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Note

First report of *Perkinsus* sp. infection in the bivalve *Magallana bilineata* (Roding, 1798) from Pulicat Lake, Tamil Nadu, south-east coast region of India

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ABSTRACT

Perkinsus sp., an important protozoan parasite of mollusca is reported in the bivalve *Magallana bilineata* (Roding, 1798) for the first time from Pulicat Lake, Thiruvallur District, Tamil Nadu, south-east coast of India. Molluscs collected from Pulicat Lake were screened for *Perkinsus* sp. as part of a state-wide aquatic animal disease surveillance programme. *Perkinsus* sp. infection was diagnosed based on culture in Ray's Fluid Thioglycolate Medium (RFTM), histology as well as by molecular analyses. Microscopic analysis of the RFTM supernatant revealed enlarged blue-black hypnospores characteristic of *Perkinsus* sp. like organisms and typical 'signet ring' stages of trophozoites. Histological preparation of the molluscan gills also revealed trophozoites. PCR amplification of genomic DNA isolated from infected specimens using specific internal transcribed spacer (ITS) 85 and ITS 750 primers resulted in 703 bp DNA fragments specific for *Perkinsus* sp. BLAST analysis of the sequence revealed 97% homology with *Perkinus marinus*.

Keywords: Dermocystidium, OIE, *Perkinsus marinus*, Protozoan parasite

Perkinsosis, a significant infection caused by the protozoan parasites of the genus *Perkinsus* causes mass mortality in the farmed and wild marine molluscs including mussels, abalones, clams, scallops, pearl oysters, cockles and oysters (Villalba *et al.,* 2004, Yadavalli *et al*., 2020). Seven species have been described, *viz., Perkinsus 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). *P. marinus* previously known as *Dermocystidium marinum* was the first species to be described (Mackin, *et al.,* 1950; Perkins, 1968; 1988). Transmission of *Perkinsus* spp. occurs without intermediate hosts (Ray, 1954; Goggin and Lester, 1995; Chu, 1996; Blackbourn *et al.,* 1998). *Perkinsus* spp. have three main life stages: trophozoites stage, which occurs in the tissues of the live host (Goggin and Lester, 1995; Perkins, 1996; Blackbourn *et al.,* 1998); hypnospore stage, where thick wall is developed by the trophozoites (Ray, 1952) and zoospore stage, where hundreds of zoosporangium are formed within the cell wall (Perkins and Menzel, 1966).

The total world production of molluscs from marine and coastal aquaculture is 16.4 million t and the contribution from Asia is 14.94 million t (FAO, 2017). Molluscan farming, which used to count for about 30% of the total farmed food fish production around the year 2000, has gradually declined to 21% in 2015 (FAO, 2017). Though India has a vast potential for molluscan culture comprising of edible oyster, mussel, pearl oyster, abalone and clams, no commercial production of any groups of molluscs is undertaken on a large scale. Throughout the world, there have been many reports of mass mortalities in the farmed and wild bivalves due to various protozoan infections which caused substantial losses for the associated industries (Soniat, 1996). Periodical disease surveillance was undertaken on a monthly basis by the State Referral Laboratory for Aquatic Animal Health of Tamil Nadu Dr. J. Jayalalithaa Fisheries University to screen the molluscs for the OIE-listed *Perkinsus* infection along the coast of Pulicat Lake, south-east coast region of India. The diagnosis revealed the presence and confirmation of *Perkinsus* sp. in the bivalve *Magallana bilineata* (Roding, 1798) from Pulicat Lake. This constitutes the first report on the detection of *Perkinsus* sp. in molluscs from the south-east coast of India.

