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Monitoring *Perkinsus olseni* Infection in Manila Clam *Ruditapes philippinarum* on Intertidal Beachs in Jeju Island

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ABSTRACT

Previous studies have shown that the apicomplexan protist *Perkinsus olseni* induces both sublethal and lethal effects on the Manila clam (*Ruditapes philippinarum*) along the west and south coasts of Korea, including Jeju Island. In this study, we assessed the prevalence and infection intensity of *P. olseni* in Manila clams from sand beaches on Jeju Island in July 2022, using Ray's fluid thioglycollate medium assay (RFTM) and histology. Condition index (CI) as the proxy of the fitness of clams (i.e., the ratio of dry or wet tissue weight to the shell weight) collected in July 2022 ranged from 74.5 (Jongdal-ri) to 122.9 (Segwipo), or 0.27 (Moseulpo) to 0.62 (Segwipo). The mean infection intensity, measured as *P. olseni* cells per gram of gill tissue in clams collected from six sites, ranged from 0 (Geumneung, Gimnyeong, and Moseulpo) to 56,000 cells/g of gills (Seongsan), with prevalence rates (i.e., the percentage of infected clams) ranging from 0 to 47%. Similarly, infection intensity in total tissue ranged from 0 (Geumneung and Gimnyeong) to 47,870 cells/g of tissue (Seongsan), with prevalence rates between 0 and 83%. Histological analysis corroborated these findings, revealing low infection intensity, with infection scores ranging from 0 to 1.0 across the six sites. Despite rising sea surface temperatures in Jeju Island over recent decades due to global warming, *P. olseni* infection prevalence and intensity appear to have remained stable. The low density of Manila clams in Jeju Island may partially explain the observed low infection intensity and prevalence.

Keywords: *Perkinsus olseni*, *Ruditapes philippinarum*, Jeju island, Infection intensity, RFTM assay, Histology

INTRODUCTION

Since its first identification in 1997, the alveolate protozoan parasite *Perkinsus olseni* has been detected in Manila clam (*Ruditapes philippinarum*) populations in Korean waters (Choi and Park, 1997; Park and Choi, 2001; Subramaniam *et al.*, 2024). A comprehensive nationwide survey revealed that most clams on sandy-mud tidal flats along the west and south coasts are infected, with infection prevalence reaching 90 to 100% in many cases. *P. olseni* is known to cause significant mortality and sublethal physiological stress in juvenile and adult clams (Park *et al.*, 2006; Lee *et al.*, 2021; Yang *et al.*, 2021). Heavily infected female clams on the west coast exhibit slow gonad maturation and reduced egg production during spawning (Park *et al.*, 2006), a phenomenon also observed by Uddin *et al.* (2010) on a tidal flat on Seonjaedo Island, west coast of Korea.

P. olseni infection in Manila clams has been diagnosed using various methods, including the traditional fluid thioglycollate medium assay invented by Ray (1966), histology, and polymerase chain

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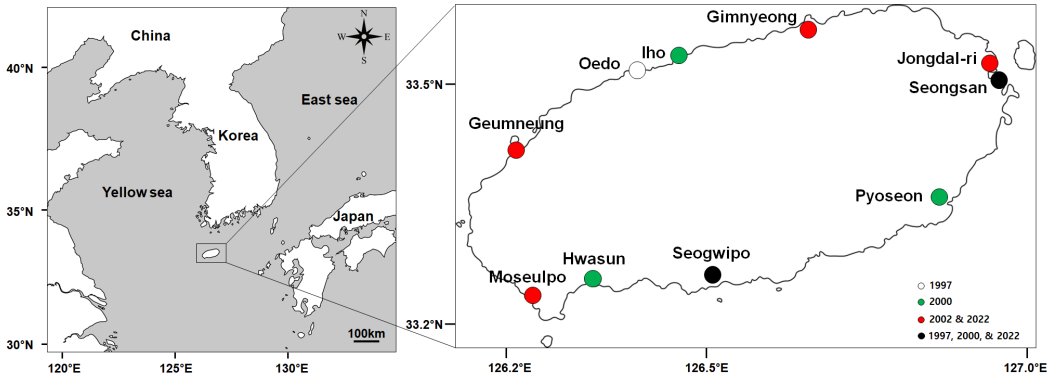

Fig.1. Location of the sampling sites on Jeju Island.

