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Perkinsus olseni in the short neck yellow clam, *Paphia malabarica* (Chemnitz, 1782) from the southwest coast of India

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ABSTRACT

Parasites of the genus *Perkinsus* predominantly infect bivalves, and two species among them, *P. olseni* and *P. marinus*, are notifiable to OIE. *P. olseni* infections are known to cause extensive damage to wild as well as farmed bivalves globally with enormous implications to its fishery. Consequent to the initiation of a surveillance programme for aquatic animal diseases in India, *Perkinsus* infections were observed in many species of bivalves. The present paper describes *P. olseni* infections in the short neck yellow clam, *Paphia malabarica* from the southwest coast of India. Diagnosis of the parasite was carried out using Ray's Fluid Thioglycollate Medium culture, histology, *in-situ* hybridisation and molecular taxonomy. Pathology of infection and development of zoospores is also described. This forms the first report of a *P. olseni* infection in *P. malabarica*. High prevalence and intensity of infection of *Perkinsus* in clams raises concerns, as clam reserves in this geographical area sustain fisheries and the livelihoods of local fishing communities.

1. Introduction

The venerid clam, *Paphia malabarica* (Chemnitz, 1782), also known as short neck yellow clam, is an important bivalve species distributed along the east and west coasts of India. It inhabits sandy, marine/brackish water stretches and estuaries, and can withstand high variations in salinity. *P. malabarica* represents 80–90% of the total clam exports from India (Mohite, 2010). Continuous exploitation and increased fishing pressure, along with other factors, contribute to the fluctuation/decline in *P. malabarica* populations along the west coast of India (Venkatesan et al., 2015).

Perkinsosis is a disease in molluscs caused by protozoan parasites of the genus *Perkinsus*. Seven species have been described, viz. *P. marinus* (Mackin et al., 1950), *P. olseni* (Lester and Davis, 1981), *P. quagwadi* (Blackbourn et al., 1998), *P. chesapeaki* (McLaughlin et al., 2000), *P. mediterraneus* (Casas et al., 2004), *P. honshuensis* (Dungan and Reece, 2006) and *P. beihaiensis* (Moss et al., 2008). Among these, *P. olseni* and *P. marinus* are notifiable to World Organisation for Animal Health (OIE). *P. olseni* was first reported by Lester and Davis (1981) in *Haliotis ruber* from Australia and has a wide geographical distribution and host range, infecting various species of bivalves and gastropods (Choi and Park, 2010; Villalba et al., 2004). *Perkinsus* infections have been reported in clams worldwide. *P. olseni* (= *atlanticus*) infections have been documented in *Tapes decussatus* from Portugal (Azevedo, 1989), *Venerupis pullastra* and *V. aureus* from Spain (Navas et al., 1992), *Tapes philippinarum* from South Korea (Choi and Park, 1997), *Tridacna crocea* from Australia (Goggin, 1995), *Austrovenus stutchburyi* from New Zealand (Hine, 2002), *Paphia undulata* from Thailand (Leethochavalit et al., 2004), *Pitar rostrata* from Uruguay (Cremonte et al., 2005), *Protothaca jedoensis* from South Korea (Park et al., 2006) and *Anadara trapezia* from Australia (Dang et al., 2015; Goggin, 1994). Information on *Perkinsus* species from India is limited to reports of *P. marinus* in *Crassostrea madrasensis* (Muthiah and Nayar, 1988), *P. olseni* in *Pinctada fucata* (Sanil et al., 2010) and *P. beihaiensis* in *C. madrasensis* (Sanil et al., 2012). The present study identified *P. olseni* infections in *P. malabarica* from the west coast of India and is the first report of a *Perkinsus* infection in *P. malabarica*.

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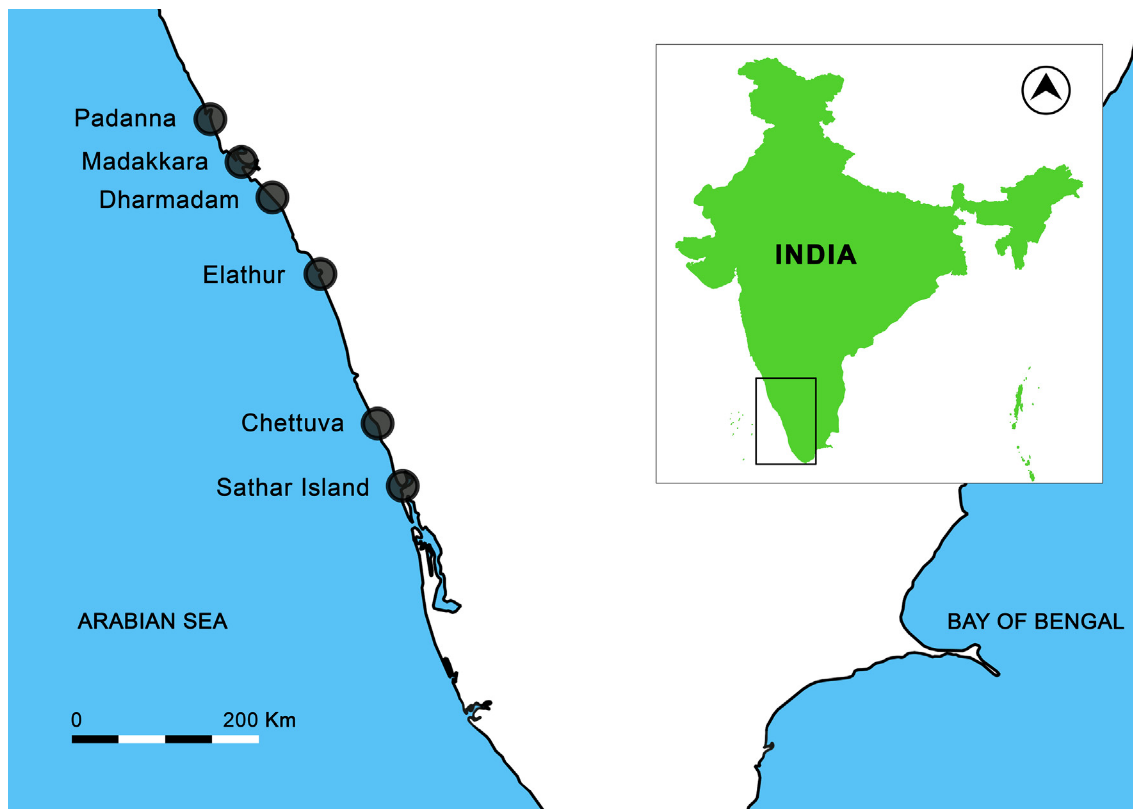

