit was $y = a*(1 - e^{-b*x})$, where a is the maximum infection level (I_{max}) and b is α/I_{max}. Alpha (α) represents the slope of the initial linear portion of the fitted curve and reflects the potential of dinospores to infect host cells. Estimates for α were derived as I_{max}*b, with SE for α determined by propagation of error terms.

Dinospore survival and infectivity. Replicate 300- to 400-mL volumes of recently formed dinospores harvested as above from each host species were held under standard growth conditions (20° C with a 14:10 light:dark cycle of cool-white fluorescent light at 100–200 μmol photons·m^{-2}·s^{-1}) and subsampled over time to estimate dinospore survival and infectivity. Persistence of green autofluoresence was taken as an indication of dinospore survival and was used to track cell abundance over time. Samples were preserved with formalin and examined for dinospore abundance using a hemocytometer and a Zeiss Axioscope (×200) (Zeiss, Jena, Germany) equipped for epifluorescence microscopy (Filter Cube 09, 450- to 490-nm excitation, 510-nm beam splitter, 520-nm barrier filter). Counts were made in duplicate, with each based on examination of 10 hemocytometer grids equivalent to 1 mm^3 of sample. Data for each parasite strain were fitted to a single two-parameter exponential decay curve. For our purposes, this model corresponds to the exponential growth function $N_t = N_0e^{kt}$, where N_0 is the dino-

spore abundance (cells·mL^{-1}) at the beginning of the experiment (T_0), N_t is the dinospore abundance at subsequent times, t is the time in days, and k is the the negative growth constant (i.e. mortality rate).

To assess the ability of dinospores of different ages to infect naive host cells, subsamples from each of the replicate 300- to 400-mL volumes of recently formed dinospores were added to triplicate scintillation vials at each sampling period and mixed with equivalent volumes of the corresponding host culture to produce 10- or 20-mL volumes of host cells at 1×10^3 mL^{-1}. This approach generated a dinospore:host ratio of 5:1 for *A. sanguinea*, 15:1 for *K. micrum*, and 22:1 for *G. instriatum* for the initial (T_0) sampling period. Ratios for subsequent sampling times varied with survival of dinospores. Scintillation vials were incubated for 36 h as above, fixed using concentrated Bouin's fluid (Coats and Heinbokel 1982), and stained for determination of parasite prevalence and parasite load (i.e. number of parasites inside each infected host). Preserved samples for *A. sanguinea* and *G. instriatum* were processed using quantitative protargol staining (Montagnes and Lynn 1993), whereas those for *K. micrum* were stained with alum hematoxylin (Galigher and Kozloff 1971). Stained samples were examined using Zeiss optics (1000–1250×), with parasite prevalence and parasite load determined following procedures of Coats et al. (1996). Dinospore success (i.e. the percent of dinospores that invaded host cells) at each sampling period was expressed relative to dinospore abundance at T_0 and at the time samples were taken.

Generation time. For each host species, replicate 200- or 300-mL cultures at initial densities of approximately 1×10^3 mL^{-1} were inoculated with recently formed dinospores, incubated under growth conditions described above, and sampled over time to determine host abundance, parasite prevalence, stage of infection, and dinospore abundance. To examine possible effects of inoculum size on parasite generation time, treatments for each host species included inoculations at low (approximately 1:1) and high dinospore:host ratios (20:1, 40:1, and 115:1 for *A. sanguinea*, *K. micrum*, and *G. instriatum*, respectively).

Estimates for host abundance were obtained from Bouin-fixed samples by enumerating cells present in microscope transects (×100) of triplicate Sedgwick-Rafter chambers. For each chamber, successive transects were examined until 100 cells had been counted or five transects (= half the chamber area) had been scanned. Parasite prevalence was determined from protargol-stained samples as described previously, with parasitized hosts partitioned by stage of infection using criteria patterned after those of Coats and Bockstahler (1994). For this analysis, early infections were classified as host cells containing parasites that had a single nucleus or multiple nuclei that were arranged in an irregular pattern. Late infections were host cells that contained parasites having multiple nuclei arranged in a circular or spiraled fashion. Dinospore abundance was obtained from formalin-fixed samples as above.

Temporal differences in the occurrence of early and late stage infections were used to estimate parasite intracellular development time. The areas defined by plots for abundance of early and late stage infections versus time were determined by integration, with the mid-point of each stage derived as half of the integrated area for the corresponding plot. The time interval between mid-points for early and late stage infections was multiplied by two to estimate the duration of the parasite's intracellular phase. Estimates for total generation times (i.e. time required for infection of host cells, intracellular development, vermiform emergence, and extracellular maturation) were derived from the temporal occurrence of new dinospores after infection of host cells. Plot areas from the appearance of new dinospores to peak concentrations were calculated by integration, with elapsed time corresponding to half of the integrated area taken as total generation time.

