

Serological Affinities of the Oyster Pathogen *Perkinsus marinus* (Apicomplexa) with Some Dinoflagellates (Dinophyceae)

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ABSTRACT. The protozoan oyster pathogen *Perkinsus marinus* is classified in the phylum Apicomplexa, although molecular-genetic and ultrastructural evidence increasingly concur on its closer phylogenetic relationship with the dinoflagellates. To test for evidence of serological epitopes common to *P. marinus* and dinoflagellates, we probed 19 free-living and 8 parasitic dinoflagellate, or dinoflagellate-like, species for cross-reactivity with polyclonal antibodies to *P. marinus*. Three of 19 free-living dinoflagellates (16%), and 7 of 8 parasitic dinoflagellates (88%) were labeled by anti-*P. marinus* antibodies. In reciprocal immunoassays using polyclonal antibodies to the *Hematodinium* sp. dinoflagellate parasite of Norway lobsters, *Nephrops norvegicus*, *P. marinus* and the same 7 parasitic dinoflagellates labeled by anti-*P. marinus* antibodies, were again labeled. The dinoflagellate-like parasite of prawns *Pandalus platyceros* was not labeled by either antibody reagent. These reciprocal results confirm the presence of shared antibody-binding epitopes on cells of *P. marinus* and several dinoflagellates. The apparent widespread serological affinity between *P. marinus* and the parasitic dinoflagellates suggests a closer phylogenetic link to the syndinean dinoflagellate lineage. The consistent failure of the dinoflagellate-like prawn parasite to bind either antibody reagent shows that this parasite is serologically distinct from both *P. marinus* and *Hematodinium*-species parasitic dinoflagellates.

Key Words. Antibody cross-reaction, dermo, epitope sharing, *Hematodinium*, immunoassay, protozoan phylogeny, oyster disease, parasitic dinoflagellates, spot prawn, Syndinidae.

DESTRUCTIVE impacts on aquatic habitats and living aquatic resources by toxic algae, parasitic dinoflagellates, and pathogenic protozoans in estuarine environments have been increasingly recognized, yet the taxonomic status of some identified agents remains controversial, and objective methods for their rapid identification unavailable. Development of polyclonal antibodies to the protozoan oyster pathogen *Perkinsus marinus* (Dungan and Roberson 1993) improved detection of this pathogen in host tissues and environmental samples, and provided a novel tool for testing long-standing hypotheses on the *P. marinus* life cycle, oyster disease transmission, and disease pathogenesis. Because *Perkinsus karlssoni* is no longer considered a valid *Perkinsus* species (Goggin et al. 1996), these polyclonal anti-*P. marinus* antibodies are now known to be broadly specific for all described, and most undescribed, *Perkinsus*-species mollusc parasites (Blackbourn, Bower, and Meyer 1998; Dungan and Roberson 1993; Maeno, Yoshinaga, and Nakajima 1999; Dungan et al. 2002), but not for other protozoan pathogens of fish and shellfish.

Perkinsus marinus is currently classified as an apicomplexan based on zoospore apical complex ultrastructures, including an atypical conoid apparatus (Levine 1978). However, recent analyses of *P. marinus* rRNA and actin gene nucleotide sequences (Fong et al. 1994; Goggin and Barker 1993; Reece et al. 1997; Siddall et al. 1997) and morphological attributes (Perkins 1996; Siddall et al. 1997; Sunila, Hamilton, and Dungan 2001; Vivier 1982) have challenged inclusion of *Perkinsus* species in the Apicomplexa. Several independent analyses that have increasingly indicated that the strongest phylogenetic affinities of *Perkinsus* species lie with dinoflagellate taxa (Siddall et al. 1997; Wolters 1991), have inspired Cavalier-Smith (1998) to propose erection of the phylum Dinozoa to house proposed subphyla Dinoflagellata and Protalveolata (*Perkinsus* et al.) together. Toward a similar taxonomic objective, Norén, Moestrup, and Rehnsam-Holm (1999) proposed separate phylum status for both the Perkinsozoa and the Dinoflagellata, within their common alveolate clade.

