# FISH Probes for the Detection of the Parasitic Dinoflagellate *Amoebophrya* sp. Infecting the Dinoflagellate *Akashiwo sanguinea* in Chesapeake Bay

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ABSTRACT. A comparison of the small subunit rRNA sequences of a Chesapeake Bay strain of the dinoflagellate *Akashiwo sanguinea* and the dinoflagellate *Amoebophrya* sp. parasitizing it revealed several potential target sites that could be used to detect the parasite through in situ hybridization. The fluorescence of probed cells under various conditions of hybridization was measured by using a spot meter on a Nikon UFX-II camera attachment so that the effect of various hybridization parameters on probe binding could be determined. Probes directed against both the junction between helices 8 and 11 and helix 46 could detect the parasite, although the helix 8/11 probe produced a stronger signal under the conditions tested. The fluorescence of the probed cells increased with increasing hybridization time up to approximately twelve hours. The background fluorescence was lower at the wavelengths used to detect Texas Red than at those used to detect fluorescein, so probed cells were more distinct when Texas Red was used as the label. Cells stored in cold paraformaldehyde for a year still bound the probes. Young stages of the parasite could be seen more readily after in situ hybridization than after protargol impregnation.

Key Words. Alveolate, dinoflagellate, fluorescein, hybridization, in situ, parasite, rRNA, small subunit, Syndiniophyceae, Texas Red.

SEVERAL free-living dinoflagellate species in Chesapeake Bay are hosts for parasitic dinoflagellates in the genus *Amoebophrya*. Since the infection is lethal, *Amoebophrya* can potentially be used as a biological control agent to suppress toxic dinoflagellate blooms. The parasite develops in the nuclei of *Akashiwo sanguinea* and *Scrippsiella trochoidea*, but in the cytoplasm of *Ceratium furca* and *Gyrodinium uncatenum*. *Amoebophrya* dinospores isolated from *A. sanguinea* will readily reinfect cells of the same host species, but will not infect *C. furca*, *G. uncatenum* nor *S. trochoidea* (Coats et al. 1996), suggesting that more than one species of *Amoebophrya* may be present. This may also be true of "*Amoebophrya ceratii*" from European waters, as Cachon (1964) noted morphological and developmental differences correlated with the host species.

Fluorescent In Situ Hybridization (FISH) probes directed against unique regions of the parasites' rRNAs could potentially be used to determine the infection rates of host dinoflagellate species in plankton samples. While this can also be accomplished by standard fixation and staining procedures (Coats and Bockstahler 1994), FISH probes offer the important advantage of discriminating between potential targets at the nucleotide sequence level (Amann et al. 1990). If rRNA sequence differences exist among *Amoebophrya* in Chesapeake Bay, FISH probes would offer a way of determining the host range of each of the genotypes found as well as the infection levels of each parasite genotype in the various host species.

FISH probes targeting prokaryotic rRNAs are now widely used in microbial ecology, but there are fewer publications describing their use in studying protistan ecology (e.g. Adachi et al. 1996; Caron et al. 1999; Knauber et al. 1996; Lange et al. 1996; Lim et al. 1996; Lim et al. 1999; Miller and Scholin 2000; Rice et al. 1997; Scholin et al. 1996; Simon et al. 2000; Stothard et al. 1999). Less is known about the accessibility to probes of potentially useful variable sites in eukaryotic small subunit and large subunit rRNAs, and about methods required to adequately fix and permeabilize eukaryotic cells. Anecdotal observations indicate that background fluorescence can be a problem when targeting eukaryotic cells. The purpose of the project was to determine whether FISH probes could be used to detect *Amoebophrya* cells parasitizing other dinoflagellates, what incubation conditions produced the best signal-to-noise ratio, whether fluorescein or the rhodamine derivative Texas

Red was more suitable as a fluor, and how the signal might be affected by storage conditions.

## MATERIALS AND METHODS

**Probe construction.** The *Amoebophrya* strain infecting *Akashiwo sanguinea* in Chesapeake Bay (Coats and Bockstahler 1994) was used to test FISH procedures, as it can be maintained in laboratory cocultures with its host. This strain was referred to as *Gymnodinium sanguineum* in earlier papers (e.g. Coats and Bockstahler 1994; Coats et al. 1996; Gunderson et al. 1999), but we are here referring to it as *Akashiwo sanguinea* in accordance with the taxonomic revision of Daugbjerg et al. (2000). In order to identify regions where the rRNA sequences from host and parasite differ, the small subunit rRNA sequence of *A. sanguinea* was determined and compared to the previously published sequence of its *Amoebophrya* parasite (Gunderson et al. 1999). *Akashiwo sanguinea* cells grown in culture as described (Gunderson et al. 1999) were collected and lysed in a solution of 4 M guanidium isothiocyanate and 0.1% beta-mercaptoethanol. Following purification of genomic DNA with a Wizard PCR Preps DNA Purification System (Promega, Madison, WI), small-subunit rRNA (SSU rRNA) sequences were amplified and then directly sequenced using a SequiTherm Cycle Sequencing Kit (Epicentre Technologies, Madison, WI) as described for *Amoebophrya* (Gunderson et al. 1999). Secondary structure diagrams of host and parasite rRNAs were prepared using RnaViz (De Rijk and De Wachter 1997) and compared with each other.

