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Occurrence of *Perkinsus* sp. in undulated surf clams *Paphia undulata* from the Gulf of Thailand

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ABSTRACT: The undulated surf clam *Paphia undulata* supports Thailand's largest shellfishery in the Gulf of Thailand, with landings in 1999 recorded at 70 000 t (metric tonnes) yr^{-1}. We report, for the first time, the prevalence of *Perkinsus* sp. in clams in the Gulf. A monthly survey from January to December 2001 utilizing the fluid thioglycollate medium (FTM) method showed that average monthly prevalence was 84.7% (n = 360). The monthly percentage of infected clams was generally 100%, with low prevalence in May (66.7%) and no infection in September. The monthly mean infection intensity in terms of *Perkinsus* sp. cells g^{-1} tissue varied from 0 in September to 187 759 ± 18 970 (x ± SE) in October. No obvious annual variation in intensity and prevalence was observed. Prezoosporangia that developed in FTM were 25 to 75 µm in diameter. A few days after incubation in aerated seawater, the prezoosporangia underwent successive binary cell division and formed motile zoospores (2 to 5 µm long). The zoospores were released into the seawater through a discharge tube formed during the 2- and 4-cell stages. Serial semi-thin sections (1 to 4 µm thickness) of clam tissue (n = 120 clams) showed developing trophozoites 3 to 6 µm in diameter within gills, connective tissue, gonads and, especially, the digestive glands. Microscopic features of different life stages indicated that *Perkinsus* sp. in Thailand closely resembled *P. olseni* (= *P. atlanticus*) reported in Australia, New Zealand, Korea, Japan, Spain and Portugal.

KEY WORDS: Clam · *Perkinsus* sp. · *Paphia undulata* · Gulf of Thailand · Zoosporulation · Infection intensity · Prevalence

INTRODUCTION

Protozoan parasites of the genus *Perkinsus* infect many species of marine bivalves throughout the world (Goggin & Lester 1987, Perkins 1988, Figueras et al. 1992, Park & Choi 2001, Blackbourn et al. 1998, Bower et al. 1998, Casas et al. 2002, Hine & Diggles 2002). Parasitized bivalve stocks are usually subject to mass mortalities from infection and result in substantial losses for the associated industries (Mackin 1953, Andrews 1988, Soniat 1996). However, most of our knowledge on protozoan parasites of the genus *Perkinsus* has come from studies conducted in temperate regions of the world, and only a few studies have been conducted in warm temperate and tropical areas (Lester & Davis 1981, Goggin & Lester 1987). Recent surveys of clams, oysters, and mussels from farms and natural harvest grounds in Thailand have shown the presence of several protozoan parasites that have the potential to cause damage to the bivalve shellfish industry in Thailand (Taveekijakarn et al. 2002).

The undulated surf clam *Paphia undulata* supports Thailand's largest shellfishery in the Gulf of Thailand and Andaman Sea. In general, the clams' harvest grounds consist of consolidated mud substrates within 3 to 7 km distance from the shoreline. Shellfish

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grounds are located off the coast of several coastal provinces (see Fig. 1) and are harvested year-round. The harvested clams are generally processed for export as canned products, and landings in 1999 reached 70 000 t, with domestic market values estimated to be around US$ 15 million (DOF 2002). Reported declines of the resource in many provinces have been attributed to overharvesting. In this study however, we report, for the first time, the prevalence of *Perkinsus* sp. in undulated surf clams in the Gulf of Thailand. The present study details prevalence and infection intensity of *Perkinsus* sp. monitored over 12 mo and includes microscopic observations of the trophozoites and zoosporulation patterns.