Cross infection. Six flasks containing 100 mL of culture at 1×10^3 cells·mL^{-1} were established for each host species. Two flasks per host species were inoculated with dinospores from infected *A. sanguinea*, two with dinospores from *G. instriatum*, and two with dinospores from *K. micrum* to give dinospore:host ratios of 10:1, 50:1, and 50:1, respectively. Flasks were incubated as above, except that light was supplied at 80 μmol photons·m^{-2}·s^{-1}. After inoculation, 2-mL subsamples were taken from each flask at 12-h intervals over 4 days and fixed with $CaCO_3$ buffered formaldehyde (2% final concentration) for epifluorescence determination of parasite prevalence as above.

Data analysis. Data are reported in the text as mean ± SE. Statistical analyses and curve fits were performed using Jandel Scientific Software (SigmaPlot 6.0 and SigmaStat 2.0) (SPSS Science, Chicago, IL, USA).

RESULTS

Nomenclature. Specimens from our cultures of *G. instriatum* were morphologically indistinguishable from *Gyrodinium instriatum* Freudenthal et Lee, except that close examination revealed the presence of a horseshoe-shaped apical groove running in an anticlockwise direction. Because an apical groove of this configuration is characteristic of the genus *Gymnodinium* Kofoid et Swezy emend. G. Hansen et Moestrup (Daugbjerg et al. 2000), we refer to this host species as *Gymnodinium instriatum* (Freudenthal et Lee) Coats comb. nov.; basionym: *Gyrodinium instriatum* Freudenthal et Lee (Freudenthal and Lee 1963).

Parasite prevalence versus inoculum size. Parasite prevalence showed an exponential increase to a maximum relative to inoculum size in all three host species (Fig. 1). Estimates for maximum infection levels (I_{max}) and initial slope of the fitted curves (α) were $I_{max} = 98.2 \pm 0.82$; $\alpha = 66.0 \pm 2.68$ ($r^2 = 0.9921$; $P < 0.0001$) for *A. sanguinea*, $I_{max} = 97.9 \pm 1.03$; $\alpha = 9.3 \pm 0.36$ ($r^2 = 0.9928$; $P < 0.0001$) for *G. instriatum*, and $I_{max} = 91.8 \pm 2.37$; $\alpha = 19.6 \pm 1.91$ ($r^2 = 0.9042$; $P < 0.0001$) for *K. micrum* (experiments 1 and 2 combined).

Dinospores of *Amoebophrya* sp. from *A. sanguinea* were most aggressive of the three parasite strains, with parasite prevalence rising sharply to near maximum levels by a dinospore:host ratio of 10:1. Inoculations above a 10:1 ratio typically showed 100% infection of

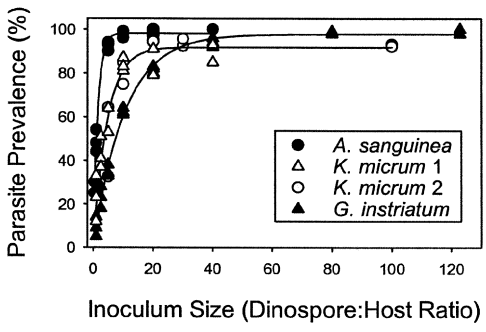

FIG. 1. Parasite prevalence as a function of inoculum size for strains of *Amoebophrya* infecting *Akashiwo sanguinea*, *Karlodinium micrum*, and *Gymnodinium instriatum*. Host density was maintained at 10^3 cells·mL^{-1}, with dinospore density varied to yield dinospore:host ratios of 1:1 to 120:1. Data represent replicate measurements for each experimental treatment. *Karlodinium micrum* 1 and *K. micrum* 2 indicate results from two separate experiments.

host cells. Dinospores from *G. instriatum* were least aggressive, with parasite prevalence showing a more gradual increase to saturation; however, 100% infection levels were detected at a dinospore:host ratio of 120:1. Parasitism in *K. micrum* saturated at an inoculum size intermediate to that of *A. sanguinea* and *G. instriatum* (dinospore: host ratio of 20:1) but failed to reach 100% infection levels, even at a dinospore:host ratio of 100:1. Parasite prevalence in *K. micrum* only averaged 91.3 ± 1.37% ($n = 12$) at dinospore:host ratios ≥20:1.