Fig. 1. Map of the southwest coast of India indicating the collection sites.

2. Materials and methods

2.1. Sampling

Samples of *P. malabarica* were collected from six locations (Padanna, Madakkara, Dharmadam, Elathur, Chettuva and Sathar Island) along the southwest coast of India during 2015–2017 (Fig. 1). Clams were collected by handpicking or hand operated dredges. Tissues from 346 clams were processed for RFTM culture assay, histopathology, molecular analysis and fluorescent *in-situ* hybridisation.

2.2. Ray's Fluid Thioglycollate Medium (RFTM) culture assay:

Gill and mantle tissues approximately 5 to 10 mm in size were excised and incubated in Ray's Fluid Thioglycollate Medium (RFTM) supplemented with penicillin (500 U ml^{-1}), streptomycin (340 μg ml^{-1}), and nystatin (200 U ml^{-1}) in dark, at ambient temperature for 5 d (Ray, 1966; OIE, 2018). The incubated tissue fragments were placed on a glass slide, macerated in a drop of Lugol's iodine solution, cover-slipped and allowed to stand for 10 min. The preparation was examined under a Nikon Eclipse 80i microscope (Nikon, Japan). Blue-black hypnospores were counted and the intensity of infection calculated as per the modified Mackin's scale (Ashton-Alcox et al., 2006). Numerical values ranging from 0 to 5.0 are assigned based on the degree of infection (0 = negative, 0.33 = very light [1–10 hypnospores], 0.67–1.33 = light infection [11- > 125 hypnospores but < 25% of the tissue], 1.67–2.33 = light/moderate infection [< 25% of tissue to > 25% but < 50% of the tissue], 2.67–3.33 = moderate infection [> 25% of the tissue but < 50% to > 50% but < 75%], 3.67–4.33 = moderately heavy [> 50% of the tissue but < 75% to > 75% but < 100%] and 4.67–5.0 = heavy infection [> 75% of the tissue to nearly 100% of the tissue].

2.3. Sporulation

The decaying tissues in RFTM positive tubes were macerated gently under a Nikon SMZ 1000 stereomicroscope (Nikon, Japan) to release hypnospores into a Petri dish containing autoclaved seawater at 28 ppt salinity (Goggin et al., 1989). Coarse tissue debris was removed and the hypnospores were isolated using a fine micropipette. Isolated hypnospores were transferred to autoclaved seawater and incubated for 5–7 d with regular change of sea water twice a day and monitored closely to study the sequence of development. Zoospores were studied in detail using a Scanning Electron Microscope (Tescan VEGA3, Brno, Czech Republic).

2.4. Histopathology

Sagittal sections of *P. malabarica* were fixed in Davidson's fixative for 48 h and transferred to 70% ethanol. The fixed samples were dehydrated through ascending series of alcohol, cleared in xylene, embedded in paraffin blocks and 3–5 μm thick sections were cut using a microtome (Leica, Germany). The sections were stained with Harris hematoxylin and counterstained with alcoholic eosin, and photomicrographs were taken using a Nikon DS Fi1c camera and Nikon - Elements BR software (Nikon, Japan).

2.5. Molecular analysis

Small portions of *P. malabarica* gill and mantle tissues were fixed in 95% ethanol for molecular analysis. Total DNA was isolated using HiPurA™ Multi-Sample DNA purification kit (HiMedia, India) following the manufacturer's protocol. PCR assays were performed following the OIE recommended protocols using *Perkinsus* genus specific primers producing a 700 bp PCR product on ITS rDNA (Casas et al., 2002).

PCR protocol included an initial denaturation at 95 °C for 4 min, 35 amplification cycles at 94 °C for 1 min, 59 °C for 1 min, 72 °C for 1 min

Table 1
Details of DNA sequences of *P. olseni* downloaded from NCBI Genbank and used in this study.