MATERIALS AND METHODS

Live commercial-sized clams of approximately 4 cm shell length were collected from local markets in Chonburi Province (Province No. 4, Fig. 1) on the eastern seaboard of the Gulf of Thailand on a monthly basis from January to December 2001. Each month, 30 whole clams were incubated in 30 ml fluid thioglycollate medium (FTM) per clam supplemented with 500 µg ml^{-1} streptomycin and 500 U ml^{-1} penicillin-G potassium at room temperature (approximately 27°C) for 7 to 14 d in darkness (Ray 1966, Almeida et al. 1999). After incubation, whole clams were digested with 30 ml NaOH (2 M) at 60°C for 3 h (Choi et al. 1989). The samples were then washed with phosphate-buffered saline (PBS) and twice centrifuged at 4500 × *g* for 15 min; 3 subsamples of 100 µl each were taken and stained with 100 µl Lugol's iodine solution, and the hypnospores were enumerated (Bushek et al. 1994). In addition to FMT diagnosis, histology was performed on 10 clams mo^{-1}. After staining with Harris' hematoxylin and counterstaining with eosin (Howard & Smith 1983), semi-thin sections (1 to 4 µm thickness) of paraffin-embedded clams were made through the gills, digestive tract, gonad, mantle and foot to determine the presence of trophozoites in each tissue.

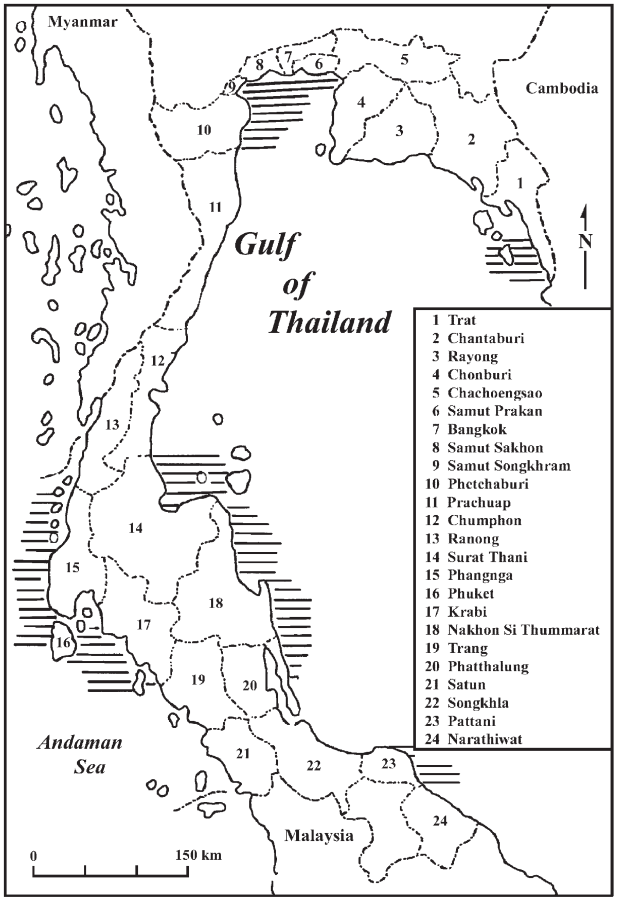

Fig. 1. Coastal provinces of Thailand. Hatched areas: areas in Gulf of Thailand and Andaman Sea where undulated surf clams *Paphia undulata* have been or are still harvested. (Modified from map by Ole Hagan, Department of Geography, University of Victoria, Canada)

To obtain prezoosporangia for inducing zoosporulation *in vitro*, the infected tissue was incubated in FTM in darkness for 3 d at 27°C. Incubated tissue was then trypsinized by trypsin powder (0.25%) for 3 to 4 h and prezoosporangia were then filtered through silk screens of 120, 70, 50, and 20 µm mesh size. The prezoosporangia were subsequently washed with filtered seawater and centrifuged twice at 450 × *g* for 8 min followed by centrifugation at 125 × *g* and finally 30 × *g* for 3 min. The prezoosporangia obtained in this way were then placed into petri dishes with aerated filtered seawater fortified with streptomycin (400 µg ml^{-1}) and penicillin-G (400 U ml^{-1}). Cultured prezoosporangia were incubated at room temperature (27°C). Zoosporulation was observed and photographed at frequent intervals under a light microscope.