Dinospore survival and infectivity. Size fractionation of infected host cultures provided stocks of recently formed dinospores (≤6 h old) with initial densities of $9.5 \pm 2.0 \times 10^3$ mL^{-1} from *A. sanguinea*, $30 \pm 3.5 \times 10^3$ mL^{-1} from *K. micrum*, and $43 \pm 5.3 \times 10^3$ mL^{-1} from *G. instriatum*. From the onset of the experiment, dinospore abundance for *Amoebophrya* strains from *G. instriatum* and *K. micrum* exhibited an exponential decay, falling below detection levels (~250 cells·mL^{-1}) by days 3 and 13, respectively (Fig. 2). By contrast, dinospores harvested from *A. sanguinea* increased in number by ~25% during the first 16 h, suggesting some cell division following size fractionation of the original culture. After 16 h, dinospores from *A. sanguinea* declined exponentially, falling below detection levels by day 5. Exponential curves fitted to data for dinospore abundance gave growth constants (k) of −1.020 ± 0.149 ($r^2 = 0.9567$; $P = 0.0001$), −0.476 ± 0.070 ($r^2 = 0.9442$; $P < 0.0001$), and −0.257 ± 0.0240 ($r^2 = 0.9572$; $P < 0.0001$) for *Amoebophrya* strains infecting *G. instriatum*, *A. sanguinea*, and *K. micrum*, respectively.

The ability of dinospores to initiate infections differed considerably among the three *Amoebophrya* strains and varied dramatically with dinospore age (Fig. 3). Only 4.7 ± 0.34% of recently formed dinospores from *G. instriatum* successfully invaded host cells, compared with 9.0 ± 0.81% and 18.0 ± 1.76% for dinospores from *K. micrum* and *A. sanguinea*, respectively. The percentage of harvested dinospores that successfully established infections (Fig. 3A) declined with dinospore age, more or less paralleling patterns in dinospore survival; however, the ability of surviving dinospores (i.e. those still detectable by epifluorescence microscopy) to infect host cells exhibited a markedly different pattern (Fig. 3B). Success of surviving dinospores declined rapidly with age for *Amoebophrya* strains infecting *G. instriatum* and *A. sanguinea*, with values dropping below detection levels (~0.1%) by days 2 and 5, respectively. By contrast, the success of surviving dinospores from *K. micrum* increased by a factor of two during the first day, stabilized at ~18% over the following 3 days, and then gradually declined with some infections still being established when dinospores were 11 days old.

Generation time. Inoculation of *A. sanguinea* and *G. instriatum* with dinospores at a 1:1 ratio produced increasing numbers of infected hosts over the first 20–24 h, with values stabilizing at 470 ± 9 cells·mL^{-1} ($n = 6$; T_{24}-T_{48}) and 100 ± 6 cells·mL^{-1} ($n = 7$; T_{20}-T_{44}), respectively, before death of host cells and formation of new dinospores (Fig. 4, A and C). Comparable inoculation of *K. micrum* resulted in a more gradual increase in parasitized hosts over 36 h, with subsequent values averaging 170 ± 7 cells·mL^{-1} (T_{36}-T_{44}; $n = 3$) before the appearance of new dinospores (Fig. 4E). By contrast, inoculation of cultures at high dinospore:host ratios resulted in maximum numbers of parasitized cells within 16 h for all three host species

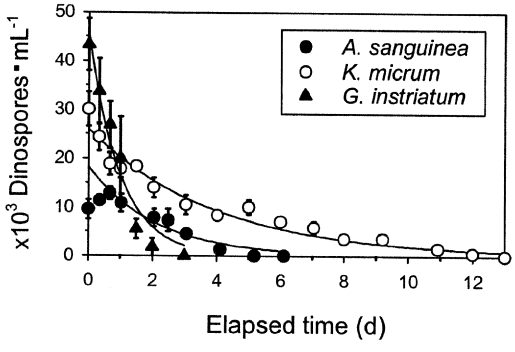

FIG. 2. Survival of *Amoebophrya* spp. dinospores from infected *Akashiwo sanguinea*, *Karlodinium micrum*, and *Gymnodinium instriatum*. Dinospores were harvested ≤6 h after formation, with abundance determined over time using epifluorescence microscopy. Error bars indicate SE.