To test for phenotypic evidence supporting or refuting phylogenetic affinities between *Perkinsus* species and the dinofla-

gellates suggested by nucleotide sequence homology data, and to localize possible shared antibody-binding epitopes, we probed a variety of free-living and parasitic dinoflagellate cells with polyclonal rabbit anti-*P. marinus* antibodies (Dungan and Roberson 1993). Upon the unexpected detection of such cross-reactivity, especially among the parasitic dinoflagellates, we also tested the parasitic dinoflagellates and *P. marinus* for reciprocal cross-reactivity with polyclonal rabbit antibodies raised against a *Hematodinium* sp. dinoflagellate parasite of Norway lobsters, *Nephrops norvegicus* (Field and Appleton 1996). We report here the results of those experiments.

MATERIALS AND METHODS

Viable and formaldehyde-fixed suspensions of free-living dinoflagellates were obtained from several sources (Table 1). Viable populations were propagated in vitro and fixed for 1 h with 4% (w/v) buffered formaldehyde or 2% (w/v) paraformaldehyde, diluted in culture-medium seawater. Fixed cells were immobilized on track-etched, 1- μ m pore-size, black polycarbonate membranes clamped in 13-mm swinnex filter housings, and fluorescence-immunostained by sequential, unidirectional passage of immunoassay reagents through the sample membranes, as described (Dungan and Roberson 1993). Membrane-immobilized cells were washed with 0.15 M phosphate-buffered saline (PBS) containing 0.05% (v/v) Tween-20 (PBST), and incubated in blocking buffer for 30 min. Blocking buffer was PBST containing 2% (w/v) bovine serum albumin (BSA) and 0.2% (v/v) normal goat serum. Sample cells were then sequentially exposed to a 10^{-3} dilution ($\sim 10 \mu\text{g IgG ml}^{-1}$) of the rabbit anti-*P. marinus* antiserum of Dungan and Roberson (1993) for 30 min, goat anti-rabbit IgG-FITC conjugate ($1 \mu\text{g IgG ml}^{-1}$) for 30 min, and 0.5% (w/v) Evan's blue counterstain. Antibody reagents were diluted in serum diluent (PBST + 1% BSA). Samples were washed with PBST between immunoassay reagent incubations, and immunostained membranes were mounted in glycerol mounting medium pH 9, for examination by epifluorescence microscopy under 480-nm blue light excitation.

Histological samples of parasitic dinoflagellates infecting a variety of hosts were obtained from cooperating colleagues worldwide (Table 2). Histological samples were sectioned and immunostained as described (Dungan and Roberson 1993), with

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the following differences. Dewaxed and rehydrated sections were incubated for 1 h in blocking buffer. Primary antibodies were protein A-affinity purified IgG from either normal rabbit serum (negative control normal IgG) or from the anti-*P. marinus* antiserum of Dungan and Roberson (1993), both incubated at 10 µg IgG ml⁻¹ for 1 h. Secondary antibodies were affinity-purified, FITC-conjugated goat anti-rabbit IgG, which were incubated with sections for 1 h at 1 µg IgG ml⁻¹. Other reagents were identical to those employed for membrane-immobilized free-living cells.

Formalin-fixed *P. marinus* hypnozooids from infected oyster tissues, which were enlarged in Ray's fluid thioglycollate medium (RFTM) and isolated after host tissue hydrolysis (Bushek, Ford, and Allen 1994), were immobilized on membranes as positive controls for cytological immunoassays of suspended cells. Sections of *P. marinus*-infected *Crassostrea virginica* oyster tissues were used as positive controls for histological immunoassays performed with anti-*P. marinus* antibodies. Replicate preparations of each tested sample omitted the primary antibody, or substituted normal rabbit IgG, to control for possible non-specific binding of either rabbit primary antibodies or FITC-conjugated secondary antibodies. Autofluorescence control replicates of each tested sample were not exposed to any antibodies, but were blocked, counterstained, and analyzed for autofluorescence under blue light excitation. Positive immunoassay results, including sites of antibody labeling, were recorded only for immunofluorescence signals detected in samples stained with both primary and secondary antibodies, but which

were absent from autofluorescence and non-specific antibody binding control replicates.