RESULTS

The cultivated whole-clam preparations stained with Lugol's iodine showed strong positive results, exhibiting a dark blue color in the gill tissue (Fig. 2). A total of 360 clams (4.74 ± 0.34 cm shell length) (x ± SD) were analyzed for prevalence and infection intensity using FTM. During the course of the study, mean prevalence was 84.7% (305 out of 360 clams). Over the 12 mo period, infection intensity varied from 0 to 578 573 *Perkinsus* sp. $clam^{-1}$ or 0 to 187 759 cells g^{-1} tissue. The highest infection intensity was observed in October at 578 573 cells g^{-1} tissue. *Perkinsus* sp. was not detected in clams examined in September (Table 1).

FTM incubation resulted in the enlargement of trophozoites to sizes ranging from 25 to 75 µm. Pre-

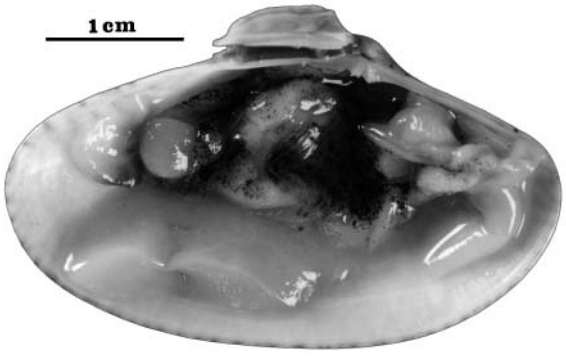

Fig. 2. *Paphia undulata.* FTM (fluid thioglycollate medium)-cultivated, infected clam stained with Lugol's iodine exhibiting dark blue color in gill tissue

zoosporangia (Fig. 3A) were round in appearance, with an eccentric nucleus and large vacuole. Once placed in aerated seawater, prezoosporangia began sporulation characterized by cells with enlarged nuclei and condensed cytoplasm (Fig. 3A). Within 48 h, these cells entered karyo- and cytokinesis, giving rise to a 2-cell stage (Fig. 3B). During this sequence of development, some prezoosporangia developed discharge tubes followed by rapid division resulting in numerous small prezoospores (Fig. 3C,D). Within 3 to 4 d after incubation in darkness, the prezoospores developed into biflagellated and elongated motile cells within the prezoosporangia (Fig. 3E). At maturation, zoospores were released through a distinct discharge tube (Fig. 3F). The resulting free-swimming zoospores ranged in size from 2 to 5 µm in length.

The trophozoites observed in histological preparations under a microscope were round in appearance, varying in diameter from 3 to 6 µm, and contained a large eccentric vacuole. Clams heavily infected with *Perkinsus* sp. often showed massive aggregations of hemocytes around the trophozoite. Of the 120 clams prepared for histology, only 28 showed the presence of trophozoites. In these clams, the main tissue of infection was the digestive gland (Fig. 4A), although trophozoites were also present in the muscle (Fig. 4B) and gill (Fig. 4C).

DISCUSSION

This is the first report of the prevalence of *Perkinsus* sp. in a commercially harvested tropical clam species. Previous reports of *Perkinsus* sp. in temperate areas in Asia have been from Korea (Choi & Park 1997, Park & Choi 2001), Japan (Hamaguchi et al. 1998, Choi et al. 2002) and China (Liang et al. 2001). In this study, we found that *Paphia undulata* harvested from natural stocks are infected almost all year round with *Perkinsus* sp., with a high percentage of infection (up to 100%) recorded in 8 of 12 mo. These values are substantially higher than the prevalence reported in *Paphia australis* from New Zealand (Hine & Diggles 2002), *Crassostrea virginica* from South Carolina (Crosby & Roberts 1990) and *Tapes semidecussatus* from the northern Mediterranean coast of Spain (Montes et al. 2001). However, these values are similar to the infection intensity in *Ruditapes philippinarum* in Korea (Park & Choi 2001). In Chonburi Province, clams that arrive in local markets are harvested from several grounds in the Gulf of Thailand, namely Chonburi, Samut Prakan and Samut Sakhon Provinces (Tharnbuppha 1996) (Province Nos. 4, 6 and 8, respectively, Fig.1). Because of Thailand's location, the average coastal sea temperatures remain around 30°C, with little seasonal variation. Coastal salinities, however, may fluctuate widely, according to the amount of rainfall. Off the coast of Chonburi Province, coastal salinities range from 20 to 28 ppt on the surface and 22 to 28 ppt near the bottom (BIMS 2002).