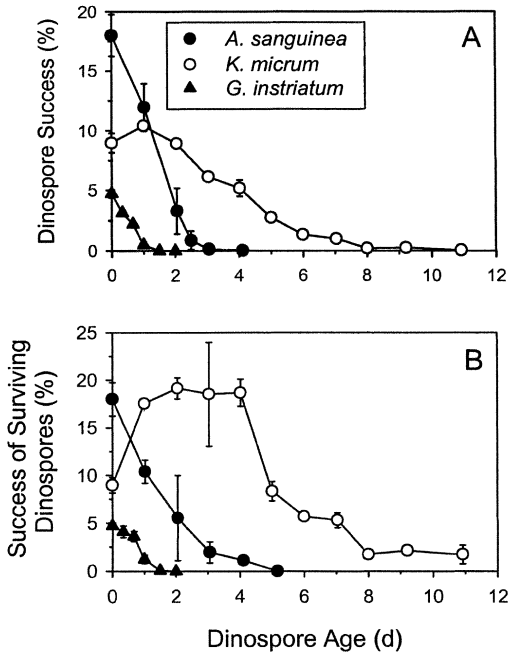

FIG. 3. Ability of dinospores of different age to infect *Akashiwo sanguinea*, *Karlodinium micrum*, and *Gymnodinium instriatum*. (A) Percent of harvested dinospores that successfully infected host cells. (B) Percent of surviving dinospores (i.e. those detectable by epifluorescence microscopy) that established infections. Error bars indicate SE.