reaction (PCR) (Choi *et al.*, 1989; Ngo and Choi, 2004; Villalba *et al.* 2004; Xie *et al.*, 2013). The Ray's Fluid Thioglycolate Medium (RFTM) assay involves culturing the whole body or a piece of gill tissue of clam in RFTM to induce the development of *Perkinsus* hypnospores, which can be visualized after staining with Lugol's iodine. Histological examination is another common diagnostic approach, as histology allows for the direct observation of *P. olseni* trophozoites within clam tissues. Based on the spatial distribution of *P. olseni* in different tissues of the host clams, Ngo and Choi (2004) categorized the infection intensity from 0 (not infected) to 4 (presented in all types of the tissues) since *P. olseni* infection is systemic when the infection progressed in the host tissues. Molecular techniques, particularly polymerase chain reaction (PCR), have become increasingly popular in *P. olseni* infection diagnosis due to their sensitivity and speed (Park *et al.*, 2005; Balseiro *et al.*, 2010; Xie *et al.*, 2013). PCR can detect *P. olseni* DNA in clam tissues with high specificity, making it suitable for screening large numbers of samples quickly. Nested PCR, in particular, is highly sensitive and less time-consuming compared to histology and RFTM. These molecular methods often combine with traditional techniques to confirm diagnoses and monitor infection prevalence in clam populations (Balseiro *et al.*, 2010).

Located off the south coast of Korea, Jeju Island features a unique coastal environment with rocky intertidal beaches and some sand beaches where Manila clams occur at lower densities compared to other regions. The first report of *P. olseni* infection intensity and prevalence of Manila clams in Jeju Island was by Park and Choi (2001), noting infections in clams from Seogwipo, Seongsan, and Jongdal-ri. Ngo and Choi (2004) also investigated seasonal changes in *P. olseni* infection in Manila clams on a sand beach on the east coast of Jeju Island using histology and reported that the infection prevalence of Manila clams on Jeju Island is much lower than the levels reported from the west and south coast.

In this study, conducted in July 2022, we surveyed *P. olseni* infection in Manila clams on Jeju Island using histology and RFTM. This research details the spatial distribution of *P. olseni* across different beaches and examines decadal changes in infection levels on Jeju Island.

MATERIALS AND METHODS

1. Sampling efforts

In July 2922, we collected Manila clams from sand beaches in Jeju Island, including Geumneung, Gimnyeong, Moseulpo, Seogwipo, Seongsan, and Jongdal-ri, where Manila clams occurred at a density of < 10 individuals/m^2 (Fig. 1). We collected 60 clams from each site except Moseulpo (N = 4) for the assay for the *P. olseni* infection and histology. The shell length (i.e., the longest axis of the shell) and wet tissue weight of each clam were recorded to mm and mg at the laboratory. The dry shell weight was measured to mg. The fitness of each clam was assessed as a condition index (CI), which is a ratio of

the wet or dry tissue weight to the shell weight as CI = wet tissue weight (wt)/dry shell weight, or CI = (dry tissue weight/dry shell weight) × 1,000).

2. Assessment of *P. olseni* Infection Using RFTM

The level of *P. olseni* infection was assessed using RFTM (Ray, 1966). For the assay, the entire tissue of each clam was placed in 10 mL of FTM, supplemented with 20 µL of Nystatin (0.2 g/mL) and Chloramphenicol (100 µg/mL) to inhibit bacterial growth during incubation. The RFTM tubes, containing the whole tissue of suspected Manila clams, were incubated at room temperature in the dark for one week. A total of 30 clams were used to assess the infection intensity. Alternatively, *P. olseni* infection was also determined using a piece of gill tissue from each clam. After weighing, the gill tissue was placed in 5 mL of FTM with the same antibiotics and incubated at room temperature in the dark for one week. An additional 30 clams from each sampling site, except Moseolpo, were used for the gill assay. Following incubation, hypnospores that developed in the gill or whole-body tissues were isolated using the method described by Choi *et al.* (1989). The tissues were digested in 2 M NaOH at 60°C, and the hypnospores were harvested via centrifugation, washed twice in phosphate-buffered saline (PBS, pH 7.6), and counted using a hemocytometer. *P. olseni* infection prevalence was expressed as the percentage of infected clams out of the total number analyzed, while infection intensity was determined as the number of *P. olseni* cells (i.e., hypnospores) per gram of gill or total tissue.