Two oligonucleotides complementary to regions in which the *Amoebophrya* and host sequences differed were synthesized with an Aminolink at the 5′-end (Operon Technologies, Inc., Alameda, CA) and labeled with either fluorescein or Texas Red (Molecular Probes, Eugene, OR) according to standard methods (DeLong 1993). A probe complementary to positions 294–311 of the *Amoebophrya* amplification product (at the junction of helices 8 and 11) had the sequence CTGAATGACGCAGCCCCG, while a probe complementary to part of helix 46 (positions 1504–1521) had the sequence TATCGGACAAGGTTGTAG.

**Fixation and hybridization.** Infected *A. sanguinea* cells were fixed by adding 16% electron microscopy grade paraformaldehyde (EM Sciences, Ft. Washington, PA) to the cell suspension to a final concentration of 6%. Aliquots of fixed cells were placed in the wells of Teflon-coated slides (Cel-Line Associates, Inc., Newfield, NJ) and allowed to air dry. Cells were post-fixed in methanol-formalin (DeLong 1993), rinsed briefly in water and dried again. Fifteen μl of hybridization solution (0.75 M NaCl, 5 mM EDTA, 100 mM Tris-HCl, pH 7.8, 0.1%

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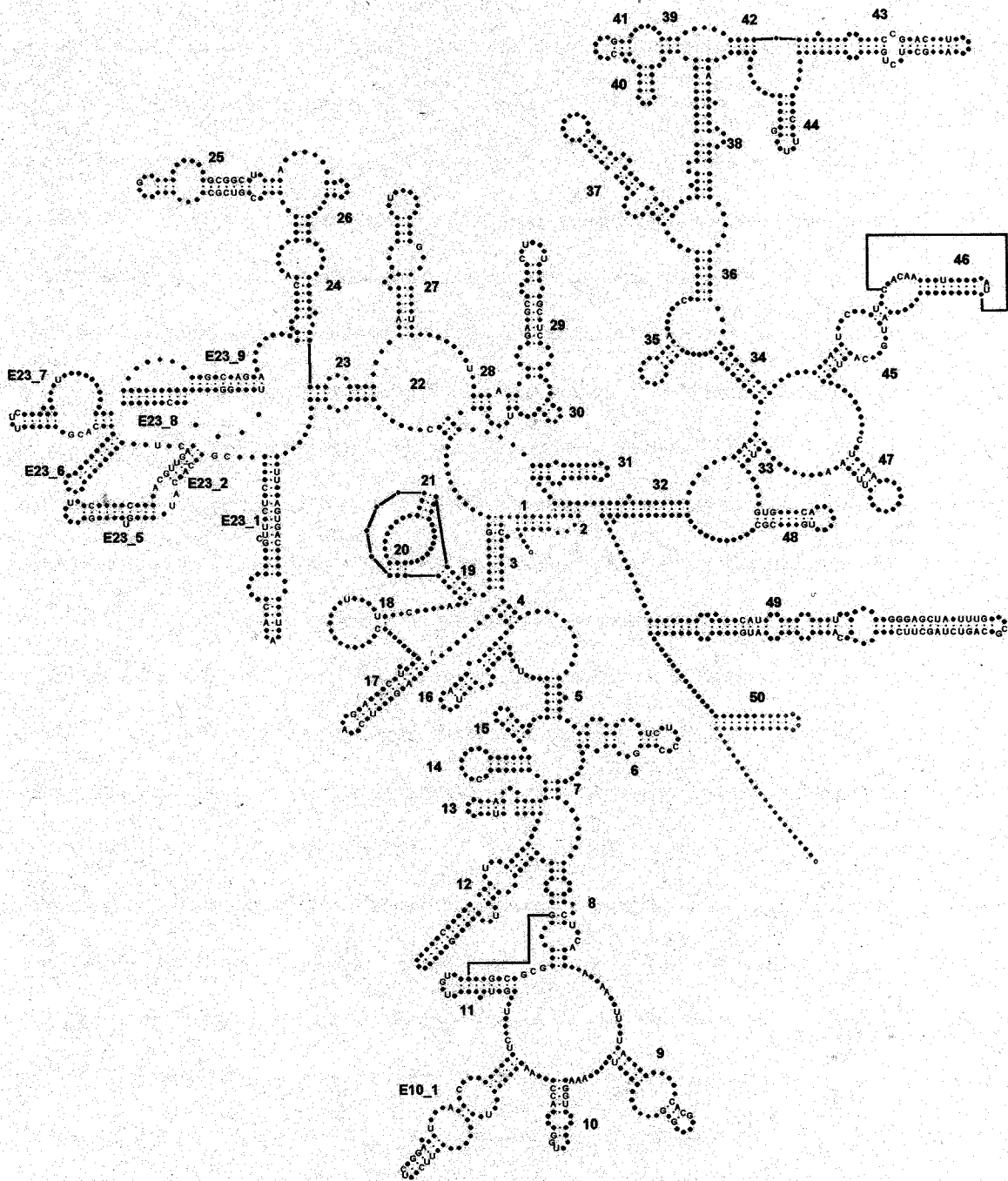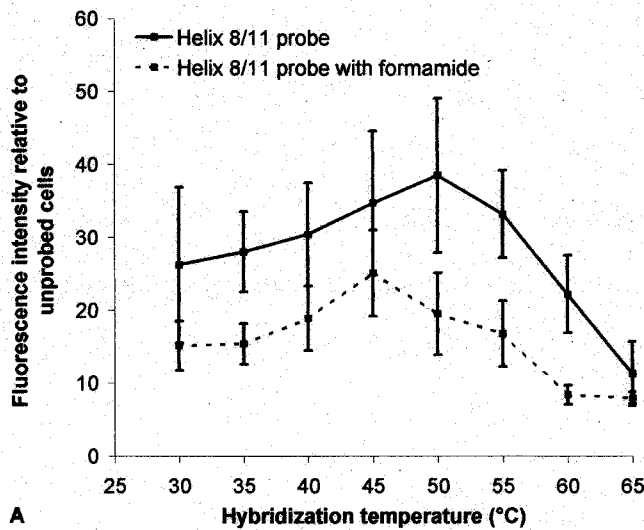The GenBank accession number for the *Akashiwo sanguinea* sequence is U41085.