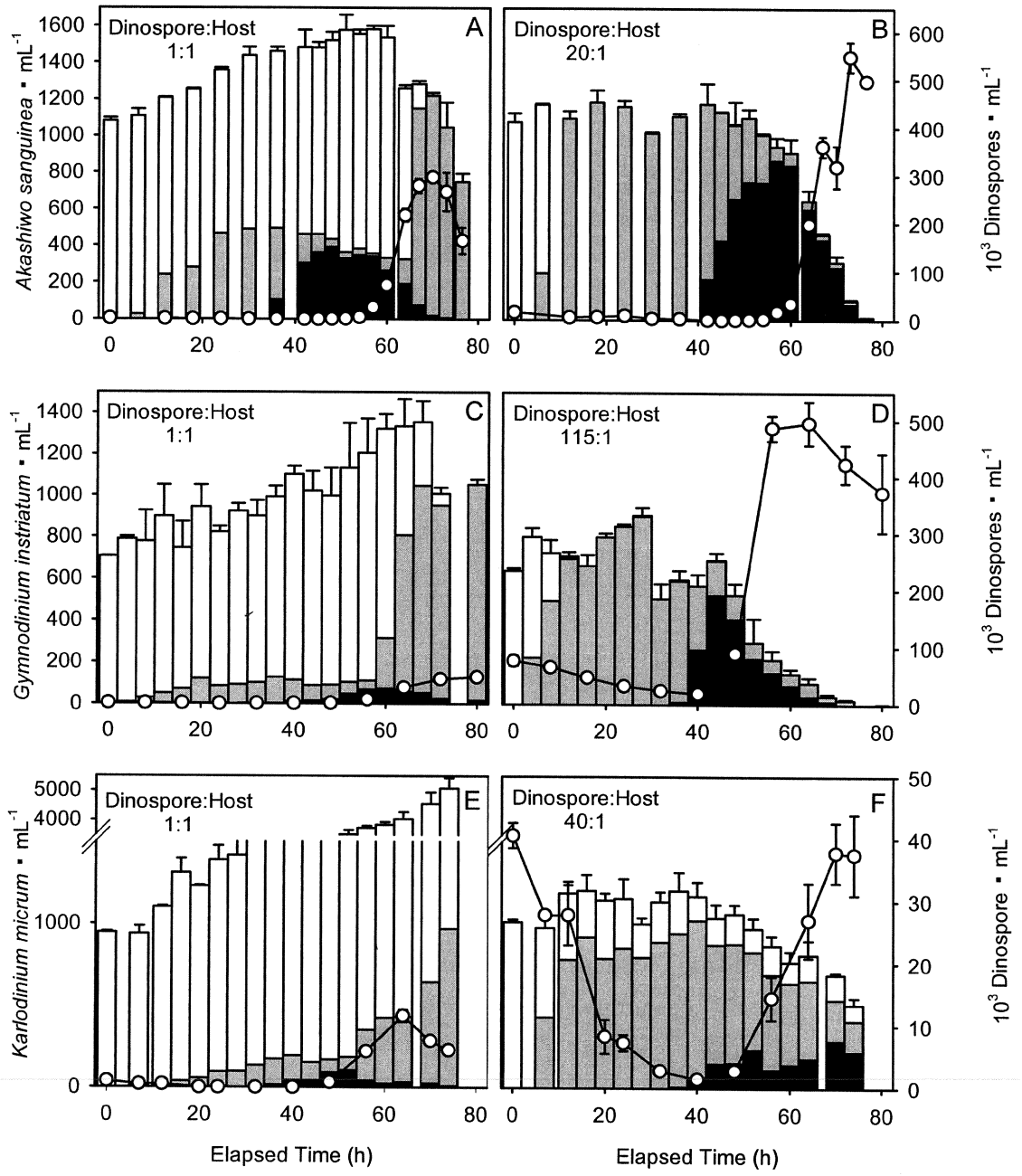

FIG. 4. Time-course studies of *Amoebophrya* spp. infecting *Akashiwo sanguinea*, *Gymnodinium instriatum*, and *Karlodinium micrum* inoculated at high and low dinospore:host ratios. Bars show cumulative host abundance with white, gray, and black regions representing uninfected hosts, early infections, and late infections, respectively. Open circles are dinospore abundance. Errors bars indicate SE.

(Fig. 4, B, D, and F), with the abundance of infected hosts averaging 1120 ± 24 (n = 8; T_{12}-T_{48}), 700 ± 47 (n = 9; T_{12}-T_{44}), and 890 ± 27 (n = 8; T_{16}-T_{44}) cells·mL^{-1} for *A. sanguinea* (20:1), *G. instriatum* (115:1), and *K. micrum* (40:1), respectively, until host densities began to decline.

Early and late stage infections showed discrete peaks during the first parasite generation in all treatments except for the 40:1 inoculation of *K. micrum* (Fig. 4, A–F). In that treatment, many parasites failed to complete the infection cycle before the end of the experiment, with high numbers of late stage infections (220 ± 12 cells·mL^{-1}) remaining in the final sample. Infections occurred in the nucleus of *A. sanguinea*, in the cytoplasm of *G. instriatum*, and in either the nucleus or cytoplasm of *K. micrum* (Fig. 5). Host cells exposed to high densities of dinospores (i.e. 20:1, 40:1, and 115:1 dinospore:host treatments) were infected by multiple dinospores. In those treatments, more than one late-stage parasite developed inside

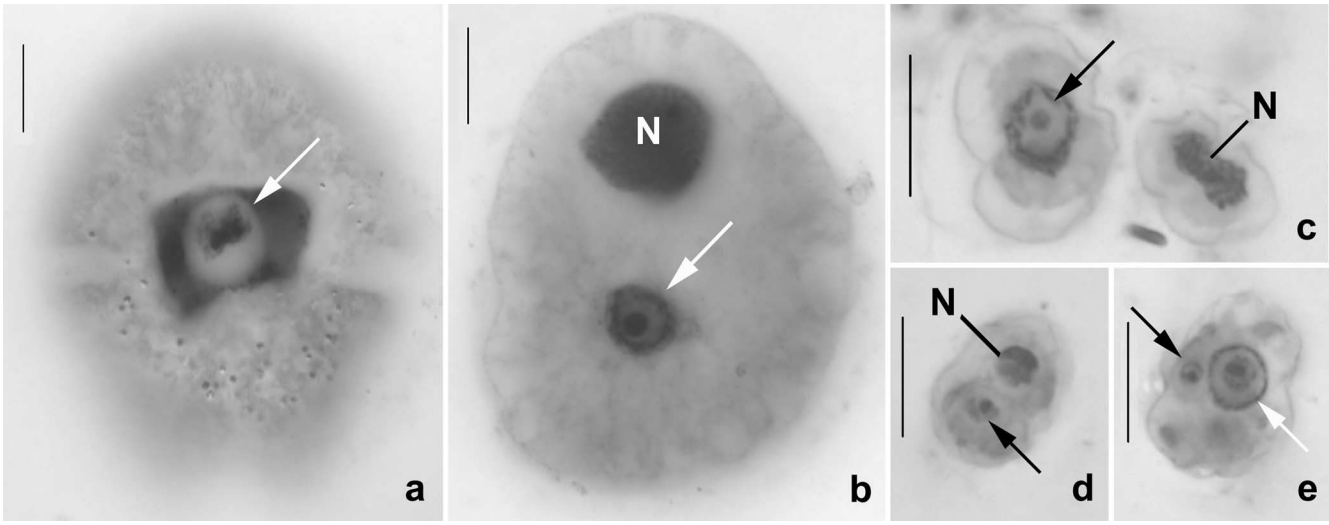

FIG. 5. Protargol-stained specimens showing location of *Amoebophrya* spp. inside host cells. Scale bars, 10 μm. (a) *Akashiwo sanguinea* with intranuclear parasite (arrow). (b) *Gymnodinium instriatum* with intracytoplasmic parasite (arrow); host nucleus (N). (c) An uninfected *Karlodinium micrum* showing the nucleus (N), and an infected *K. micrum* with an intranuclear parasite (arrow). (d) *Karlodinium micrum* with intracytoplasmic parasite (arrow); host nucleus (N). (e) *Karlodinium micrum* with two parasites, one in the nucleus (white arrow) and one in the cytoplasm (black arrow).