Sl no.	Accession number	Host	Geographical Location
1.	GQ896504	*Pinctada fucata*	India
2.	AF522321	*Paphia undulata*	Thailand
3.	AF473840	*Ruditapes philippinarum*	South Korea
4.	DQ516715	*Venerupis philippinarum*	Japan
5.	GU377511	*Ruditapes philippinarum*	China
6.	EU871715	*Tridacna crocea*	Vietnam
7.	JQ669641	*Ruditapes philippinarum*	France
8.	DQ194974	*Tapes decussatus*	Spain
9.	KJ701595	*Venerupis aurea*	Italy
10.	AF509333	*Ruditapes decussatus*	Portugal
11.	U07701	*Haliotis laevigata*	Australia
12.	PAU07697	*Ruditapes decussatus*	Australia
13.	EF204089	*Austrovenus stutchburyi*	New Zealand
14.	KJ608210	*Crassostrea gasar*	Brazil
15.	KU172677	*Isognomon alatus*	Carribean sea

followed by a final extension at 72 °C for 10 min. PCR products were visualized in 1% agarose gel incorporated with ethidium bromide. Corresponding PCR products of positive bands were excised, purified and sequenced (Sanger sequencing) by commercial firms. Contigs formed from the sequence data were curated manually and submitted to NCBI GenBank. The sequences were analysed by BLAST and hits were downloaded giving due consideration for identity scores, host species and geographic distribution of the isolates (Table 1). Phylogenetic analysis was carried out using *Perkinsus* genus-specific sequences generated in this study and downloaded BLAST sequences with the sequence of *P. quagwadi* as outgroup. The best fit model for phylogenetic analysis was calculated using MEGA 6 software. Maximum Likelihood (ML) and Neighbor Joining (NJ) (Tamura et al., 2013) trees using the best fit model were constructed with 1000 resamplings each in MEGA 6 software. The pairwise genetic distance was also calculated between the selected species using Kimura-2 parameter method (Kimura, 1980).

2.6. Fluorescent in-situ hybridization (FISH)

Nucleotide probe (5′CTCACAAGTGCCAAACAACTG3′) specific for *P. olseni* (Moss et al., 2006; OIE, 2018) was 5′end labelled with Alexafluor 488 by chemical synthesis (Sigma, Singapore) and a stock solution of 1.0 $\mu g\,\mu l^{-1}$ was prepared by reconstitution in nuclease free water. Tissue sections for FISH were prepared as in the case of histopathology studies on silane coated glass slides and processed following Carnegie et al. (2006) with slight modifications. The sections were dewaxed in three changes of xylene for 2 min each and rehydrated in descending series of alcohol. Subsequently, the sections were immersed in tap water and then in PBS for 1 min each. The sections were treated with 400 U ml^{-1} pepsin for 10 min at 37 °C, washed in 0.2% glycine in PBS, followed by 5X SET buffer for 5 min each. The sections were incubated in hybridization buffer for 10 min at 42 °C followed by overnight incubation with labelled probe at a concentration of 0.04 $\mu g\,\mu l^{-1}$. The sections were then washed in three changes of 0.2 X SET buffer at 42 °C for 1 min each, air dried, mounted in glycerol PBS (9:1) and observed under fluorescent microscope.

3. Results

3.1. Prevalence and intensity

Perkinsus infections were observed in clams from all six sampling locations. Prevalence of infection ranged from 75% to 100% with an overall prevalence of 87.86% in RFTM assay (Table 2). The intensity of infection varied with locations and ranged from 0 to 5 in modified Mackin's scale.

3.2. Ray's Fluid Thioglycollate Medium (RFTM) culture assay

Numerous enlarged, blue-black spheres typical of *Perkinsus* hypnospores were observed in gill and mantle tissues. In gill tissues, hypnospores were arranged in rows along the margin of the gill lamellae or as aggregates. In mantle tissues, they were observed as isolated clusters when the intensity was low or completely dispersed in heavy infections (Fig. 2a, b).

3.3. Histology

Numerous trophozoites of *P. olseni* were observed in the gill and mantle tissues of *P. malabarica* samples collected from all locations. Trophozoites exhibited the characteristic signet ring shape with an eccentric nucleus and a large vacuole that occupied most of the cytoplasm; trophozoites measured 2.56–3.96 µm (mean, 3.28 ± 0.38 µm). In the connective tissues of gill and mantle, groups of trophozoites were arranged in clusters, demarcated by a clear zone from surrounding tissues (Fig. 2c, d). Trophozoites were also observed in muscle tissues (Fig. 2e). Schizonts exhibiting typical rosette-like arrangement were also observed (Fig. 2f). Pathological changes included histological lesions, host cell disintegration, pyknosis of nuclei and hemocytic infiltration. Ceroid bodies were also present in infected mantle tissues (Fig. 2g, h).

3.4. Fluorescent in-situ hybridization

Alexafluor 488-specific fluorescence was observed in the tissues indicating *P. olseni* infections. Fluorescence corresponded to areas of infection observed in the histology slides (Fig. 2i).

3.5. Molecular analysis

Genus-specific PCR assays produced a ca 700 bp amplicon, confirming the presence of *Perkinsus* spp. infection. The species identity of the parasite was confirmed by nucleotide sequencing followed by BLAST analysis (www.ncbi.com). The sequences showed 99–100% identity with *P. olseni* sequences. The sequence information generated was submitted to NCBI database (GenBank Accession Nos. MG733365, MG733366, MG733367, MG733368, MG733369 and MG733370). The calculated best fit model for phylogenetic analysis was Kimura-2 parameter method. The pairwise genetic distance between the five isolates from Padanna (MG733365), Madakkara (MG733366), Dharmadam (MG733367), Elathur (MG733368) and Chettuva (MG733369) was 0.000, while the sixth from Sathar Island (MG733370) was 0.002. The *P. olseni* sequence (GQ896504) from *P. fucata* reported earlier from India showed a genetic distance ranging from 0.002 to 0.005 from the *P. malabarica* isolates. The genetic distance between these isolates and other isolates of *P. olseni* from different geographic locations ranged from 0.000 to 0.024. Sequence distances of *P. olseni* from Thailand (AF522321) and Australia (U07701) were 0.000, while the sequence from Vietnam (EU871715) was 0.024. The pairwise genetic distance between the *P. olseni* isolates from *P. malabarica* with those of other species of *Perkinsus* varied from 0. 031 to 0.537 (Table 3). Phylogenetic trees constructed with Maximum Likelihood and Neighbour Joining methods were similar and sequences generated in the present study clustered along with sequences of *P. olseni* with high bootstrap value of 96 (Fig. 3).