Mollusc samples were randomly collected irrespective of the genus, comprising of *Volegalea cochlidium*, *Magallana bilineata, Mytilus* sp*., Babylonia zeylanica* and *Pugilina conchlidium* from the Karimanal Lagoon located in Pulicat Lake, Thiruvallur District (13°26'43.41"N; 80°19'32.05"E) (Fig. 1). The collected samples were transported live in aerated bags to the laboratory for diagnosis. Of the 60 individuals collected

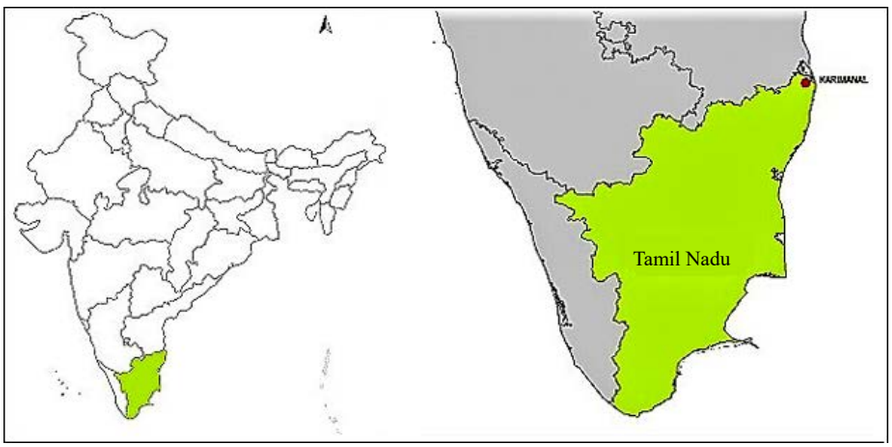


Fig. 1. Map showing the sampling site at Karimanal, Pulicat Lake, Tiruvallur District, Tamil Nadu (lat. 13.44123; long. 80.31782)

comprising of the aforesaid genus, five individuals were of adult *M. bilineata.* The samples were examined under stereomicroscope (Accu-Scope, USA) for any abnormality, fouling, shell damage, gaping, retraction of mantle, fluid accumulation, abscess and lesions.

About 5 mm of the gill and mantle tissue samples were excised and placed in individual 30 ml tubes containing 15 ml Ray's fluid thioglycollate medium (RFTM) supplemented with antibiotics to inhibit the growth of bacterial and fungal organisms (OIE, 2006; Ray, 1966). The tubes were incubated in the dark at 24°C for 7 days. After incubation, the fragments of tissue and the supernatant from each tube were collected and placed on a glass slide, stained directly with both Lugol's iodine and haematoxylin solution and examined under the microscope (100x) for the presence of spores.

Mantle tissue and gills were fixed in Davidson's fixative for 24 h, transferred to 70% alcohol, dehydrated in ethanol series, cleared, embedded in paraffin and 5 µm thick sections were made using a Microtome (Leica, Wetzlar, Germany) and processed for histopathology following standard methods (Roberts, 2012). The sections were stained using Harris haematoxylin and eosin (H&E) and examined under a light microscope (Leica DMLS, Wetzlar, Germany).

The mantle tissue and the gills were aseptically excised and fixed in absolute ethanol for DNA extraction. PCR screening of *Perkinsus* sp. was carried out using primers targeting ITS 85 and ITS 750 (Casas *et al*., 2002). PCR amplification reaction was carried out in a thermal cycler (Bio-rad T100 Thermal cycler, USA) in a total volume of 25 µl reaction mixture containing 10 µl of master mix (Ampliqon Taq DNA polymerase, 2.0 x Master mix red, $MgCl_2$ 2.0 mM), 12 µl of nuclease free water, 1.0 µl (10 pmol) of forward and reverse primer each and 1.0 µl (50 ng) of sample DNA. The PCR cycling conditions followed was initial denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 94°C for 20 s, annealing of primers at 58°C for 20 s and extension at 72°C for 45 s with a final extension at 72°C for 5 min. The PCR products were separated by electrophoresis in an agarose gel (1.2%) stained with ethidium bromide (0.5 µg ml^{-1}) and visualised using a gel documentation system (Bio-Rad, USA). The amplified PCR products were sequenced (Eurofins, Bangalore) and homology of the generated sequence was analysed using the Basic Local Alignment Search Tool (BLAST) program in the National Centre for Biotechnology Information (NCBI) following Altschul *et al.* (1990).