Table 1. *Perkinsus* sp. infecting *Paphia undulata*. Results of FTM (fluid thioglycollate medium) quantification of *Perkinsus* sp. in undulated surf clams in Gulf of Thailand; 30 individuals analyzed on each sampling date. Data are means ± SE

Sampling date (2001)	Shell length (cm)	Wet tissue wt (g)	% infection	No. *Perkinsus* clam^{-1}	No *Perkinsus* g^{-1} tissue
25 Jan	4.61 ± 0.24	3.10 ± 0.54	100	141270 ± 65807	45477 ± 20214
25 Feb	4.39 ± 0.16	4.02 ± 0.9	100	89943 ± 21987	26436 ± 8281
19 Mar	4.96 ± 0.22	6.41 ± 1.48	93.33	40210 ± 6255	6595 ± 967
30 Apr	4.76 ± 0.19	5.69 ± 1.75	100	53240 ± 11959	9904 ± 1927
26 May	4.15 ± 0.13	3.03 ± 0.45	66.66	24 ± 10	7 ± 3
20 Jun	4.75 ± 0.26	5.44 ± 1.28	93.33	381026 ± 69056	68408 ± 12686
12 Jul	5.27 ± 0.27	8.69 ± 1.73	100	134127 ± 21905	14705 ± 2065
10 Aug	4.93 ± 0.18	3.84 ± 0.34	100	2301 ± 402	596 ± 102
14 Sep	4.71 ± 0.15	3.25 ± 035	0	0	0
15 Oct	4.81 ± 0.20	3.10 ± 0.52	100	578573 ± 60861	187759 ± 18970
14 Nov	4.80 ± 0.19	3.18 ± 0.52	100	275713 ± 47981	86691 ± 14768
8 Dec	4.72 ± 0.20	6.95 ± 1.34	100	518930 ± 61986	75801 ± 8991
Average	4.74 ± 0.34	1.73 ± 2.09	84.72	184605 ± 15607	43530 ± 4072