3.6. Sporulation studies

Sporulation of uninucleated hynospores was not uniform and began as early as the first day of incubation in seawater. A discharge tube appeared followed by a series of mitotic cell divisions on day 2, producing hundreds of biflagellate zoospores by the fourth day. On days 4–5, actively swimming zoospores were released through the discharge

Table 2
Details of sampling locations, prevalence and intensity of *P. olseni* infections in *P. malabarica*.

Sampling locations	Geographical co-ordinates	Samplings	Numbers screened	Numbers infected (RFTM)	Prevalence (%)	Intensity (range)
Padanna	N 12°11′34.0″ E 75°08′3.80″	7	80	60	75.00	0.00–5.00
Madakkara	N 11°57′43.80″ E 75°18′10.54″	2	30	30	100.00	0.33–5.00
Dharmadam	N 11°46′04.52″ E 75°28′22.16″	6	96	85	88.54	0.00–5.00
Elathur	N 11°20′56.64″ E 75°44′05.75″	5	70	68	97.14	0.00–5.00
Chettuva	N 10°31′53.66″ E 76°02′51.71″	2	40	35	87.50	0.00–4.67
Sathar Island	N 10°11′23.32″ E 76°11′09.67″	2	30	26	86.67	0.00–4.67
Total	–	**24**	**346**	**304**	**87.86**	**0.00–5.00**

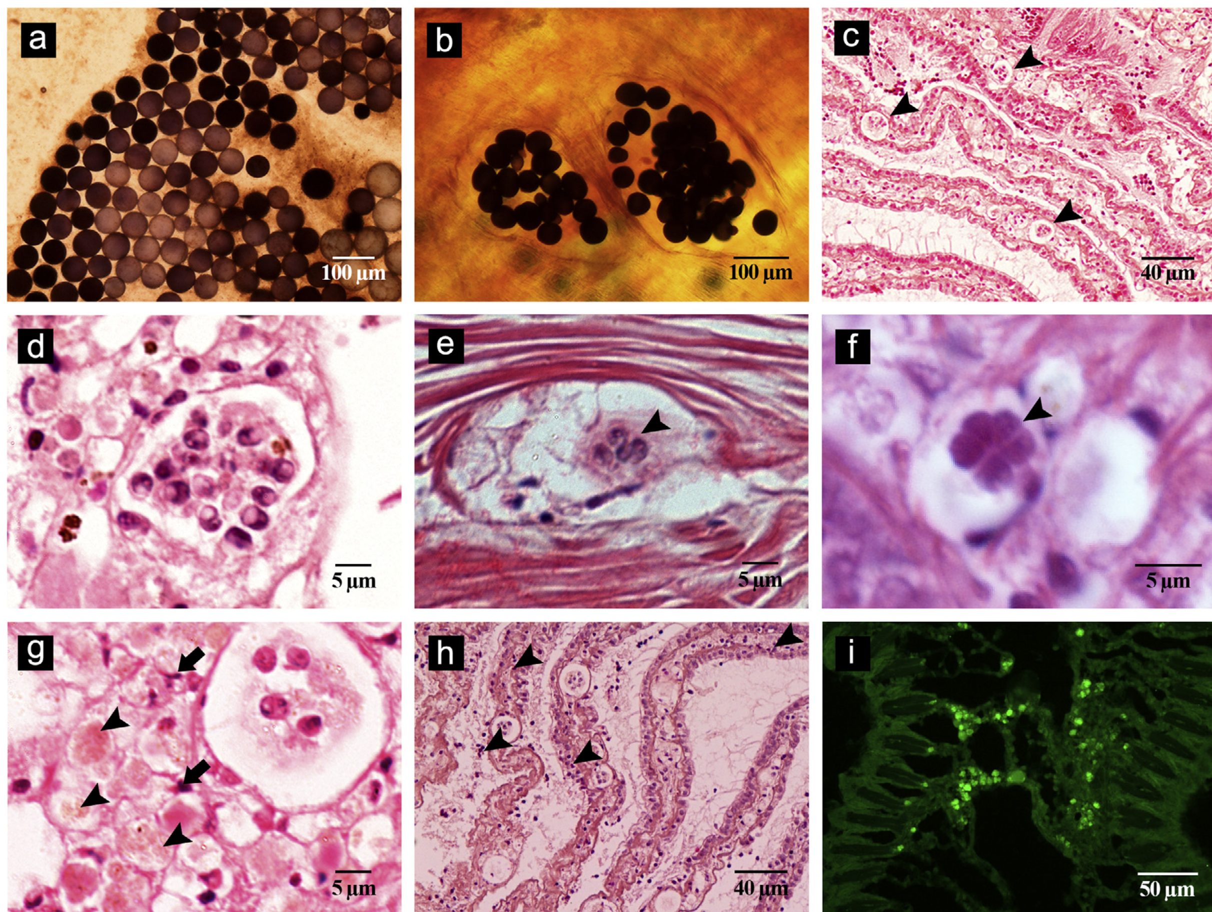

Fig. 2. (a, b) Hypnospores in gill and mantle tissues; (c) lesions in gill tissues; arrowheads indicate cluster of *P. olseni* trophozoites; (d) Cluster of trophozoites in mantle tissues; (e) trophozoites in muscle tissues; (f) schizont (g) ceroid bodies (arrowheads) and pyknotic nuclei (arrows) adjacent to trophozoite cluster; (h) hemocytic infiltration in gill tissues; (i) *P. olseni* in gill tissues – fluorescent *in-situ* hybridization.

tubes. The development of spores was not simultaneous and 5–6 days were required for the complete sporulation of all mature spores (Fig. 4).