Similar histological immunoassay methods were used to stain *P. marinus*-infected oyster tissues, and fish and crustacean tissues infected by dinoflagellate, or dinoflagellate-like, parasites, using the anti-*Hematodinium* sp. antibodies of Field and Appleton (1996). This rabbit antiserum, raised against a lysate from in vitro propagated *Hematodinium* sp. vegetative cells, was diluted 1:500 in serum diluent, to an estimated concentration of 10 µg IgG ml⁻¹. Positive control sections were from *Hematodinium* sp.-infected *N. norvegicus* tissues. Otherwise, immunoassays were identical to those performed with anti-*P. marinus* antibodies.

RESULTS

The rabbit polyclonal antibodies to *P. marinus* used in this study label all described, and several undescribed, *Perkinsus*-species mollusc parasites (Blackbourn, Bower, and Meyer 1998; Dungan and Roberson 1993; Maeno, Yoshinaga, and Nakajima 1999; Dungan et al. 2002). Using the methods described here for histological immunoassays, these antibodies were previously shown not to label the protistan pathogens of finfish and shellfish, *Dermocystidium salmonis*, *Haplosporidium nelsoni*, and a *Dermocystidium*-like systemic salmon pathogen (Dungan and Roberson 1993). We have subsequently determined that these antibodies are also unreactive with the protists *Labyrinthuloides halitidis* (Bower 1987) and the undescribed

Table 1. Antibody binding by free-living dinoflagellates assayed with anti-*Perkinsus marinus* antibodies.

Dinoflagellate species	Isolate code	Isolate source	Antibody binding
phototroph, toxic			
<i>Alexandrium affine</i> Balech	AABB01/2	a	—
<i>Alexandrium catenella</i> Balech	GCRUX	a	—
<i>Alexandrium catenella</i>	OF878-C2	a	—
<i>Alexandrium fundyense</i> Balech	AFNS85	a	—
<i>Alexandrium fundyense</i>	GTCA29	a	—
<i>Alexandrium minutum</i> Balech	GTPORT	a	—
<i>Alexandrium minutum</i>	AMD01	a	—
<i>Alexandrium tamarense</i> Balech	OF84423-03	a	—
<i>Alexandrium tamarense</i>	AT-9	a	—
<i>Alexandrium tamarense</i>	PGT183	a	—
<i>Alexandrium tamarense</i>	ATGHOPE1	a	—
<i>Amphidinium carterae</i> Hulbert	CCMP1314	b	—
<i>Gymnodinium breve</i> Davis	GB, Willon	c	—
<i>Gymnodinium breve</i>		d	—
<i>Gymnodinium catenatum</i> Graham	CCMP414	b	+
<i>Gyrodinium mikimotoi</i> Miyake & Kominari	CCMP430	b	—
<i>Gyrodinium galatheanum</i> Baarud	CCMP416	b	+
<i>Lingulodinium polyedra</i> Dodge	CCMP407	b	—
<i>Prorocentrum minimum</i> Schiller	CCMP698	b	—
<i>Prorocentrum minimum</i>	PROR6 17-2	e	—
<i>Prorocentrum minimum</i>	P. min. 2A	e	—
phototroph, nontoxic			
<i>Ceratium fusus</i> Dujardin	CCMP1758	b	—
<i>Gyrodinium uncatenum</i> Hulbert	CCMP1310	b	+
<i>Gyrodinium</i> sp. Kofoid & Swezey	G. est.	e	—
<i>Heterocapsa pygmaea</i> Loeblich, Schmidt & Sherley	2421	f	—
<i>Peridinium foliaceum</i> Lemmerman	1688	f	—
<i>Symbiodinium</i> sp. Freudenthal	CCMP832	b	—
heterotroph, nontoxic			
<i>Oxyrrhis marina</i> Dujardin	1974	f	—

Isolate Sources: a. G. Doucette, NOAA/NOS, Charleston, SC, USA; b. Provasoli-Guillard Center for Culture of Marine Phytoplankton, West Boothbay Harbor, ME, USA; c. F. Van Dolah, NOAA/NOS, Charleston, SC, USA; d. P. Tester, NOAA/NMFS, Beaufort, NC, USA; e. A. Lewitus, Belle W. Baruch IMBCR, University of South Carolina, Georgetown, SC, USA; f. The Culture Collection of Algae at the University of Texas, Austin, TX, USA.