Fig. 1. Superimposed secondary structure diagrams of the small subunit rRNA sequences from the host dinoflagellate *Akashiwo sanguinea* and its *Amoebophrya* parasite. Identical positions are represented by black dots, while letters represent the *Amoebophrya* sequence at the positions that differ between the two sequences. The gray dots at the 5'- and 3'-ends represent the rRNA sequences contained in the amplification primers. Black lines by helices 8, 11, and 46 indicate the 5'- and 3'-ends of the probe target sequences.
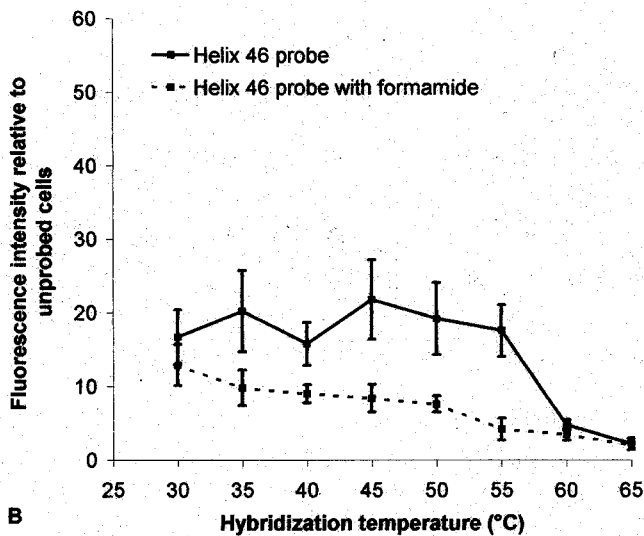
SDS, 10 ng/μl probe, with or without formamide to a final concentration of 10%) were added to each well. Slides were placed in a post-hybridization solution (0.75 M NaCl, 5 mM EDTA, 100 mM Tris-HCl, pH 7.8) and held at the hybridization temperature for 20 min. The slides were then dipped briefly in water and air-dried.

**Determination of relative fluorescence.** Prolong Antifade reagent (Molecular Probes, Eugene, OR) was added to each well, and the cells were then examined with a Nikon Microphot epifluorescence microscope using the appropriate barrier filters (Omega Optical, Brattleboro, VT) for observing either fluorescein or Texas Red fluorescence. Fluorescence was recorded by using a spot meter on a Nikon UFX-II camera attachment, which records light emission from a spot slightly smaller than a mature trophont and gives a value corresponding to the time required to expose film of a given ASA value. The values in the graphs represent fluorescence intensity compared to the fluorescence of unprobed infected *A. sanguinea* cells examined with barrier filters suitable for examining Texas Red fluorescence. That is, a cell twice as fluorescent as an unprobed cell