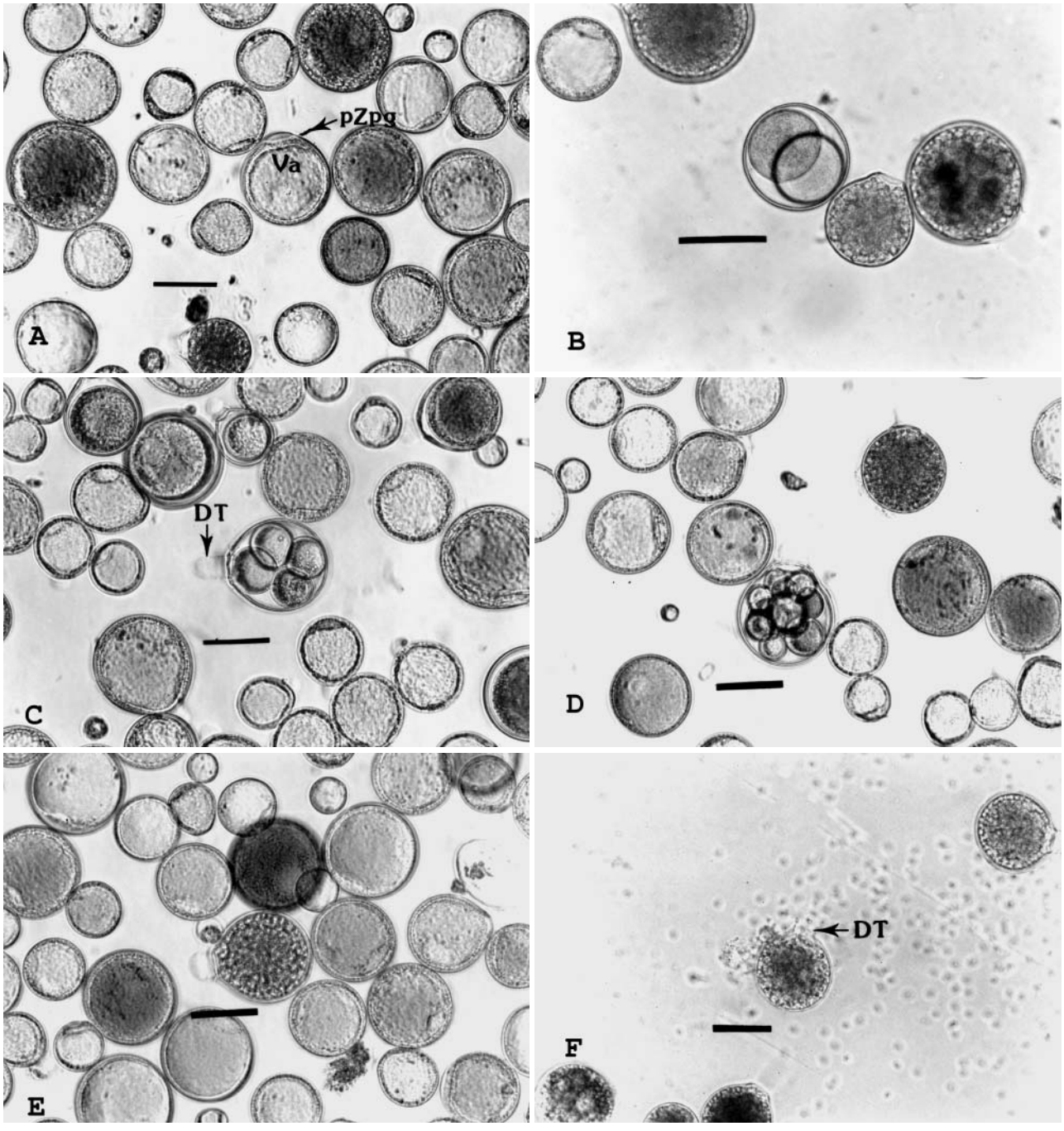

Fig. 3. Light micrograph of zoosporulation of *Perkinsus* sp. in filtered seawater. (A) Prezoosporangia (pZpg) at beginning of vacuolar (Va) subdivision; (B) 2-cell stage; (C) 4-cell stage showing discharge tube (DT); (D) 8-cell stage; (E) prezoosporangia enclosing numerous motile zoospores; (F) free zoospores released through discharge tube (DT). All scale bars = 50 µm

Numerous studies have documented the relationship between infection intensity and pathogenicity in relation to salinity for *Perkinsus* spp. (Andrews 1988, Chu et al. 1993, Burreson & Ragone Calvo 1996). In the eastern oyster *Crassostrea virginica*, a critical range for *P. marinus* pathogenicity apparently exists in salinity ranging between 9 and 12 ppt. Salinities as low as 6 ppt are tolerated, but virulence decreases at salinities below 9 ppt (Ragone & Burreson 1993). Chu & Greene (1989) reported a salinity range of infection from 6 to 35 ppt for *P. marinus*. The Thai populations of *Paphia undulata* are primarily located at sites where salinity and seawater temperatures are high, suggesting a high risk situation for damage to clam stocks from disease. It is not clear,