The internal transcribed spacer (ITS) sequences for the known *Perkinsus* spp. sequences retrieved from NCBI GenBank were used for constructing the phylogenetic tree (see appendix for the list of sequences used). The retrieved sequences along with the sequence generated in the present study were aligned using clustalW (Thompson *et al*., 1994). Pairwise genetic distances between the present species and other *Perkinsus* sp. were calculated based on Kimura 2 parameter model (Kimura, 1980), using MEGA7 (Kumar *et al*., 2016). The Best fit nucleotide substitution model was selected from 24 models, based on the one with the lowest BIC scores (Bayesian Information Criterion) which are considered to describe the substitution pattern the best (Nei and Kumar, 2000). The phylogenetic tree was constructed using maximum likelihood model present in MEGA7. Reliability of the phylogenetic tree was estimated using bootstrap values run for 1000 iterations.

Visual observation of all the samples with the aid of a stereozoom microscope revealed remarkably emaciated muscle mass only in the mantle of *M. bilineata,* whereas other bivalves and gastropods did not show up any significant difference.

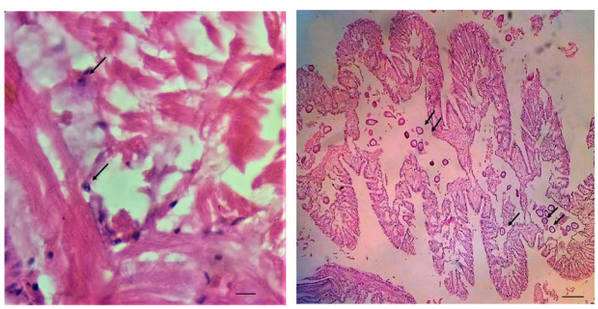
The supernatant from RFTM inoculated with *M. bilineata* mantle and gill tissues showed enlarged blue-black hypnospores characteristic of *Perkinsus* sp. like organisms (Fig. 2a). Typical 'signet ring' stages of trophozoites were observed from 4 to 8th day in the slides stained with Lugol's iodine (Fig. 2b, c, d). From day12 onwards, maturing zoosporangia containing numerous internal zoosporoblast cells that are subdividing towards maturation as motile zoospores were observed (Fig. 2e). On day 13 and 14, zoosporangium containing numerous zoospores with some leaving the sporangium were observed (Fig. 2f).

Trophozoites with vacoulation of the muscle fibre and degenerative necrosis were observed in the muscle of the infected *M. bilineata* (Fig. 3a). Degenerative necrotic changes of the gill filaments with trophozoites of the parasite were observed in the histological section of the gills of the infected samples (Fig. 3b).

The PCR screening of the samples with *Perkinsus* sp. specific primers targeting ITS amplified a DNA product of 703 bp (Fig. 4). The PCR product was further confirmed by sequencing and BLAST analysis in NCBI. The BLAST results showed 97% identity to *P. marinus* sequences present in the NCBI GenBank. The nucleotide sequence generated in the present study was deposited in NCBI GenBank with Accession No. MH542429.

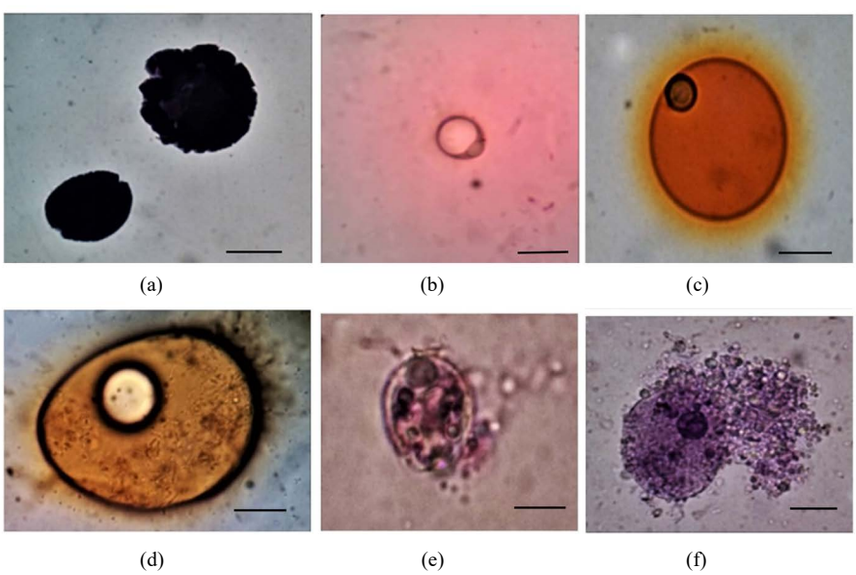
The Best-fit model test for phylogenetic analysis suggested the Kimura 2-parameter model (K2-P) as the best nucleotide substitution model with the lowest BIC scores (Bayesian Information Criterion), corrected AIC value (Akaike information criterion) and a Gamma parameter (HKY +G+I, BIC= 5070.844, AICc=4717.066, lnL= -2311.369, (+G) =1.18). The maximum likelihood tree based on the K2-P model suggests that genetically the closet relative for the present *Perkinsus* sp. is *P. marinus* from which it differs from a pair-wise sequence of 3.7-3.9% (Fig. 5).