**A**  Hybridization temperature (°C)

— Helix 8/11 probe
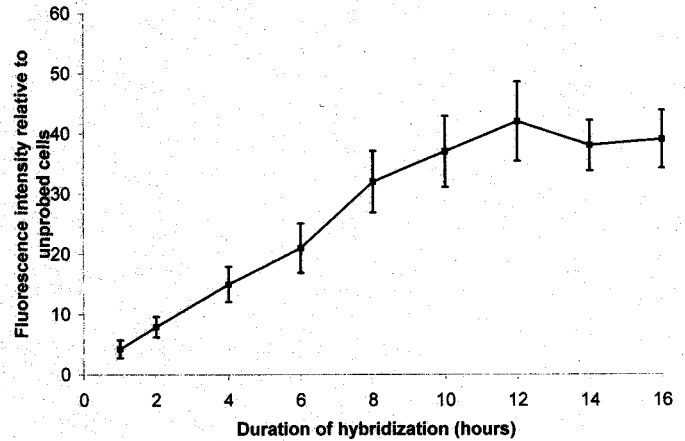- ■ - Helix 8/11 probe with formamide



Fig. 3. The effect of hybridization time on the fluorescence of probed *Amoebophrya* cells. Fluorescence increased with the length of hybridization time up to approximately 10–12 h (Texas Red-labelled helix 8/11 probe, 45 °C incubation).

length of time necessary for obtaining a strong signal. Since formamide is sometimes used in hybridization solutions to increase stringency without having to raise the incubation temperature, we tested its effect on the fluorescent signal by adding formamide to a final concentration of 10% to the hybridization solution. Hybridizations were initially conducted in parallel with Texas Red and fluorescein-labelled probes in order to determine whether one probe produced a better signal than the other.

The effect of storage on signal intensity was tested with cells kept in fixative for varying lengths of time and with cells that had been stored dried on slides for varying lengths of time. Infected *Akashiwo* cells fixed in 6% paraformaldehyde were divided into two aliquots. One aliquot was kept in paraformal-
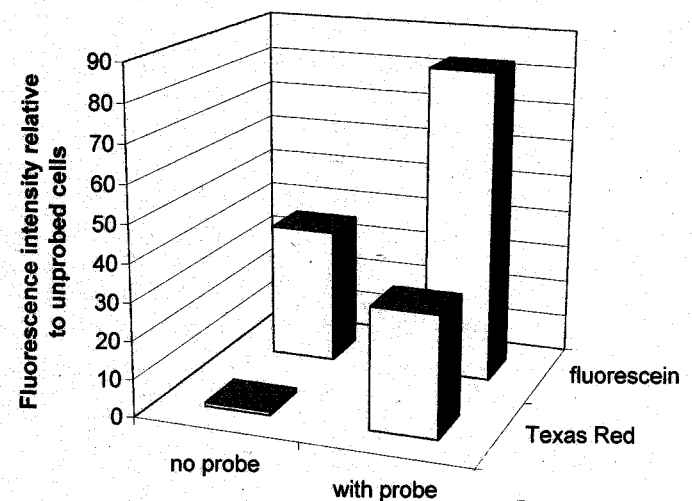


**B**  Hybridization temperature (°C)

— Helix 46 probe
- ■ - Helix 46 probe with formamide

Fig. 2. The effect of temperature and formamide on binding of the probes for the parasitic dinoflagellate *Amoebophrya*. **A.** Binding of the helix 8/11 probe. Binding of the probe began to decrease at an incubation temperature above 45 °C in the presence of 10% formamide, and above a temperature of 50 °C in its absence. The fluorescence was weaker in the presence of formamide. **B.** Binding of the helix 46 probe. The probe did not produce as strong a signal as the helix 8/11 probe, and formamide further reduced its fluorescence (Texas Red-labelled probe, 8 h hybridization).

is given a value of "2". For each set of hybridization parameters tested, the fluorescence of 10 cells was determined and compared to the average fluorescence value of 10 cells held under the same incubation conditions, but without probe. Exceptions to this procedure are described in the relevant figure captions.

**Parameters tested.** Hybridization temperatures of 30–65°C were used in order to determine the range of temperatures over which a suitably strong signal could be obtained and over which the probe would bind to parasite but not host rRNA. One- to16-hour hybridization periods were used in order to determine the