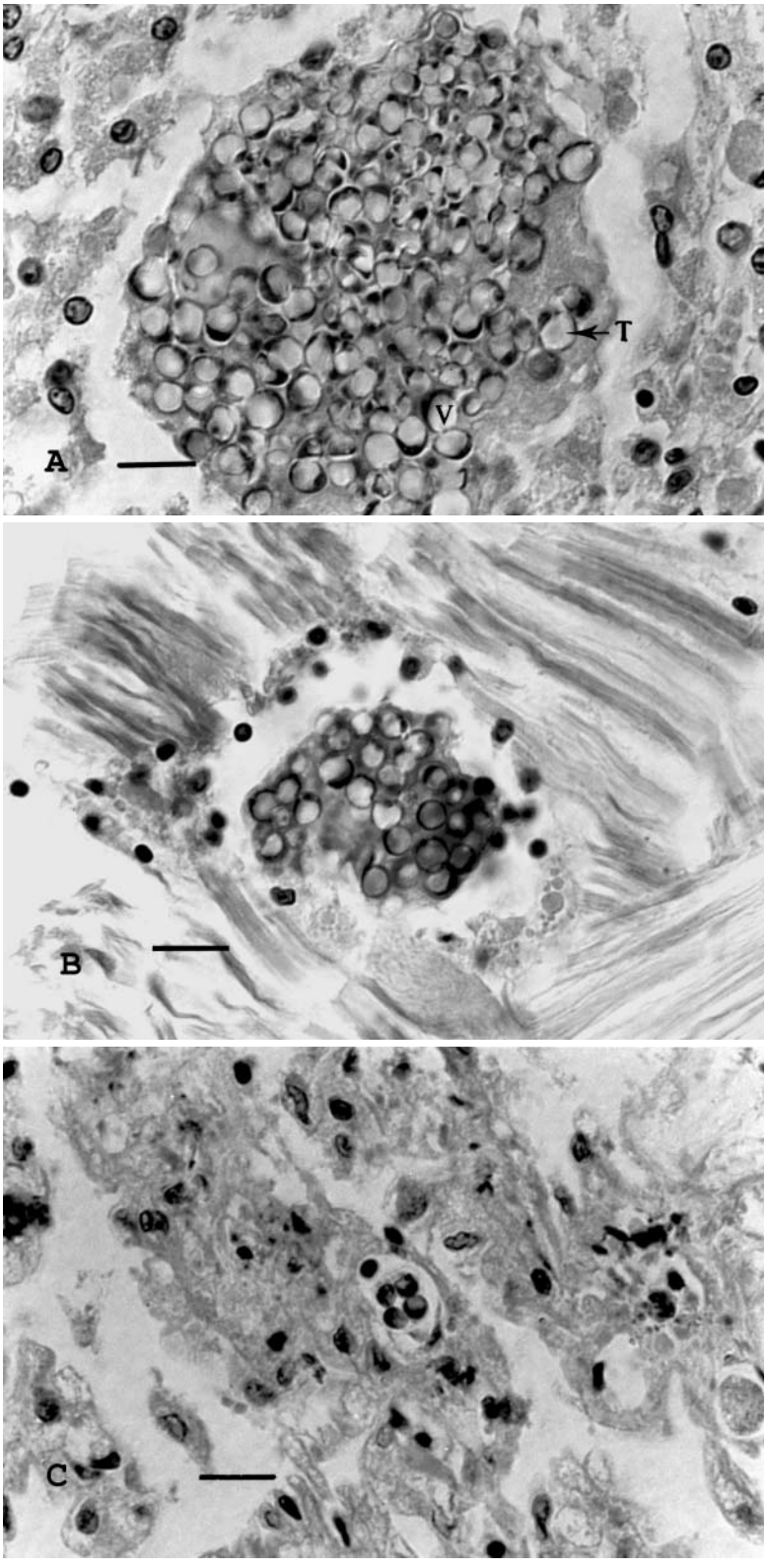

Fig. 4. *Perkinsus* sp. infecting *Paphia undulata*. Light micrograph of trophozoites in clam tissue. (A) Eccentric vacuole; (B) trophozoites in muscle tissue; (C) encapsulation of *Perkinsus* cells in gill tissue. V: vacuole; T: trophozoites. All scale bars = 10 µm

however, if clam stocks in Thailand are subject to mass mortality from *Perkinsus* sp. infection, and clarification of this issue warrants further investigation.