The present study confirms the presence of the protozoan parasite *Perkinsus* sp. in *M. bilineata* collected from Pulicat Lake of Tamil Nadu, India based on RFTM culture as well as by histopathological, PCR and sequence analyss. This is the first report of *Perkinsus* sp.



(a) (b)

Fig. 3. Histological sections of mantle/gill tissues of *M. bilineata*. a. Arrows show trophozoites with vacoulation of the muscle fibre (H&E; scale bar =20 µm); b. Arrows show trophozoites in the gills (H&E; scale bar=150 µm)



(a) (b) (c)

(d) (e) (f)

Fig. 2. Microscopic observation of *Perkinsus* sp. cultured by RFTM assay. a: Hypnospores in the RFTM supernatant; b: Trophozoite showing the typical signet ring stage on 4th day; c and d: Signet ring stage on 5th and 6th day; e: Maturing zoosporangia containing numerous internal zoospores; f: Zoosporangium containing numerous zoospores, some leaving the sporangium by the discharge tube (Scale bar=20 µm)

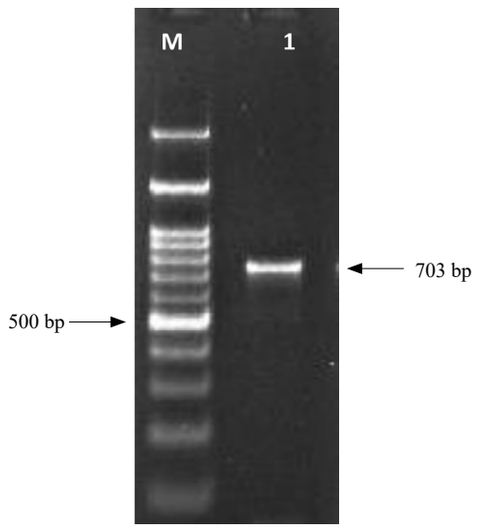


Fig. 4. PCR amplification of ITS region of *Perkinsus* sp. from *M. bilineata.* Lanes M - 100 bp DNA marker, 1 - PCR amplified product (703 bp)

infecting *M. bilineata* from Pulicat Lake. At present two species of Perkinsus have been reported from India *viz., P. olseni* in *Pinctada fucata* (Sanil *et al.,* 2010)*, Paphia malabarica* (Shamal *et al.,* 2018) and *P. beihaiensis* in *M. bilineata* (=*C. madrasensis*) (Sanil *et al.,* 2012). While there is a preliminary report of *P. marinus* infection in *M. bilineata* (=*C. madrasensis*) from Tuticorin (Muthiah and Nayar, 1988), Sanil *et al.* (2010) speculated that the report could be a possible misidentification for *P. olseni* or *P. beihaiensis* (Indian/Brazilian, Group B). The report of *P. marinus* by Muthiah and Nayar (1988) was based only upon RFTM analysis and lacked molecular confirmation. RFTM analysis is a well established preliminary diagnostic method for identification of *Perkinsus* sp. (Villalba *et al*., 2004). In the present study, the hypnospore, 'signet ring' stage of the trophozoites and zoospores observed in the RFTM analysis is supportive for the above fact. Histological analysis also revealed the presence of trophozoites in the gills and muscles. Parasite stages, schizonts and trophozoites were mostly observed in histological sections