Fig. 4. Comparison of fluorescein and Texas Red-labeled probes for detection of the parasitic dinoflagellate *Amoebophrya*. Unprobed cells examined with the Texas Red barrier filters have a relative fluorescence value of one (since all fluorescence values given are in comparison with unprobed cells examined with barrier filters for Texas Red). Cells probed using a fluorescein label were brighter than those probed using a Texas Red label. However, the intrinsic fluorescence of unprobed cells was much higher at the wavelengths used to detect fluorescein, so the signal-to-noise ratio was better when using a Texas Red-labeled probe (helix 8/11 probe, 10 h hybridization at 45 °C).
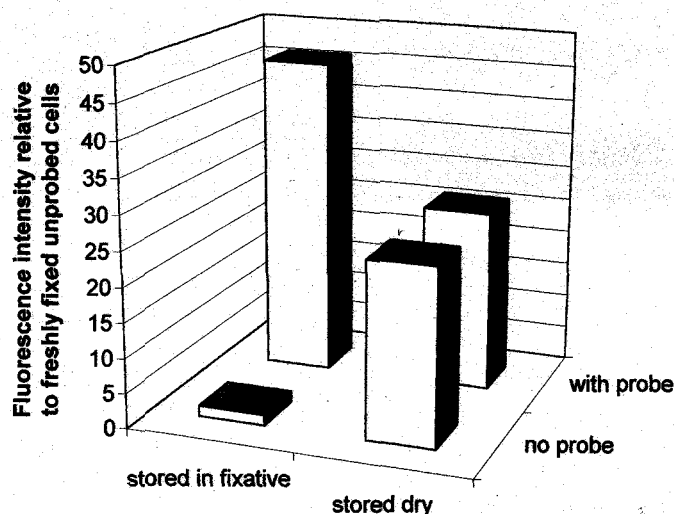
Fig. 5. The effect of storage for a year on the fluorescence of *Amoebophrya* cells during in situ hybridization. Fluorescence values represent the fluorescence intensities relative to those of freshly fixed unprobed cells examined with Texas Red barrier filters. Unprobed cells that were stored in fixative for a year before being examined with barrier filters for Texas Red were 1.5 times as fluorescent as freshly fixed unprobed cells examined in the same way. Unprobed cells that were stored dry on a slide for a year were as fluorescent as cells stored dry and then incubated with Texas Red-labeled probes. Unprobed cells that were stored in fixative for a year were much less fluorescent than cells stored in fixative for a year and then incubated with Texas Red-labeled probes. Dry storage increased the intrinsic fluorescence of the cells and reduced their ability to bind probe (Texas Red-labeled helix 8/11 probe, 10 h hybridization at 45 °C).
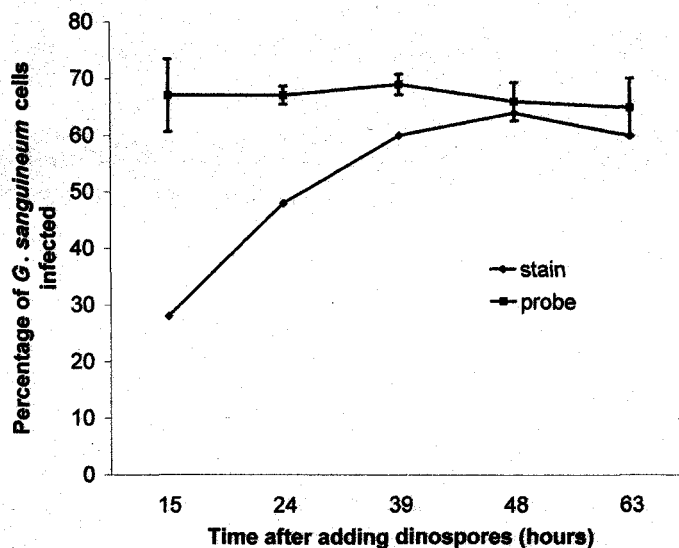


Fig. 6. More of the small, young intracellular stages (i.e. those that infected a host less than 39 h previously) of *Amoebophrya* can be detected with FISH probes than with protargol impregnation (Texas Red-labelled helix 8/11 probe, 10 h hybridization at 45 °C).

dehyde at 4°C, while cells from the second aliquot were immediately attached to slides, post-fixed in methanol-formalin, and stored dry. At approximately monthly intervals over the course of a year, cells stored in fixative were attached to slides and cells from both aliquots were then probed.

FISH probes and protargol impregnation were compared for their efficacy in detecting *Amoebophrya* infections. *Akashiwo sanguinea* cells were collected 15, 24, 39, 48, and 63 h after *Amoebophrya* dinospores had been added to their culture. Aliquots from each sample were then impregnated with protargol (Coats and Bockstahler 1994) or incubated with FISH probes and examined for parasites. One hundred protargol-impregnated host cells were examined for parasites at each time point. Four samples from each time point were examined by FISH, and 100–232 host cells were examined from each sample.