most cells of *G. instriatum* and *K. micrum*, but with very rare exception, only a single mature parasite was generated in *A. sanguinea*.

Parasite intracellular development time averaged 57 ± 3.3 h, 46 ± 1.1 h, and 34 ± 3.8 h, respectively, for *G. instriatum*, *A. sanguinea* and *K. micrum* cultures inoculated at a dinospore:host ratio of 1:1. One-way analysis of variance (ANOVA) revealed significant difference among means ($P = 0.03$), with pair-wise comparisons indicating longer development time for infections of *G. instriatum* relative to those of *K. micrum* ($P < 0.05$; Tukey test). Estimates of parasite development time for high dinospore inoculations of *A. sanguinea* (58 ± 0.4 h; 20:1 dinospore:host ratio) were consistently longer than for low inoculations, whereas those of *G. instriatum* (46 ± 0.01 h; 115 dinospore: host ratio) were consistently shorter than for corresponding low inoculations. Comparison of data using two-way ANOVA showed no significant difference for host species or inoculum level (low versus high dinospore:host ratio) but did show a significant interactive effect ($P = 0.003$), with pair-wise analysis indicating a significant increase in development time for high versus low inoculations of *A. sanguinea* ($P = 0.004$) and a significant decrease in development time for high versus low inoculations of *G. instriatum* ($P = 0.004$; Tukey test). High dinospore inoculations also appeared to increase the infection cycle of *Amoebophrya* sp. in *K. micrum* (cf. temporal patterns for late-stage infections; Fig. 4, E and F); however, the persistence of large numbers of infected cells in that treatment prevented accurate determination of parasite intracellular development time.

Dinospore densities exhibited distinct peaks or reached stationary levels by the end of the experiment in all low dinospore:host inoculations and in the high inoculum treatment for *G. instriatum*, thus permitting calculation of total parasite generation time (i.e. time required for infection of hosts, intracellular development, vermiform emergence, and extracellular maturation). The persistence of late stage infections and/or lack of a clear peak in dinospore density prevented accurate estimates of parasite generation time for high dinospore:host inoculations of *A. sanguinea* and *K. micrum*. Estimates for parasite generation time averaged 71 ± 0.6 h, 67 ± 0.7 h, and 59 ± 0.02 h in 1:1 treatments for *G. instriatum*, *A. sanguinea*, and *K. micrum*, respectively, with all means being significantly different (one-way ANOVA; Tukey test; $P < 0.05$). Data for the 115:1 dinospore:host inoculation of *G. instriatum* indicated a significantly shorter parasite generation (55 ± 2.5 h; $P = 0.025$, *t*-test) than that for the corresponding 1:1 treatment.

Cross infection. Dinospores of *Amoebophrya* sp. from *K. micrum* failed to infect *A. sanguinea* and *G. instriatum*, even though inoculum size (dinospore:host ratio of 50:1) was sufficient to produce high parasite prevalence (93.1 ± 1.22% from 12 h to 96 h after inoculation; n = 8) in the primary host species (Fig. 6A). Inoculation of *G. instriatum* with complementary dinospores (dinospore:host ratio of 50:1) generated infection levels of 83%–98% during the first parasite generation and 100% thereafter (Fig. 6B). The same inoculum produced no infections in *A. sanguinea* but did result in 26.5 ± 4.50% infection of *K. micrum* at T_{12}. Interestingly, parasite prevalence in *K. micrum* declined to zero over the following 36 h, and there was no indication that a second generation of infections developed in subsequent samples. Similarly, inoculation of *A. sanguinea* with complementary dinospores