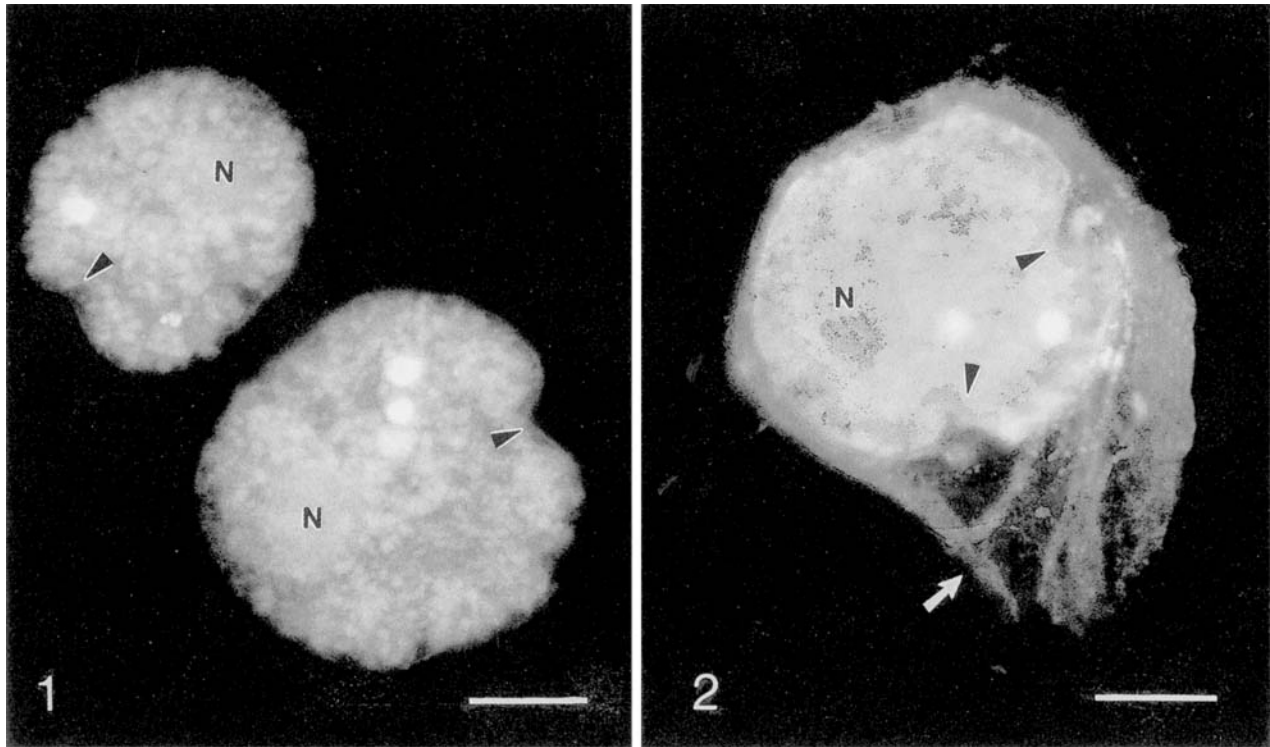


Fig. 1. Phototrophic dinoflagellate *Gyrodinium uncatenum* immunostained on black polycarbonate membrane with normal rabbit serum. Only cytoplasmic chlorophyll (orange) autofluorescence is seen in this non-specific antibody-binding negative control treatment. Digital gray scale conversion from original color print. N, nucleus; arrowhead, sulcus. Bar = 20 μ m.