## RESULTS AND DISCUSSION

The *A. sanguinea* amplification product was 1,827 bases long. The sequence from our Chesapeake Bay strain of *A. sanguinea* (GenBank accession number AF276818) differs from that of a previously determined *A. sanguinea* sequence (Gast and Caron 1996; GenBank accession number U41085) at three positions. The SSU rRNA of *Amoebophrya* is identical with that of its host at 83% of the positions between the amplification primer sites (Fig. 1). The two 18-base regions in the *Amoebophrya* sequence chosen as probe targets (positions 294–311 and 1504–1521 of the amplification product) differ from the homologous regions of *A. sanguinea* by six and eight nucleotides, respectively. Other potential target sites could also have been used, but these were chosen based on prior success using these regions as target sites in other organisms (Gunderson and Goss, unpubl.).

Both probes were bound by *Amoebophrya* rRNA, and either probe served to reveal the parasite in host cells. However, the helix 8/11 probe (Fig. 2A) produced a stronger signal than the helix 46 probe (Fig. 2B). Under the conditions tested, the strongest signal obtained with the helix 8/11 probe in the absence of formamide was at a temperature of approximately 50 °C, while the strongest signal obtained with the helix 46 probe was at approximately 45 °C. Including formamide decreased the optimum temperature approximately 5 °C and also decreased the intensity of the signal somewhat (Fig. 2).

Even when hybridization was conducted at temperatures well below the optimum, parasites were readily visible in host cells. The sequence differences between host and parasite rRNAs in the target regions are so great that there was no significant binding of probe by host cells, and it was not necessary to carefully adjust the hybridization temperature in order to selectively reveal the parasites. The intensity of the signal increased with the length of hybridization up to approximately twelve hours (Fig. 3), which is a longer hybridization time than is generally thought necessary (e.g. DeLong 1993). Although the fluorescence of probed cells was already easily detectable after two hours of hybridization, and well above background, cells incubated with the probe for longer periods were several times as bright. Factors contributing to differences in optimum hybridization times for different cell types might include differences in permeability of the cells, differences in the thickness of the fixed cytoplasm traversed by probes before reaching their targets or the relative proportion of probe and target in the samples. Several hundred *Amoebophrya* cells were usually present in each slide well, and much more target rRNA would therefore have been present in our system than on slides containing an equivalent number of bacteria, for which in situ hybridization procedures were originally worked out. In any case, it was advantageous to use an atypically long hybridization period for best results.

Texas Red is superior to fluorescein as a label for these organisms (Fig. 4). Although cells that bound a fluorescein-labelled probe were brighter than cells that bound a Texas Red-labelled probe, unprobed host and parasite cells had a strong intrinsic fluorescence at the wavelengths used for fluorescein
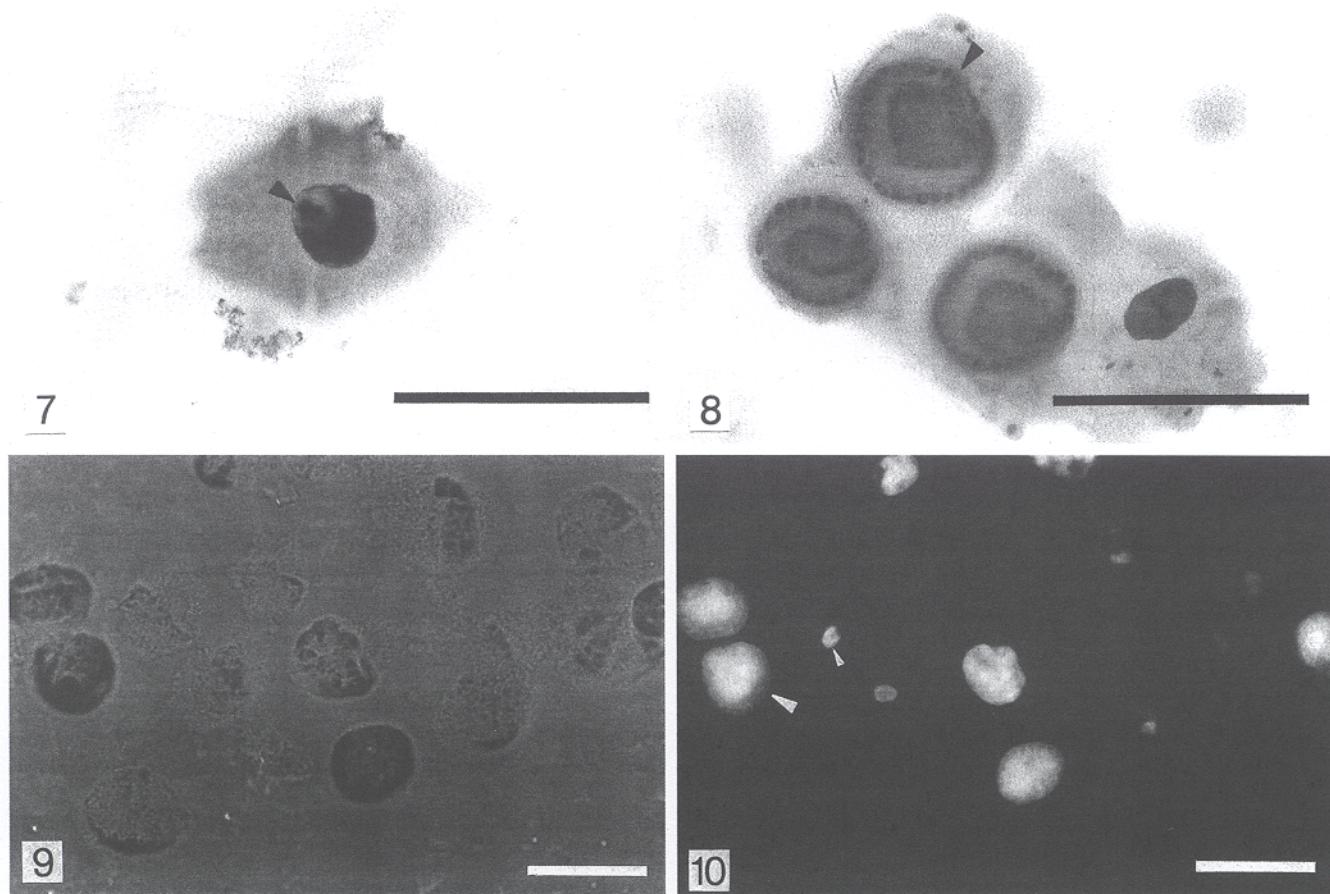
Fig. **7–10**.   Cells of the dinoflagellate *Akashiwo sanguinea* infected by the dinoflagellate *Amoebophrya*. **7.** *Amoebophrya* (arrowhead) beginning to grow in the host cell nucleus (protargol impregnation). **8.** Three mature *Amoebophrya* trophonts in three host cells (one indicated by arrowhead). The trophonts contain numerous nuclei and the host nuclei have been destroyed (protargol impregnation). **9.** Field of infected *A. sanguinea* cells seen with transmitted light following in-situ hybridization (Texas Red-labelled helix 8/11 probe, 10-h hybridization at 45 °C). **10.** The same field of cells seen through barrier filters for detecting Texas Red fluorescence. Parasites just beginning to grow in host nuclei may be seen (one indicated by small arrowhead) as well as large multinucleate trophonts (one indicated by large arrowhead). In situ hybridization reveals the cytoplasm of the parasite, whereas protargol impregnation stains the nuclei most darkly (In all figures, bar = 50 μm).