3. Histology

After excising the gills for the RFTM assay, a 2 to 3 mm-thick central tissue section, including the gonad, digestive gland, mantle, gill, and foot, was removed for histological analysis. The remaining body was frozen and lyophilized to determine the dry weight-based condition index. The longitudinal tissue sections were immediately fixed in Davidson's fixative. Once fixed, the Manila clam tissues were transferred to 70% ethanol and gradually dehydrated in a series of ethanol solutions ranging from 80% to 100% using a tissue processor (TP1020, LEICA, Germany). The paraffin-infiltrated tissues were then embedded in paraffin, and the paraffin blocks were sectioned into six µm-thick slices. The sections were stained with Harris's hematoxylin and eosin Y. The presence of *P. olseni* in the histological preparations was examined under a light microscope. Infection intensity was graded according to the criteria of Ngo and Choi (2004), with the following scale: 0) no infection, 1) *P. olseni* limited to the gill and mantle, 2) infection present in the gill, mantle, digestive tubules, and connective tissues, 3) infection spread to the gill, mantle, digestive tissues, and gonads, and 4) infection present in the gill, mantle, digestive tissue, gonad, and foot.

RESULTS

1. The sampling effort and CI

Table 1 summarizes the sampling effort. In July 2022, a total of 304 clams, with shell lengths ranging from 26.7 to 35.3 mm, were collected from beaches and a lagoon in Seongsan, Jeju Island. The shell sizes

Table 1. The mean and standard error of shell length (mm) of Manila clams used in this study. N, number of clams collected

Sites	N	Shell Length (mm)
Geumneung	60	26.7 ± 1.8
Gimnyeong	60	28.7 ± 3.8
Moseulpo	4	29.7 ± 2.1
Seogwipo	60	29.8 ± 4.2
Seongsan	60	33.8 ± 6.2
Jongdal-ri	60	35.3 ± 4.5

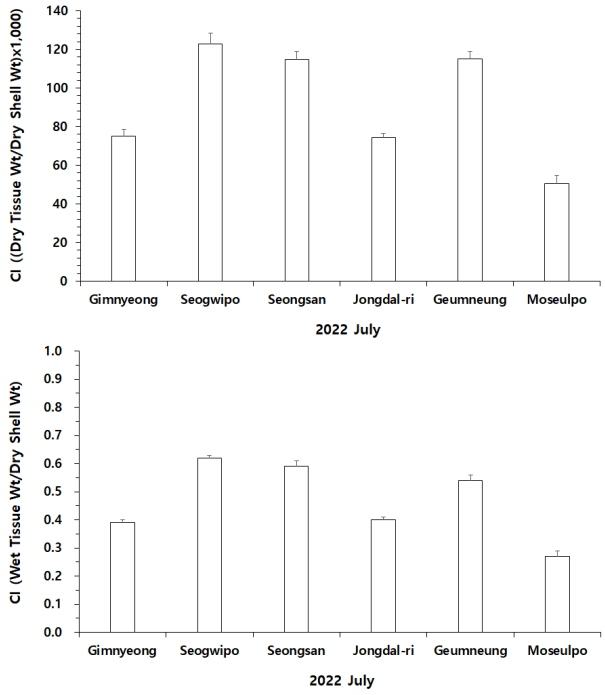

Fig. 2. The mean (bar) and standard error (vertical line) of the condition index (CI) of Manila clams collected from 6 sites in Jeju Island in July 2022.

suggested that these clams were two- to three-year-old adults. Histological analysis revealed that most of the clams collected in July 2022 were reproductively mature, either ready to spawn or had partially spawned. The condition index, defined as the ratio of dry tissue weight to dry shell weight, ranged from 50.7 (Moseolpo) to 122.9 (Seogwipo), while the ratio of wet tissue weight to dry shell weight ranged from 0.27 (Moseolpo) to 0.62 (Seogwipo).

2. RFTM assay

In the gill RFTM assay, *P. olseni* was not detected in Manila clams collected from Geumneung, Gimnyong, Moseolpo, or Jongdal-ri (Fig. 3). The mean intensity of *P. olseni* infection in the gill tissue from Seogwipo and Seongsan was 41,600 ± 10,000 cells/g and 56,000 ± 17,000 cells/g, respectively, with infection prevalences of 43% and 47%.