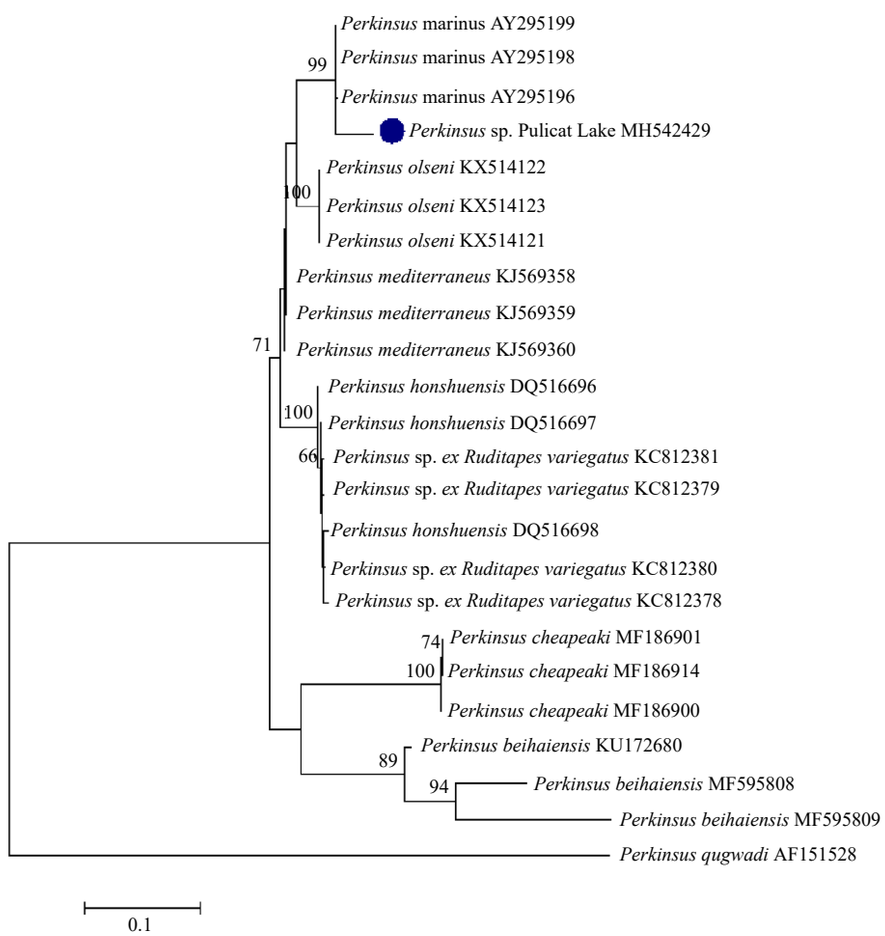


Fig. 5. Phylogenetic position of *Perkinsus* sp. from Pulicat Lake based on maximum likelihood analysis. Values along the nodes are percent bootstraps for 1000 iterations. (Blue circle indicates the GenBank accession number generated in the present study)

of the connective tissue, especially adjacent to the epithelial lining of the stomach, among the digestive tubules and in the muscles of *M. bilineata* (=*C. madrasensis*) (Sanil *et al*., 2012). Clusters of trophozoites of *P. olseni* were observed in the gill and mantle tissues of *Paphia malabarica,* which was demarcated by a clear zone from surrounding tissues (Shamal *et al.,* 2018).

Brown *et al.* (2004) reported that the intra-specific variation among sequences within a *Perkinsus* sp. ranges from 0 to 3% and among species, differences range from approximately 4-14%. Similar findings were noted in the present study where a pair-wise sequence difference of 3% with the known *P. marinus* sequences was observed and the phylogenetic tree demonstrates the present species to be forming a clade together with *P. marinus*. The present findings need further research on the study of the ultrastructure and different stages of the parasite in comparison with the known described species of *Perkinsus*. As there is pair-wise sequence distance of 3% with the known *P. marinus* sequences, the isolated *Perkinsu*s sp. may indicate an undescribed species.

Pulicat Lake is the second largest brackishwater lake in India and rich diversity of cultivable molluscan species occur in this lake, comprising of 4 families and 6 species (Mohan *et al.,* 2013). The edible oyster *M. bilineata* (=*C. madrasensis*) is the most extensively distributed bivalve in this lake and is ideally suited for the culture of this oyster (Sanjeeva Raj, 2006). The report of *Perkinsus* sp. from Pulicat is a significant finding, when considering the health of the commercial molluscs inhabiting the region, as the presence of these parasites may pose a serious threat to their population.

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