detection. Intrinsic fluorescence is sometimes attributed to chlorophyll or its breakdown products, but this cannot explain the fluorescence of *Amoebophrya*. Treating cells with sodium borohydride (DeLong 1993) did not reduce this background fluorescence. This fluorescence is lower at the wavelengths used for Texas Red detection, which made the parasites more distinguishable when using a Texas Red label. Cells that bound a fluorescein-labelled probe were two to three times as fluorescent as unprobed cells, whereas cells that bound a Texas Red-labelled probe were usually 30–40 times as fluorescent as unprobed cells.

Cells may be stored in 6% paraformaldehyde at 4 °C for long periods of time. There was no significant loss of signal or increase in background fluorescence in cells stored for a year under these conditions (Fig. 5). However, cells stored dry on slides for a year had a high intrinsic fluorescence; unprobed cells were as fluorescent as probed cells. The fluorescence of cells probed after being stored dry was not as great as the fluorescence of probed cells that had been stored in fixative. Therefore, cells that have been stored dry display not only a higher background fluorescence, but also a reduced capacity to bind probe. The degradative processes responsible for these changes are unknown. It is clearly preferable to store cells in cold fixative rather than dry on slides if they cannot be examined immediately after collection (Fig. 5).

This result was quite unexpected, since most reviews of in situ hybridization procedures indicate that cells retain probe-binding capacity for a longer period if they are stored dry on slides (e.g. DeLong 1993), and that cells stored in fixative for even a few weeks begin to lose their capacity to produce a signal. However, others have also noted that samples stored in fixative retained their capacity for binding probe longer than dried samples (Miller and Scholin 2000), although the fixatives employed were different and the times tested much shorter. We do not know why our cells were successfully preserved for so long. We used a slightly higher concentration of paraformaldehyde than is normally recommended and this might have affected preservation. It is also possible that the commercial preparation of paraformaldehyde we used is especially well-suited for the long-term preservation of cells.

Counting parasites on slides stained with protargol and in FISH preparations demonstrated that FISH is a very suitable way of detecting *Amoebophrya* (Fig. 6). Large parasites, which develop approximately two days after adding dinospores, were detected equally well by protargol impregnation and FISH, while

FISH probes seemed better at revealing the smaller developmental stages characteristic of infections younger than 39 h.