P. olseni infection was also assessed using whole-body incubation in FTM. As shown in Fig. 4, *P. olseni* was detected in clams from Geumneung, Seogwipo, Seongsan, and Jongdal-ri, with prevalence rates ranging from 10 (Geumneung) to 87% (Seongsan). The highest infection intensity was recorded in Seongsan at 47,900 *P. olseni* cells/g tissue, followed by Seogwipo (21,700 cells/g), Geumneung (11,300 cells/g), and Jongdal-ri (3,800 cells/g).

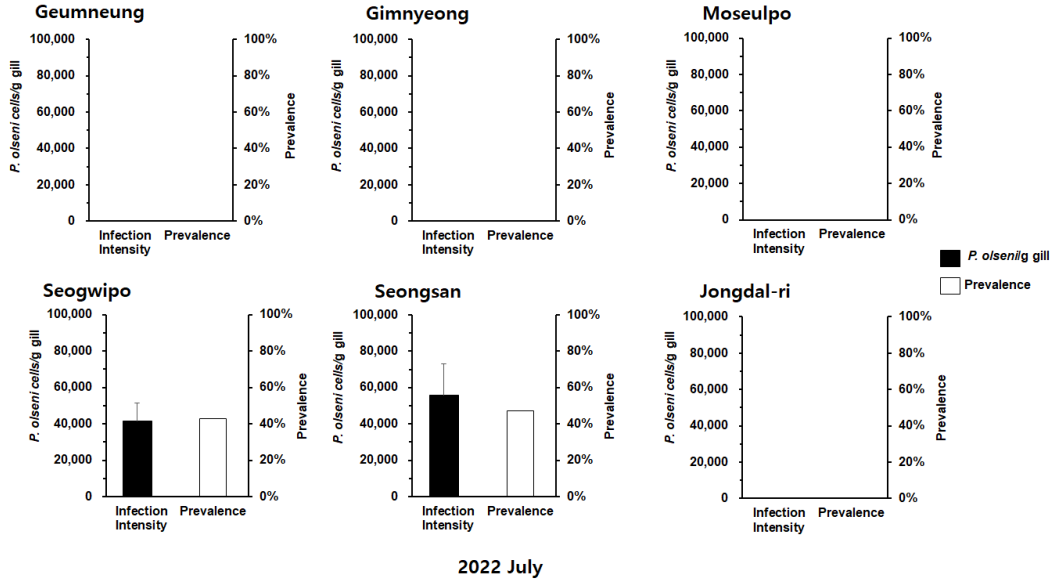

Fig. 3. The mean and standard error of *P. olseni* infection intensity and prevalence in Manila clams analyzed by the gill RFTM assay.

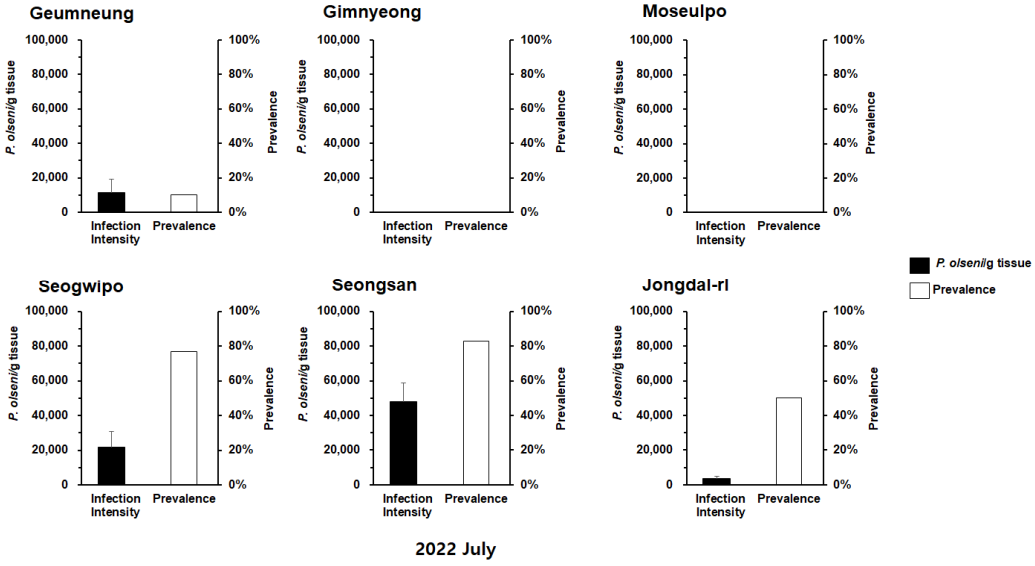

Fig. 4. The mean and standard error of *P. olseni* infection intensity and prevalence in Manila clams analyzed by the whole clam RFTM assay.