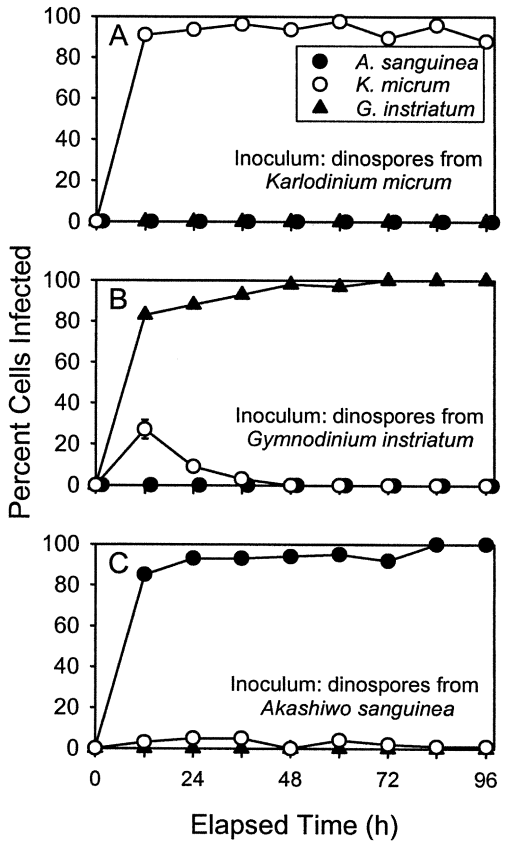

FIG. 6. Infection of *Akashiwo sanguinea*, *Gymnodinium sanguineum*, and *Karlodinium micrum* when exposed to dinospores from three strains of *Amoebophrya*. Dinospore inoculations were adjusted to give 80%–90% infection levels in the primary host species. Error bars indicate SE.

(inoculum size 10:1) produced high parasite prevalence (91.8 ± 1.41% until T_{72}) that increased to 100% in the second parasite generation (Fig. 6C). Equivalent inoculations did not stimulate parasitism of *G. instriatum* but generated low infection levels (≤5%) in *K. micrum*. Parasites that formed in *K. micrum* from dinospores derived from *A. sanguinea* also appear unable to produce a second generation of infections.

DISCUSSION

Cachon (1964) noted that *Amoebophrya ceratii* formed cytoplasmic infections in athecate dinoflagellates and nuclear infections in all thecate species, except *Prorocentrum micans* Ehrenberg. Subsequent reports demonstrated that nuclear infections can occur in some athecate dinoflagellates but continued to supported the idea that infections were site specific within each host species (Coats and Bockstahler 1994, Coats et al. 1996). *Amoebophrya* sp. from *K. micrum* is a clear exception to the general pattern of parasites being specific to particular regions of host cells, as infections occurred in both the nucleus and cytoplasm. Prior studies have also shown that individual host cells can be infected by multiple dinospores; however, the fate of those infections was not determined (Coats et al. 1996, Yih and Coats 2000). Multiple infection of *G. instriatum* and *K. micrum* in our high dinospore:host treatments resulted in the formation of more than one "beehive" stage, with parasites restricted to the cytoplasm of *G. instriatum* but occurring simultaneously in the cytoplasm and nucleus of *K. micrum*. Interestingly, infection of *A. sanguinea* by multiple dinospores produced a single mature parasite, suggesting either that one of the trophonts became dominant and prevented growth of the others or that several trophonts fused to form a syncytium before maturation of the parasite. Although sexuality has not been reported for *Amoebophrya* spp., development of a syncytium might provide a mechanism for genetic recombination via fusion of nuclei from different dinospores.

Infectivity and survival of dinospores exhibited distinct differences among our three strains of *Amoebophrya*. Recently formed dinospores from *A. sanguinea* were roughly twice as likely to infect host cells as were dinospores from *K. micrum* and four times more likely than dinospores from *G. instriatum*. Furthermore, dinospores from *G. instriatum* and *A. sanguinea* were short lived and unable to initiate infections after 2–5 days, respectively, whereas dinospores from *K. micrum* maintained high infectivity for 4–5 days, with some being able to parasitize hosts after 11 days. The prolonged survival of dinospores from *K. micrum* is surprising, given that *Amoebophrya* spp. are thought to be strictly parasitic. Our observations suggest that dinospores of *Amoebophrya* sp. from *K. micrum* may be able to use bacteria and/or dissolved organic substances as a source of nutrition or may be able to form resting stages that become active after a short period of dormancy.

Susceptibility of host cells to infection also differed among the three host–parasite systems. *Akashiwo sanguinea* and *G. instriatum* appeared completely susceptible to parasitism, as high dinospore:host treatments showed 100% infection levels. By contrast, approximately 10% of the *K. micrum* cells remained uninfected at intermediate to high dinospore:host treatments. Although we cannot rule out the possibility that higher inoculations would have produced 100% parasite prevalence, available data suggest that some specimens of *K. micrum* are resistant to infection. The source of this apparent resistance is unknown but may be related to cell cycle events that render *K. micrum* unattractive to dinospores or capable of fending off invading parasites.