Texas Red-labelled probes targeting the region joining helices 8 and 11 are very suitable for detecting *Amoebophrya* cells (Fig. 7–10). There are no problems of cell permeability, background fluorescence, or non-specific binding of probes that prevent the use of FISH probes for this purpose.

## ACKNOWLEDGMENTS

## LITERATURE CITED

Adachi, M., Sako, Y. & Ishida, Y. 1996. Identification of the toxic dinoflagellates *Alexandrium catenella* and *A. tamarense* (Dinophyceae) using DNA probes and whole-cell hybridization. *J. Phycol.*, 32: 1049–1052.

Amann, R. I., Krumholz, L. & Stahl, D. A. 1990. Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic and environmental studies in microbiology. *J. Bacteriol.*, 172:762–770.

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

Caron, D. A., Lim, E. L., Dennett, M. R., Gast, R. J., Kosman, C. & DeLong, E. F. 1999. Molecular phylogenetic analysis of the heterotrophic chrysophyte genus *Paraphysomonas* (Chrysophyceae), and the design of rRNA-targeted oligonucleotide probes for two species. *J. Phycol.*, 35:824–837.

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

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

Daugbjerg, N., Hansen, G., Larsen, J. & Moestrup, Ø. 2000. Phylogeny of some of the major genera of dinoflagellates based on ultrastructure and partial LSU rDNA sequence data, including the erection of three new genera of unarmoured dinoflagellates. *Phycologia*, 39:302–317.

DeLong, E. F. 1993. Single-cell identification using fluorescently-labeled, ribosomal RNA-specific probes. *In*: Kemp, P. F., Sherr, B. F., Sherr, E. B. & Cole, J. J. (ed.), Handbook of Methods in Aquatic Microbial Ecology. Lewis Publ., Boca Raton, Florida. p. 285–294.

De Rijk, P. & De Wachter, R. 1997. RnaViz, a program for the visualisation of RNA secondary structure. *Nucleic Acids Res.*, 25:4679–4684.

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

Gunderson, J. H., Goss, S. H. & Coats, D. W. 1999. The phylogenetic position of *Amoebophrya* sp. infecting *Gymnodinium sanguineum*. *J. Eukaryot. Microbiol.*, 46:194–197.

Knauber, D. C., Berry, E. S. & Fawley, M. W. 1996. Ribosomal RNA-based oligonucleotide probes to identify marine green ultraphytoplankton. *J. Eukaryot. Microbiol.*, 43:89–94.

Lange, M., Guillou, L., Vaulot, D., Simon, N., Amann, R. I., Ludwig, W. & Medlin, L. K. 1996. Identification of the class Prymnesiophyceae and the genus *Phaeocystis* with ribosomal RNA-targeted nucleic acid probes detected by flow cytometry. *J. Phycol.*, 32:858–868.

Lim, E. L., Caron, D. A. & DeLong, E. F. 1996. Development and field application of a quantitative method for examining natural assemblages of protists with oligonucleotide probes. *Appl. Environ. Microbiol.*, 62:1416–1423.

Lim, E. L., Dennett, M. R. & Caron, D. A. 1999. The ecology of *Paraphysomonas imperforata* based on studies employing oligonucleotide probe identification in coastal water samples and enrichment cultures. *Limnol. Oceanogr.*, 44:37–51.

Miller, P. E. & Scholin, C. A. 2000. On detection of *Pseudo-nitzschia* (Bacillariophyceae) species using whole cell hybridization: sample fixation and stability. *J. Phycol.*, 36:238–250.

Rice, J., O'Connor, C. D., Sleigh, M. A., Burkhill, P. H., Giles, I. G. & Zubkov, M. V. 1997. Fluorescent oligonucleotide rDNA probes that specifically bind to a common nanoflagellate, *Paraphysomonas vestita*. *Microbiology*, 143:1717–1727.

Scholin, C. A., Buck, K. R., Britschgi, T., Cangelosi, G. & Chavez, F. P. 1996. Identification of *Pseudo-nitzschia australis* (Bacillariophyceae) using rRNA-targeted probes in whole cell and sandwich hybridization formats. *Phycologia*, 35:190–197.

Simon, N., Campbell, L., Örnólfsdóttir, E., Groben, R., Guillou, L., Lange, M. & Medlin, L. K. 2000. Oligonucleotide probes for the identification of three algal groups by dot blot and fluorescent whole-cell hybridization. *J. Eukaryot. Microbiol.*, 47:76–84.

Stothard, D. R., Hay, J., Schroeder-Diedrich, J. M., Seal, D. V. & Byers, T. J. 1999. Fluorescent oligonucleotide probes for clinical and environmental detection of *Acanthamoeba* and the T4 18S rRNA gene sequence type. *J. Clin. Microbiol.*, 37:2687–2693.