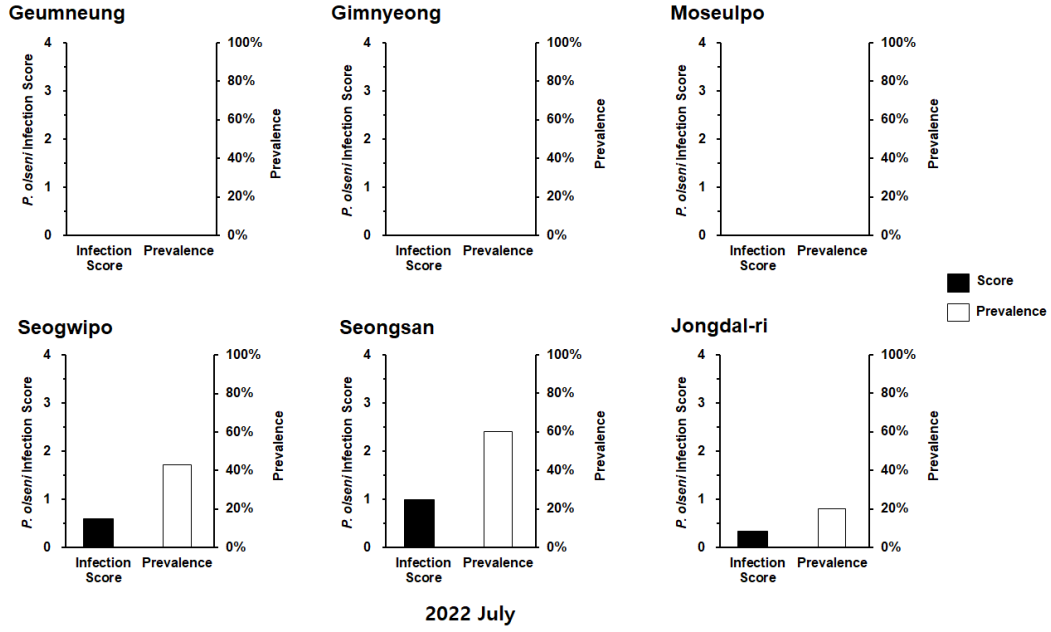

Fig. 5. The mean and standard error of *P. olseni* infection score and prevalence in Manila clams collected from 6 sites on Jeju Island in July 2022. The infection score was determined from histology.

3. P. olseni infection score based on histology

Histological analysis also confirmed *P. olseni* infections in Manila clams from Jeju Island, but these were limited to Seogwipo (43%), Seongsan (60%), and Jongdal-ri (20%) (Fig. 5). The infection scores, used as a proxy for infection intensity, ranged from 0.3 (Jongdal-ri) to 1.0 (Seongsan), suggesting that infections were low and restricted mainly to the gill and mantle tissues.

DISCUSSION

The histology revealed that *P. olseni* infection in Manila clams from Seongsan and Seogwipo was limited to the gills and mantle tissues, exhibiting the early phase of the infection. In contrast, *P. olseni* can be observed from the gonadal connective tissues and foot of clams when the clams are heavily infected, suggesting that *P. olseni* is systemic as the infection starts at the gills or mantle and extends to other tissue (Yang 2010). According to Wang *et al.* (2018), the clam gills and labial palps serve as the primary portal for *P. olseni*, as these organs are directly exposed to the surrounding environment, making them accessible to the zoospores. As the gills and labial palps are involved in feeding and respiration, clams may accidentally filter *P. olseni* zoospores or hypnospores. Using histology, Ngo and Choi (2004) also surveyed *P. olseni* infection in Manila clam on Jongdal-ri beach in Jeju Island over 12 months. In July 2001, *P. olseni* prevalence in Manila clams at Jongdal-ri Beach was 26% with an infection intensity of 0.4, suggesting that *P. olseni* infection level measured in this study is comparable to 2001.