Fig. 2. Phototrophic dinoflagellate *Gyrodinium uncatenum* immunostained on black polycarbonate membrane with anti-*Perkinsus marinus* antiserum. Besides cytoplasmic chlorophyll (orange) autofluorescence, the feeding veil (arrow) and cell wall were fluorescence-labeled (green) by antibodies. Digital gray-scale conversion from original color print. N, nucleus; arrowhead, sulcus. Bar = 20 μ m.

labyrinthomorphid quahog clam parasite *QPX* (Whyte, Cawthorn, and McGladdery 1994).

Twenty-eight strains of free-living dinoflagellates, representing 19 phototrophic, heterotrophic, toxic, or non-toxic species, were tested for labeling by anti-*P. marinus* antibodies (Table 1). Three planktonic phototrophs (16% of tested species) showed immunofluorescence signal intensities similar to positive control *P. marinus* hypospores. Both cell walls/membranes and feeding veils of *Gymnodinium catenatum* and *Gyrodinium uncatenum* bound anti-*P. marinus* antibodies (Fig. 1, 2). Cell walls or membranes of *Gyrodinium galatheanum* were stained, as were cytoplasmic epitopes of *G. uncatenum*. Nuclear immunostaining in free-living suspended dinoflagellate cells was never observed. Sixteen species (84%) of free-living dinoflagellates failed to bind anti-*P. marinus* antibodies, indicating that *P. marinus*-like epitopes labeled in immunopositive species are neither broadly conserved nor generally expressed by free-living dinoflagellates.

Seven of eight (88%) tested dinoflagellate, or dinoflagellate-like parasites of marine crustacea and fish were labeled by anti-*P. marinus* antibodies (Table 2), and intracellular locations of labeled epitopes varied between parasites. Like anti-*P. marinus* antibody labeling in cognate *P. marinus* cells (Fig. 3), a *Hematodinium* sp. infecting Alaska tanner crabs *Chionoecetes bairdi* (Meyers et al. 1987) showed strong labeling of cell wall, cytoplasmic and nuclear epitopes. Parasite cell wall and cytoplasmic epitopes, but not nuclear epitopes, were labeled in a *Hematodinium* sp. infecting Scottish Norway lobsters *N. norvegicus* (Field et al. 1992) (Fig. 5). Another *Hematodinium* sp. infecting Chesapeake Bay blue crabs *Callinectes sapidus* (Mes-

sick 1994; Newman and Johnson 1975) showed strong labeling of both cytoplasmic and nuclear epitopes. The plasmalemma, nuclear membrane, and nucleoplasm were labeled in *Hematodinium australis* infecting Australian sand crabs *Portunus pelagicus* (Hudson and Shields 1994). Nuclear and cytoplasmic epitopes were labeled in a *Hematodinium* sp. parasite of velvet swimming crabs *Necora puber* from France (Wilhelm and Mialhe 1996). Nuclear, cell wall, and cytoplasmic epitopes were labeled in a *Hematodinium* sp. infecting an unidentified gammaridean amphipod from Maryland coastal bays (Johnson 1986). In *Amyloodinium ocellatum* ectoparasites infesting gills in Gulf of Mexico red drum *Sciaenops ocellatus* (Brown and Hovasse 1946; Nigrelli 1936), cell walls, reticular cytoplasm, nuclear membranes, and non-chromosomal nucleoplasm were labeled. Repeated efforts to immunostain a dinoflagellate-like parasite in histological samples of infected spot prawns *P. platyceros* from both southeast Alaska (Meyers, Lightner, and Redman 1994) and British Columbia (Bower, McGladdery, and Price 1994), consistently failed.

Tests for reciprocal immuno-staining by anti-*Hematodinium* sp. rabbit antiserum were performed only on histological sections from *P. marinus*-infected oysters and from dinoflagellate-infected hosts, using the antiserum described by Field and Appleton (1996). These antibodies label *Hematodinium* sp. parasite cells in infected Norway lobster tissues, but not host lobster tissues, or a co-infecting *Paranophrys*-like ciliate also present in some affected lobsters.