3.6.1. Morphology of zoospores

Scanning electron microscopy revealed that the zoospores were elongate-oval in shape with a pointed anterior end, round posterior region and smooth surface (Fig. 3h, i). Ventrally, the zoospores possessed a pair of unequal, uniformly thick flagella located one-third distance from the anterior end. Zoospores measured 2.46–3.56 µm (mean, 3.21 ± 0.30 µm) in length, 1.20–1.66 µm (mean, 1.43 ± 0.15 µm) in width and 1.23–1.56 µm (mean, 1.45 ± 0.10 µm) in thickness. Short and long flagella measured 2.51 – 3.68 µm (mean, 3.12 ± 0.34 µm) and 6.23 – 12.27 µm (mean, 8.84 ± 1.62 µm) respectively, with 0.17 – 0.21 µm (mean, 0.19 ± 0.01 µm) thickness.

4. Discussion

Enlarged hypnospores in gill and mantle tissues in RFTM culture assay and presence of signet ring-shaped trophozoites and rosette-shaped schizonts in histological preparations indicated the presence of *Perkinsus* infection. PCR studies using genus-specific *Perkinsus* primers corroborated the results of RFTM and histology and confirmed *Perkinsus* infections in the tissues of *P. malabarica*. In BLAST analysis, the sequences showed close affinities with sequences of *P. olseni*. Fluorescent

Table 3
Genetic distances between the *P. malabarica* isolates and isolates of *P. olseni* from different geographic locations.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
MG733365 - *P. olseni* (Present isolate)		0.000	0.000	0.000	0.000	0.002	0.002	0.000	0.000	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.003	0.004	0.004	0.007	0.012	0.009	0.010	0.026	0.025	0.066
MG733368 - *P. olseni* (Present isolate)	0.000		0.000	0.000	0.000	0.002	0.002	0.000	0.000	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.003	0.004	0.004	0.007	0.012	0.009	0.010	0.026	0.025	0.066
MG733366 - *P. olseni* (Present isolate)	0.000	0.000		0.000	0.000	0.002	0.002	0.000	0.000	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.003	0.004	0.004	0.007	0.012	0.009	0.010	0.026	0.025	0.066
MG733367 - *P. olseni* (Present isolate)	0.000	0.000	0.000		0.000	0.002	0.002	0.000	0.000	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.003	0.004	0.004	0.007	0.012	0.009	0.010	0.026	0.025	0.066
MG733369 - *P. olseni* (Present isolate)	0.000	0.000	0.000	0.000		0.002	0.002	0.000	0.000	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.003	0.004	0.004	0.007	0.012	0.009	0.010	0.026	0.025	0.066
MG733370 - *P. olseni* (Present isolate)	0.002	0.002	0.002	0.002	0.002		0.003	0.002	0.002	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.004	0.004	0.005	0.008	0.012	0.009	0.011	0.027	0.025	0.065
GQ896504 - *P. olseni* – (India)	0.002	0.002	0.002	0.002	0.002	0.005		0.002	0.002	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.002	0.004	0.003	0.008	0.013	0.009	0.011	0.027	0.026	0.067
AF522321 - *P. olseni* (Thailand)	0.000	0.000	0.000	0.000	0.000	0.002	0.002		0.000	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.003	0.004	0.004	0.007	0.012	0.009	0.010	0.026	0.025	0.066
U07701.1 - *P. olseni* (Australia)	0.000	0.000	0.000	0.000	0.000	0.002	0.002	0.000		0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.003	0.004	0.004	0.007	0.012	0.009	0.010	0.026	0.025	0.066
AF473840 - *P. olseni* (South Korea)	0.002	0.002	0.002	0.002	0.002	0.005	0.005	0.002	0.002		0.000	0.000	0.000	0.000	0.000	0.000	0.004	0.003	0.004	0.008	0.012	0.009	0.011	0.027	0.026	0.065
DQ516715 - *P. olseni* (Japan)	0.002	0.002	0.002	0.002	0.002	0.005	0.005	0.002	0.002	0.000		0.000	0.000	0.000	0.000	0.000	0.004	0.003	0.004	0.008	0.012	0.009	0.011	0.027	0.026	0.065
DQ194974 - *P. olseni* (Spain)	0.002	0.002	0.002	0.002	0.002	0.005	0.005	0.002	0.002	0.000	0.000		0.000	0.000	0.000	0.000	0.004	0.003	0.004	0.008	0.012	0.009	0.011	0.027	0.026	0.065
GU377511 - *P. olseni* (China)	0.002	0.002	0.002	0.002	0.002	0.005	0.005	0.002	0.002	0.000	0.000	0.000		0.000	0.000	0.000	0.004	0.003	0.004	0.008	0.012	0.009	0.011	0.027	0.026	0.065
KJ701595 - *P. olseni* (Italy)	0.002	0.002	0.002	0.002	0.002	0.005	0.005	0.002	0.002	0.000	0.000	0.000	0.000		0.000	0.000	0.004	0.003	0.004	0.008	0.012	0.009	0.011	0.027	0.026	0.065
AF509333 - *P. atlanticus* (Portugal)	0.002	0.002	0.002	0.002	0.002	0.005	0.005	0.002	0.002	0.000	0.000	0.000	0.000	0.000		0.000	0.004	0.003	0.004	0.008	0.012	0.009	0.011	0.027	0.026	0.065
KJ608210 - *P. olseni* (Brazil)	0.002	0.002	0.002	0.002	0.002	0.005	0.005	0.002	0.002	0.000	0.000	0.000	0.000	0.000	0.000		0.004	0.003	0.004	0.008	0.012	0.009	0.011	0.027	0.026	0.065
EF204089 - *P. olseni* (New Zealand)	0.005	0.005	0.005	0.005	0.005	0.007	0.002	0.005	0.005	0.007	0.007	0.007	0.007	0.007	0.007	0.007		0.005	0.004	0.008	0.013	0.009	0.011	0.027	0.026	0.068
JQ669641 - *P. olseni* (France)	0.007	0.007	0.007	0.007	0.007	0.009	0.009	0.007	0.007	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.012		0.006	0.009	0.013	0.010	0.011	0.028	0.027	0.066
KU172677 - *P. olseni* (Carribean sea)	0.007	0.007	0.007	0.007	0.007	0.009	0.005	0.007	0.007	0.009	0.009	0.009	0.009	0.009	0.009	0.009	0.007	0.014		0.008	0.012	0.010	0.011	0.028	0.027	0.068
EU871715 - *P. olseni* (Vietnam)	0.024	0.024	0.024	0.024	0.024	0.026	0.026	0.024	0.024	0.026	0.026	0.026	0.026	0.026	0.026	0.026	0.029	0.031	0.029		0.013	0.010	0.012	0.026	0.027	0.071
JX144358 - *P. marinus*	0.057	0.057	0.057	0.057	0.057	0.060	0.060	0.057	0.057	0.060	0.060	0.060	0.060	0.060	0.060	0.060	0.062	0.065	0.060	0.065		0.010	0.011	0.025	0.025	0.066
KJ701589 - *P. mediterraneus*	0.031	0.031	0.031	0.031	0.031	0.034	0.034	0.031	0.031	0.034	0.034	0.034	0.034	0.034	0.034	0.034	0.036	0.039	0.039	0.039	0.046		0.006	0.021	0.022	0.069
DQ516697 - *P. honshuensis*	0.041	0.041	0.041	0.041	0.041	0.044	0.044	0.041	0.041	0.044	0.044	0.044	0.044	0.044	0.044	0.044	0.047	0.049	0.049	0.052	0.051	0.019		0.022	0.021	0.068
JN054744 - *P. beihaiensis*	0.179	0.179	0.179	0.179	0.179	0.183	0.183	0.179	0.179	0.183	0.183	0.183	0.183	0.183	0.183	0.183	0.183	0.191	0.188	0.182	0.173	0.143	0.147		0.031	0.100
EU919501 - *P. chesapeaki*	0.180	0.180	0.180	0.180	0.180	0.179	0.184	0.180	0.180	0.184	0.184	0.184	0.184	0.184	0.184	0.184	0.183	0.192	0.191	0.194	0.178	0.157	0.150	0.230		0.079
AB973175 - *P. qugwadi*	0.537	0.537	0.537	0.537	0.537	0.531	0.545	0.537	0.537	0.529	0.529	0.529	0.529	0.529	0.529	0.529	0.551	0.529	0.546	0.569	0.546	0.550	0.542	0.767	0.615	