P. olseni infection in Manila clams occurring on sand beaches in Jeju Island was surveyed in this study using RFTM, which provides truly quantitative information. The RFTM revealed that Manila clams on Gimnyeong on northern Jeju Island are free from *P. olseni*. Park and Choi (1997) also investigated *P. olseni* infection in Manila clams on Gimnyeong Beach using RFTM. Of thirty clams (SL 30.4 mm) collected from Gimnyeong Beach, none of the clams incubated in FTM developed the hypnospores, indicating that the clams are not infected by *P. olseni*, as was observed in this study (Table 2). In contrast, Choi and Park (2001) examined *P. olseni* infection in Manila clams collected from Gimnyeong Bay using RFTM and reported the infection intensity as 376 cells/g tissue. The absence or negligible level of *P. olseni* infection on Gimnyeong Beach was believed to be partly associated with salinity. During the low tide, the sampling site at Gimnyeong Beach is strongly influenced by freshwater springs. At the sampling site, the freshwater springs create areas of low-salinity brackish water, which may depress the proliferation and growth of *P. olseni*, as *P. olseni* prefers high-salinity and high-temperature environments (Villalba *et al.*, 2004).

The RFTM assay indicated that Manila clams in Seogwipo and Seongsan areas are infected by *P. olseni*, although the infection levels as *P. olseni* cells per gram gill or body tissue are much lower than the levels recorded on tidal flats on the west and south

Table 2. The infection intensity and prevalence of *P. olseni* infection in Manila clam *Ruditapes phlippinarum* determined in Jeju Island. The infection intensity vales represent the mean and standard deviation or error. N, the number of clams used in the analysis

Sites	1997 (Park and Choi. 2001)			2000 (Choi and Park. 2001)			2022 (Present Study)		
	N	Infection Intensity (cells/g tissue)	Prevalence (%)	N	Infection Intensity (cells/g tissue)	Prevalence (%)	N	Infection Intensity (cells/g tissue)	Prevalence (%)
Geumneung	-	-	-	30	18,980 ± 7,114	60	30	11,345 ± 7,883	10
Gimnyong	-	-	-	30	376 ± 327	7	30	0	0
Moseopo	-	-	-	30	1,069 ± 647	17	-	-	-
Seogwipo	23	165,718 ± 81,660	70	22	426 ± 276	14	30	21,683 ± 9,147	77
Seongsan	20	410,797 ± 351,217	90	30	78,553 ± 21,763	70	30	47,870 ± 10,983	83
Oedo	34	0	0	-	-	-	-	-	-
Iho	-	-	-	29	1,527 ± 686	21	-	-	-
Jongdal-ri	-	-	-	30	8,146 ± 5,058	33	30	3,813 ± 1,075	50
Pyoseon	-	-	-	20	98,431 ± 48,388	100	-	-	-
Hwasun	-	-	-	30	0	0	-	-	-

coasts. The whole-body RFTM revealed that 83% of Manila clams occurring on a lagoon in Seongsan on the east coast of Jeju Island are infected by *P. olseni*, although the infection intensity of 47,800 cells/g tissue is considered low. Similarly, the infection prevalence of Manila clams occurring on a sandy mud flat in the inner part of the Seogwipo Harbor reached 77%, with the infection intensity of 21,600 cells/g tissue, which is the second highest in this survey. As Table 2 shows, the *P. olseni* infection prevalence and intensity of Seongsan clams determined in 2000 by Choi and Park (2001) is comparable to this study, as the prevalence and intensity recorded as 70% and 78,500 cells/g tissue. However, the prevalence and intensity level determined from Manila clams on Seaongsan lagoon, as the intensity and prevalence of 410,800 cells/g tissue 90% respectively by Park and Choi (2001) is much higher than the level observed in this study. Such differences in intensity and prevalence could be explained by the seasonal variability in *P. olseni* infection. On the west coast of Korea, *P. olseni* infection intensity shows an annual maximum during the post-spawning period in September and October, when the host clams are physiologically exhausted due to spawning (Park *et al*. 2006; Yang *et al*. 2012; Hong *et al*. 2014).

In summary, we surveyed *P. olseni* infection among different Manila clam *R. philippinarum* populations along the coastal Jeju Island using RFTM and histology. The assay indicated that *P. olseni* infection levels in Manila clams in Jeju Island collected in July 2022 were similar to the levels determined in 2001.

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