Perkinsus marinus (Fig. 4) and the same seven parasitic dinoflagellates that were labeled by anti-*P. marinus* antibodies, were also reciprocally labeled by antibodies to the *Hematodi-*

nium sp. parasite of *N. norvegicus* (Table 2). Immunostaining of *Hematodinium* sp. cells by their cognate antibodies included cell wall and cytoplasmic epitopes (Fig. 6), strikingly similar to the result obtained in *Hematodinium* sp. cells immunostained with anti-*P. marinus* antibodies (Fig. 5). The dinoflagellate-like parasite infecting spot prawns *P. platyceros*, also failed to label with anti-*Hematodinium* sp. antibodies. Although the resolution of light microscopy for epitope localization and differentiation is low, the subcellular locations of binding epitopes for both antibody reagents appeared similar, and both antibodies gave similar reciprocal staining reactions when reacted with each others' cognate antigens (Fig. 3–6). These similar reciprocal staining results confirm that *P. marinus* and several parasitic dinoflagellates share common antibody binding epitopes.

DISCUSSION

Binding of anti-*P. marinus* antibodies to the majority of tested dinoflagellate parasites of crustacea and fish collected worldwide, indicates a widespread presence of shared epitopes consistent with phylogenetic affinities suggested by DNA nucleotide sequence homologies among members of these different parasitic taxa. Nearly identical reciprocal labeling of epitopes on *P. marinus* and the same seven parasitic dinoflagellates, by antibodies to the Norway lobster *Hematodinium* sp. parasite, confirms epitope sharing between these groups as the basis for serological cross-reactions. Several phototrophic dinoflagellates were also labeled by anti-*P. marinus* antibodies, indicating that *P. marinus*-like epitopes also occur among some free-living dinoflagellates, as well. Together, these results provide strong phenotypic evidence supporting a proposed phylogenetic link between these taxonomically diverse protists (Siddall et al. 1997).

Although the chemical identities of shared epitopes were not investigated during this study, it is possible that one or more represent conserved phenotypic elements common to all im-

muno-positive samples, and broadly prevalent among the parasitic dinoflagellates. The relatively high apparent frequency of shared epitopes among parasitic dinoflagellates further suggests that *Perkinsus* species protozoan parasites may reflect a stronger phylogenetic affinity with an evolutionary lineage common to the parasitic dinoflagellates. Alternatively, expression and development of these shared epitopes may reflect phenotypic convergence associated merely with common parasitism of marine hosts. This interpretation seems unlikely, in light of the lack of reactivity documented for the anti-*P. marinus* antibodies used during this investigation with a broad range of protistan parasites of marine invertebrates and finfish.

Despite assertions that described *P. marinus* ultrastructural characteristics merely limit its taxonomic assignment to a diverse alveolate protozoan clade, and the suggestion that *P. marinus* is a dinoflagellate, per se (Siddall et al. 1997), unique dinokaryon nuclear morphology common in all parasitic and free-living dinoflagellates tested during this study, are absent in *P. marinus*. Unlike the dinoflagellates, and despite numerous observations of proliferating cell populations, condensed chromosomes are never reported in ultrastructural or histological studies of *Perkinsus* species nuclei (Sunila, Hamilton, and Dungan 2001), indicating that chromosome condensation during *Perkinsus* species cell division is either absent or highly transient. If demonstration of common antibody-binding epitopes supports the hypothesis that *P. marinus* evolved from a common basal lineage shared by dinoflagellates, and particularly by parasitic dinoflagellates, then its complete lack of dinokaryon nuclear structures clearly differentiates *P. marinus* from most dinoflagellates.

By its consistent failure to label with antibodies to either *P. marinus* or the Norway lobster *Hematodinium* sp. parasite, the dinoflagellate-like parasite of northeast Pacific pandalid shrimp *Pandalus borealis* and *P. platyceros* was clearly differentiated serologically from both *P. marinus* and the universally immu-

Table 2. Labeling of dinoflagellate, and dinoflagellate-like parasites of fish and crustaceans by anti-*Perkinsus marinus* (+, -) and anti-*Hematodinium* sp. (+, -) antibodies.