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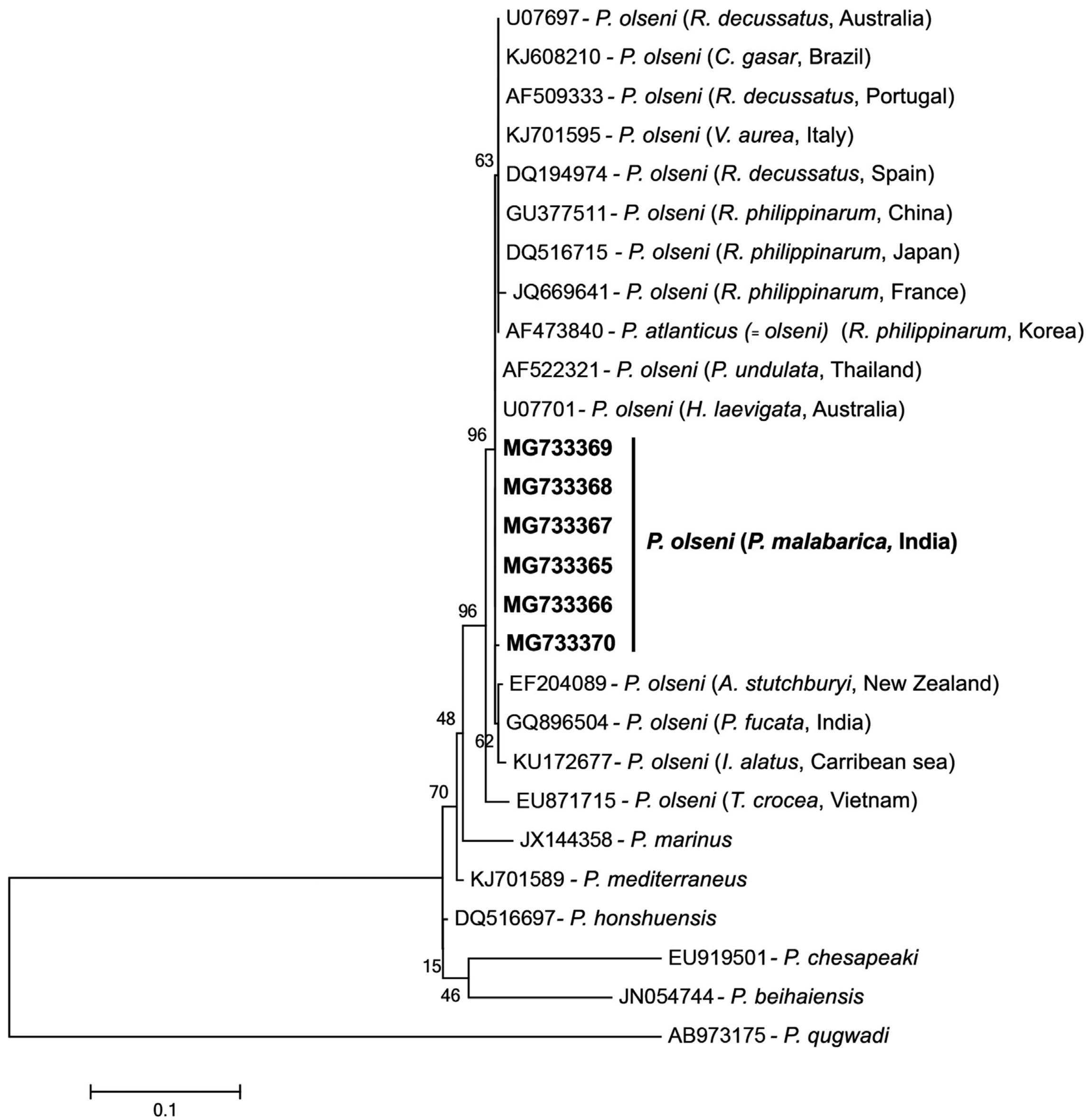