The *in vitro* zoosporulation process of *Perkinsus* sp. from *Paphia undulata* appears to be similar to karyo- and cytokinesis in *Perkinsus marinus* (Perkins 1976) and *P. olseni* (= *P. atlanticus*) (Azevedo et al. 1990, Sagrista et al. 1996). Molecular studies have shown that *P. atlanticus* is conspecific with *P. olseni* (Robledo et al. 2000, Murrell et al. 2002). *P. olseni* was described first, and therefore takes precedence, and *P. olseni* will be used here to mean *P. olseni* and the species formerly called *P. atlanticus*. Under light microscopy, the prezoosporangia of Thai *Perkinsus* sp. varied in size from 25 to 75 µm, being smaller than size ranges reported for *P. marinus* (30 to 80 µm) (Perkins 1996), *P. olseni* (= *P. atlanticus*) (30 to 40 µm) (Azevedo 1989), and *P. olseni* (56 to 94 µm) (Lester & Davis 1981) during sporulation. Several studies have reported that temperature and salinity are also the 2 major factors that regulate the zoosporulation process. Ahn & Kim (2001) reported that development of free zoospores of *Perkinsus* sp. in *Ruditapes philippinarum* is temperature- and salinity-dependent; the process being favored by high temperature and high-salinity conditions. Similar results were also reported for the carpet shell clam *R. decussatus* in Galicia, NW Spain (Casas et al. 2002).

Histological examination of clam tissue from *Perkinsus* sp. in this study detected fewer infected clams than the FTM method. Our results support findings by Rodriguez & Navas (1995), who concluded that positive results obtained by histological analysis may underestimate real infection levels in the field. Almeida et al. (1999) also reported that histological examination detected fewer infected clams than that revealed by whole-clam culture.

The microscopic appearance of the *Perkinsus* sp. infection in the clams include hemocyte infiltration, encapsulation and necrosis similar to those reported for *P. olseni* infecting the giant clam *Tridacna maxima* (Goggin & Lester 1989),

and *P. olseni* found in the carpet shell *Ruditapes decussatus* (Azevedo 1989) and the Manila clam *R. philippinarum* (Hamaguchi et al. 1998, Park & Choi 2001, Choi et al. 2002). The size of trophozoites reported in this study is comparable to that for *P. olseni* (2 to 6 µm) (Goggin & Lester 1989) and smaller than that for *P. olseni* found in *R. philippinarun* in Korea (5 to 14 µm) and Japan (5.3 to 32.5 µm) (Hamaguchi et al. 1998, Park & Choi 2001).

In the present study, *Paphia undulata* infection by *Perkinsus* sp. was heaviest in the digestive gland. This finding is supported by the results of Park & Choi (2001), who found that trophozoites were mostly concentrated in the gills and visceral mass, which includes the digestive gland. Several studies also reported that heavy infection of *Perkinsus* spp. in clams often results in milky-white pustule formation on clam mantle and gill tissues as a consequence of inflammation (Almeida et al. 1999, Park & Choi 2001, Choi et al. 2002). However, no pustule or nodules were observed on the gill and mantle surfaces of clams in this study.

We found that some Thai *Paphia undulata* stocks appear to be heavily infected by *Perkinsus* sp. Heavy infections of this parasite have been reported to cause considerable damage to clam and oyster fisheries in many parts of the world (Goggin & Lester 1987, Perkins 1988, Figueras et al. 1992, Choi & Park 1997, Blackbourn et al. 1998, Bower et al. 1998, Casas et al. 2002, Hine & Diggles 2002).

In conclusion, infection by the protozoan parasite *Perkinsus* sp. is highly prevalent in Thai populations of *Paphia undulata*, and there does not seem to be a clear seasonal (monsoonal) pattern of infection. The distribution of *Perkinsus* sp. infection in Thai clam harvesting grounds remains to be determined. Further investigation of these clams in natural harvesting grounds in conjunction with time and environmental factors will be necessary to determine patterns of infection and the potential effect of *Perkinsus* sp. on this clam. Microscopic features of different life stages indicate that *Perkinsus* sp. in Thailand closely resembles *P. olseni* (= *P. atlanticus*) reported from Australia, New Zealand, Korea, Japan, Spain and Portugal. This finding suggests that *P. olseni* (= *P. atlanticus*) is enzootic in the Southeast Asian/ Australasian or Indo-Pacific region.

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