Parasite (host)	Sample code	Antibody binding		Antibody binding site					
				Wall	Cytoplasm	Nucleus			
<i>Perkinsus marinus</i> (<i>Crassostrea virginica</i>)	SMCC-18-14	+	+	+	+	+	+	+	+
<i>Hematodinium</i> sp. (<i>Nephrops norvegicus</i>)	990427 Nnor-1 ^a	+	+	+	+	+	+	-	-
<i>Hematodinium</i> sp. (<i>Chionoecetes bairdi</i>)	98-513-1 ^b	+	+	+	+	+	+	+	+
<i>Hematodinium</i> sp. (<i>Callinectes sapidus</i>)	CS 2809 ^c	+	+	-	+	+	-	+	+
<i>Hematodinium australis</i> (<i>Portunus pelagicus</i>)	205 ^d	+	+	+	+	-	+	+	+
<i>Hematodinium</i> sp. (<i>Necora puber</i>)	475 ^e	+	+	-	-	+	+	+	+
<i>Hematodinium</i> sp. (gammaridean amphipod)	OC819T019 ^c	+	+	+	-	+	+	+	+
<i>Amyloodinium ocellatum</i> (<i>Sciaenops ocellatus</i>)	990409 Rdrum-2b ^f	+	+	+	+	+	+	+	+
dinoflagellate-like sp. (<i>Pandalus platyceros</i>)	6668-3 ^g	-	-	-	-	-	-	-	-
	90-5685 LMI ^h	-	-	-	-	-	-	-	-

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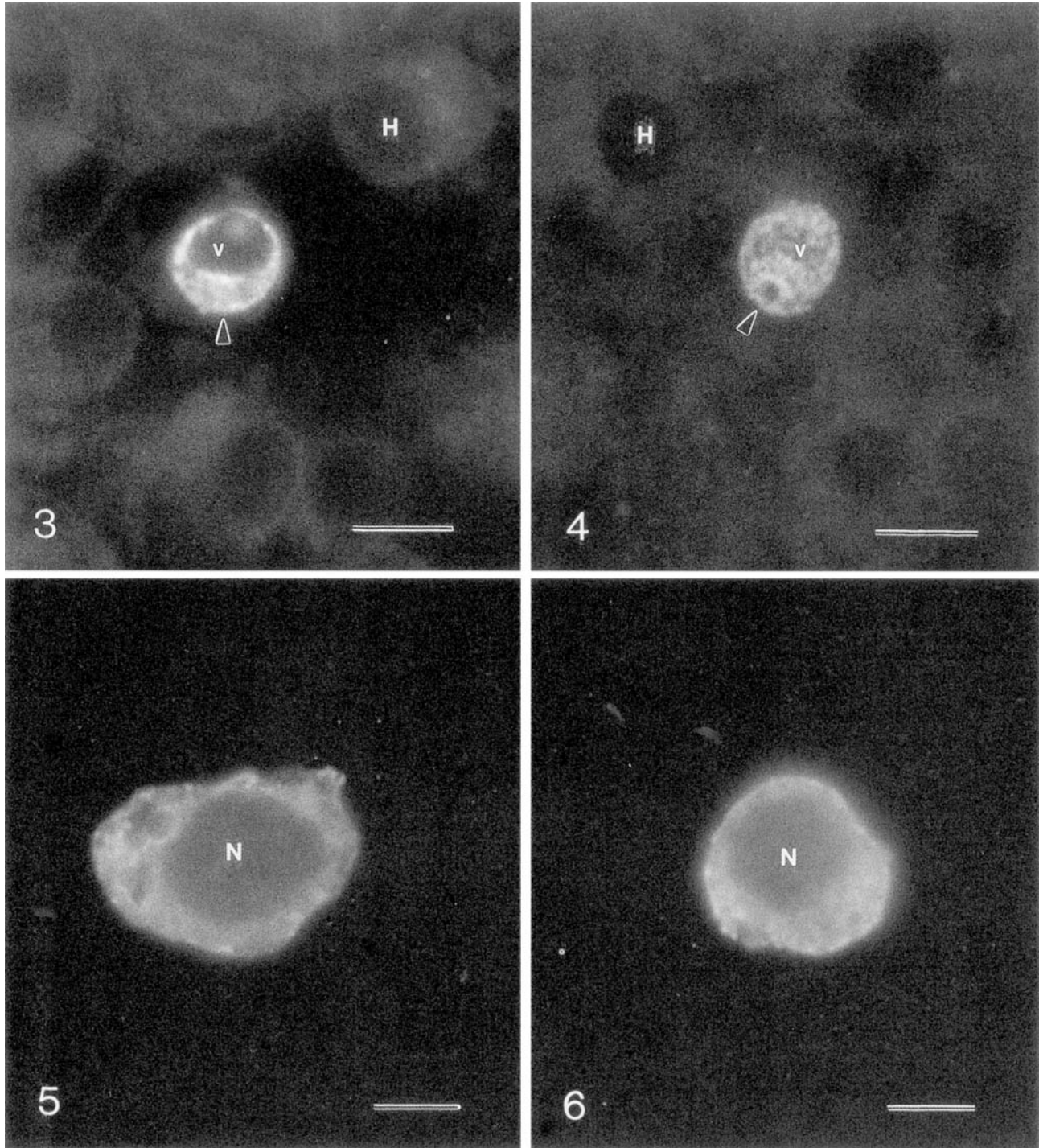