Observed differences in endurance and infectivity of dinospores among *Amoebophrya* strains, along with discrepancies in the susceptibility of host species to infection, suggest that the three host–parasite systems have evolved somewhat divergent survival strategies. Although *A. sanguinea* and *G. instriatum* appear wholly susceptible to infection, dinospores of their parasites are short lived and quickly loose the ability to infect host cells. Thus, these strains of *Amoebophrya* seem capable of rapidly exploiting dense host populations but less likely to maintain high infection levels under non-

bloom conditions, due to limited survival and time constraints in encountering host cells. By contrast, dinospores of *Amoebophrya* sp. from *K. micrum* are very long lived, providing increased opportunity to find hosts cells even when present at low densities. Nonetheless, persistence of *K. micrum* in the face of high parasite prevalence seems assured, as some host cells appear resistant to infection.

The life cycle of *Amoebophrya* sp. consists of an intracellular phase during which parasites grow to maturity inside host cells and an extracellular phase in which the mature parasite emerges from the host cell, divides to produce dinospores, and then infects new hosts. Earlier studies have estimated the duration of the intracellular phase of *Amoebophrya* sp. ex *A. sanguinea* and provided information on the timing of dinospore formation but have not determined the entire generation time of the parasite (Coats and Bockstahler 1994, Yih and Coats 2000). Data for our three host–parasite systems indicate that the intracellular phase only represents 60%–80% of total parasite generation time. In addition, the duration of the intracellular phase appeared to be influenced by parasite load. Inoculation of cultures at a 1:1 dinospore:host ratio typically generated one parasite per infected host cell, with intracellular development time averaging 34 ± 3.8 h, 46 ± 1.1 h, 57 ± 3.3 h in *K. micrum*, *A. sanguinea*, and *G. instriatum*, respectively. Inoculation of cultures at high dinospore:host ratios stimulated infection of host cells by several dinospores, with parasite generation time in *A. sanguinea* increasing by 12 h (to 58 ± 0.4 h) but that of *G. instriatum* decreasing by 11 h (to 46 ± 0.01 h). High dinospore inoculations also result in a significantly shorter total parasite generation time for *G. instriatum* infections relative to low dinospore: host treatments. That multiple infection by dinospores had opposite effects on intracellular development times of *A. sanguinea* and *G. instriatum* may be related to corresponding differences in parasite interaction. Competition between invading dinospores or fusion of trophonts into a syncytium may retard parasite maturation in *A. sanguinea*, whereass independent growth of multiple parasites in *G. instriatum* may result in more rapid utilization of host resources.

Amoebophrya strains from *A. sanguinea*, *G. instriatum*, and *K. micrum* either failed to establish infections or were unable to successfully complete their life cycle in the presence of nonprimary host species. Results are consistent with the parasites being host specific and also suggest that host specificity in *Amoebophrya* strains involves more than parasite recognition of host cells, as *Amoebophrya* spp. from *A. sanguinea* and *G. instriatum* were able to invade *K. micrum* but did not develop to maturity. *Karlodinium micrum* is considered to be a toxic dinoflagellate (Li et al. 2000) and thus may represent a very different environment for *Amoebophrya* spp. than the other two nontoxic host species. Dinospores of *Amoebophrya* strains from *A. sanguinea* and *G. instriatum* that did invade *K. micrum* may have succumbed to inappropriate growth conditions. Alternatively, *K. micrum* may be able to successfully mount a defense against dinospores from the other two host species. As mentioned above, some cells of *K. micrum* appear resistant to dinospores from their corresponding *Amoebophrya* sp. Perhaps all *K. micrum* have the ability to defend against invading parasites to some degree, with the response simply being more successful when mounted against dinospores from a "foreign" *Amoebophrya* sp.

Amoebophrya strains cultured in association with *A. sanguinea*, *G. instriatum*, and *K. micrum* exhibited marked differences in host–parasite biology. These differences along with results of our cross-infections experiments and reported genetic divergence among the parasite strains (Gunderson et al. 2000) support the contention that *Amoebophrya ceratii* is a species complex consisting of several host-specific taxa (Coats et al. 1996). Host specificity may make *Amoebophrya* spp. more attractive than nonspecific parasites like *Parvilucifera infectans* (Erard-Le Denn et al. 2000) in the biological control of bloom-forming dinoflagellate.

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