Fig. 3. Phylogenetic tree (ML) constructed using ITS region of *P. olseni* showing boot strap support values. Isolates from the present study are indicated in bold letters.

in-situ hybridization studies using *P. olseni* specific probes also substantiated the above results.

Phylogenetic analysis validated the results of PCR diagnosis and confirmed the taxonomic status of the parasite as *P. olseni*. In Maximum Likelihood tree, all six isolates from the present study clustered together with other isolates of *P. olseni* from different geographical locations with high bootstrap value. The pairwise genetic distance among the six *P. malabarica* isolates varied from 0.000 to 0.002 and are well within the range of accepted intraspecific variations for *P. olseni* (Moss et al., 2008). The genetic distances of the *P. malabarica* isolates with isolates of *P. olseni* from different global geographical regions also showed similar results. Sequences of *P. olseni* infecting *P. undulata*, Thailand (AF522321) and *H. laevigata*, Australia (U07701.1) showed minimum variations with the *P. malabarica* isolates despite their distant geographic locations. On the contrary, an isolate from Vietnam (EU871715), which is geographically closer, exhibited maximum genetic distance of 0.024, while the remaining isolates from Asia, Europe and America showed values ranging from 0.002 to 0.007, indicating low genetic variation.

P. olseni develop as clusters of trophozoites in gills and connective tissues of bivalve hosts (Arzul et al., 2012; Park and Choi, 2001; Villalba et al., 2004). Morphology of the trophozoites and developing schizonts observed in the present study correspond with the descriptions of *P. olseni* (Lester and Davis, 1981; Villalba et al., 2004). Trophozoites of *P. olseni* have been reported with a wide range of variations in morphometry (Dang et al., 2015; Dungan et al., 2007; Hamaguchi et al., 1998; Lester and Davis, 1981; Park and Choi, 2001). In morphometry, trophozoites observed in the present study are similar to those reported from *P. undulata,* Thailand (Leethochavalit et al., 2004).

The pattern of sporulation observed in the present study conforms to descriptions by earlier authors (Azevedo, 1989; Casas et al., 2002; Choi and Park, 2010; Perkins and Menzel, 1966; Lester and Davis, 1981; McLaughlin et al., 2000). The zoospores were similar to those of *P. olseni* from Korean waters but were smaller than those described from European waters. The flagella of the *P. malabarica* isolates were comparatively smaller and were uniform in thickness, while that of the Korean and European forms were longer with the posterior flagellum possessing thick basal and thin apical regions (Azevedo, 1989; Kim et al., 2012).

Pathological manifestations observed in the present study are in agreement with previous descriptions (Arzul et al., 2012; Balseiro et al., 2010; Dang et al., 2015; Dungan et al., 2007; Leethochavalit et al.,