Fig. 3. *Perkinsus marinus* trophozoite labeled by anti-*P. marinus* antibodies in histological section from infected *Crassostrea virginica*. Fluorescent parasite cell phagocytized by host hemocyte, inside oyster mantle vein. Parasite nucleus (arrowhead) and cytoplasm are labeled. H, hemocyte nucleus; v, parasite vacuole. Bar = 5 μ m.

Fig. 4. *Perkinsus marinus* trophozoite labeled by anti-*Hematodinium* sp. antibodies in histological section from infected *Crassostrea virginica*. Fluorescent parasite cell in oyster visceral connective tissue. Non-nucleolar parasite nucleoplasm (arrowhead) and cytoplasm are labeled. H, hemocyte nucleus; v, parasite vacuole; Bar = 5 μ m.

Fig. 5. *Hematodinium* sp. labeled by anti-*Perkinsus marinus* antibodies in histological section from infected *Nephrops norvegicus*. Isolated parasite cell in lobster gill. Parasite nucleus (N) is not labeled, but cytoplasm and cell wall are. Bar = 5 μ m.

Fig. 6. *Hematodinium* sp. labeled by anti-*Hematodinium* sp. antibodies in histological section from infected *Nephrops norvegicus*. Isolated parasite cell in lobster muscle. Parasite nucleus (N) is not labeled, but cytoplasm and cell wall are. Bar = 5 μ m.

nopositive parasitic syndinean dinoflagellates infecting other crustacean hosts. These results challenge previous descriptions of this prawn parasite as dinoflagellate-like (Meyers, Lightner, and Redman 1994), and *Hematodinium*-like (Bower, McGladdery, and Price 1994), but provide no guidance on its true taxonomic affinities.

These findings confirm the presence of shared antibody-binding epitopes on cells of the apicomplexan oyster pathogen, *P. marinus*, and several dinoflagellates. The phenomenon was observed especially, but not exclusively, among the heterotrophic, parasitic Syndinidae. These results provide phenotypic evidence supporting recent molecular genetic nucleotide sequence data suggesting phylogenetic affinities between *Perkinsus* species mollusc pathogens and dinoflagellates. Identification of this cross-reactivity by antibodies previously determined to react only with members of the genus *Perkinsus*, cautions diagnosticians to employ independent confirmatory or differential criteria, such as nuclear morphology, photosynthetic pigment contents, and cell ornamentation, when interpreting results of immunoassays using these or similar reagents for differentiation of organisms in these groups.

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