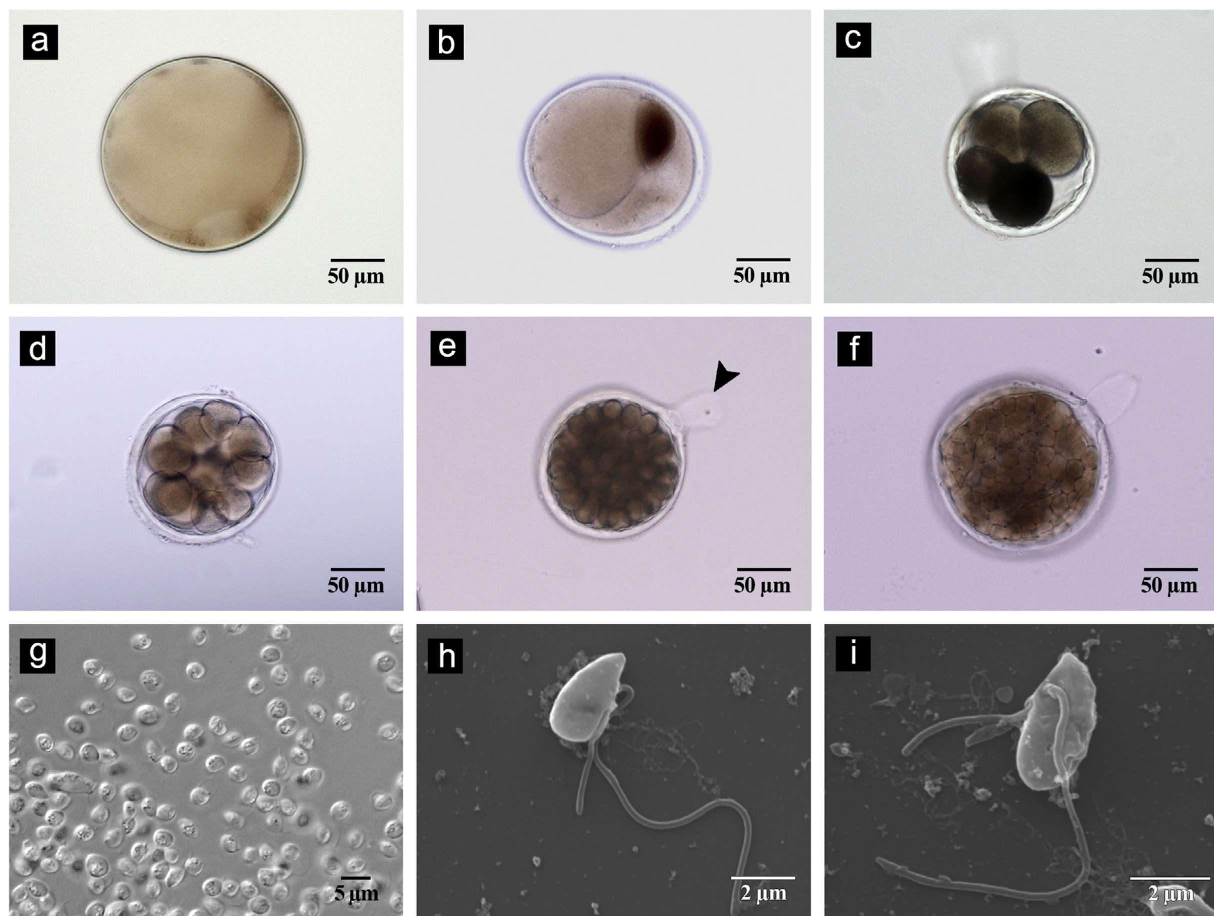

Fig. 4. Sporulation of hypnospores: (a) uninucleated hypnospore; (b) two-cell stage; (c) four-cell stage; (d) sixteen-cell stage; (e, f) numerous cells, germ tube visible; (g) zoospores liberated from hypnospore; (h, i) SEM images of zoospores showing the flagella. Arrowhead indicates germ tube.

2004; Park and Choi, 2001; Park et al., 2006; Yang et al., 2010). Though ceroid bodies were found in connective tissues surrounding *P. olseni* trophozoites, there is no direct evidence linking these to *Perkinsus* infections. Ceroid bodies are generally considered as indicators of stress or cell aging (Apeti et al., 2014). Nodule formations associated with *P. olseni* infections have been described in various bivalve hosts (Almeida et al., 1999; Blackbourn et al., 1998; Choi and Park, 1997; Hamaguchi et al., 1998; Lester and Davis, 1981; Montes et al., 1996; Navas et al., 1992; Park and Choi, 2001), however, despite heavy infections, external manifestations such as nodules or other macroscopic clinical signs were not apparent and the infected clams appeared normal. Brandão et al. (2013) reported that *Crassostrea rhizophorae* exhibited minimal tissue damage under high prevalence and intensity of *Perkinsus* infection. Similarly, Goggin (1996) observed that *P. olseni* did not cause weight loss in infected tridacnid clams. Infected *P. malabarica* may remain in the ecosystem, disseminating and serving as carriers/ reservoirs of infection. Our studies revealed that, in addition to *P. malabarica*, other species of bivalves (*Anadara granosa, Arca* sp.*, Perna viridis* and *Saccostrea cucullata*) also harbor *P. olseni* infections (unpublished), which could very well alter the epidemiology of *Perkinsus* infections in the region.

Numerous instances of mass mortalities and population declines have been reported in clams (Azevedo et al., 1990; Choi and Park, 2005; Figueras et al., 1992; Hamaguchi et al., 1998; Park et al., 1999; Sagristà et al., 1995; Wu et al., 2011; Nam et al., 2018), but Arzul et al. (2012) did not observe any mortality or population decline in clams infected with *P. olseni* and *P. chesapeaki* in France. No mass mortalities were observed in natural populations of *P. malabarica* in the region during the course of this study. *P. malabarica* is not cultured and is not generally observable in its natural habitat, thus, the possibility of under-reporting mortalities cannot be ruled out. Venkatesan et al. (2015) reported a decline in the natural populations of *P. malabarica* from the west coast of India in the past several years, raising serious concerns about the future of clam fishery in the region. Periodic fluctuations in the natural distribution of *P. malabarica* are common and may be attributed to poor spat settlement and mortality of settled spat due to unfavorable environmental conditions, overexploitation, habitat destruction and/or anthropogenic factors. Environmental factors like temperature and salinity are known to affect the immune status of bivalves and thereby influence the dynamics of *Perkinsus* infections (Villalba et al., 2004). Studies by Nam et al. (2018) suggest that physiological (thermal) stress accelerates the development of *P. olseni* by suppressing immune responses in Manila clams, leading to mortality. Although there is no direct evidence, the role of *P. olseni* in the decline/ fluctuation of clam populations in the region remains a possibility and requires further detailed investigation. The high prevalence and intensity of *P. olseni* infections observed in the present study raises concerns, as clam reserves in this geographical area sustain fishery and livelihood of local fishing communities.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jip.2018.10.001.

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