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# Detection of an amoebic parasite in cultured Pacific white shrimp

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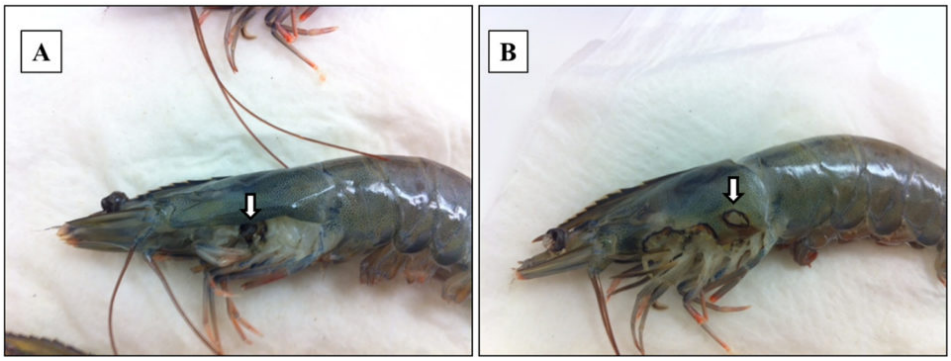
## Study results useful as initial screening method




Gross signs of Pacific white shrimp (Litopenaeus vannamei) cultured in a closed recirculation farm and naturally infected with an amoeboid protozoan. Shrimp exhibited serious damage to the gills and carapace; (A) black gills, (B) missing lamellae and eroded carapaces.

The protozoan *Paramoeba* sp. (synonymous to *Neoparamoeba* sp.) is ubiquitous and normally free-living in marine waters, and it is the most pathogenic amoeboid protozoan parasite in cultured fish. The species *P. perurans* colonizes gills and results in amoebic gill disease (AGD) in farm-raised salmonids and most notably affects the Tasmanian Atlantic salmon industry, with typical losses of 10 to 20 percent of production. So far, AGD has been recorded in 15 finfish species of 11 different genera, and in fish, *P. perurans* infection leads to proliferative cell reactions in the gills, including epithelial hyperplasia, hypertrophy, edema and inter-lamellar vesicle formation, which grossly appear as white mucoid spots and plaques on the gill surface.

Gross pathological assessment of the gill has been widely used for AGD diagnosis, but a subsequent histological examination is recommended to improve diagnostic accuracy. Additionally, molecular diagnostic methods based on conventional PCR assay targeting the small subunit rRNA (SSU rRNA) sequence are available for the diagnosis of AGD.

Various species of *Paramoeba* have also been reported in some crustacean and echinoderm species, such as lobsters, crabs and sea urchins. In these hosts, severe infections were associated with the death of the host, resulting in significant economic losses. The infections were also reported in bivalve mollusks, such as mussels, but these were considered more likely to be potential environmental reservoirs for this protozoan.

This article – adapted and summarized from the **original publication** (https://doi.org/10.1016/j.aquaculture.2019.04.036) – is the first report of this amoeboid protozoan parasite in Pacific white shrimp (*Litopenaeus vannamei*). This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIT) (grant number NRF-2018R1C1B5086350).



## Study setup

Adult Pacific shrimp (avg. weight 30 grams) from an anonymous shrimp hatchery in North America showed reduced appetite, lethargic, and respiratory distress. Cumulative mortality was 62.75 percent at 120 days after stocking, and diseased shrimp had black gills, missing lamellae and eroded carapace (see picture above). From these data, we suspected a fungal infection caused by *Fusarium*species, which has been previously reported as the causative agent of black gill disease in crustaceans, like prawns, shrimp and lobsters. However, amoebic protozoan parasites were observed by direct observation of a wet mount (Fig. 1). Moribund shrimp samples were collected at irregular intervals, fixed in Davidson's AFA fixative or 95 percent ethanol, and further examined by histopathology, PCR and ISH.

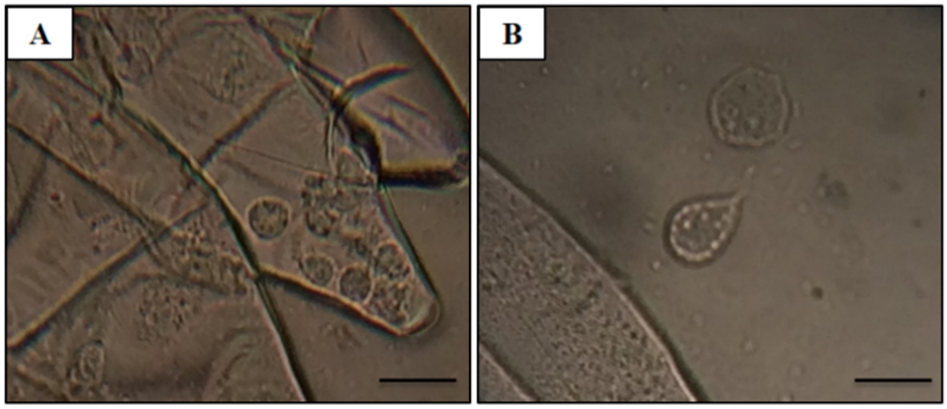



Fig. 1: Samples obtained from wet mount squashes of the gills. (A) Variations in morphology of amoeba and (B) individual free-living amoeba. Scale bars = 20 µm.

For additional, detailed information on shrimp sampling, histopathology examination and sequence analysis, PCR assay and in situ hybridization (ISH), please consult the original publication.

## Results and discussion: Histopathology examination (H&E)

The most significant finding in the microscopic examination of the studied samples was the presence of an amoebic parasite. Infestation by this parasite was found mainly in the gills, with severity grade G4 (Figs. 2A–C), and extensive hyperplasia, bridging of lamellae, and forming of inter-lamellar spaces were observed. According to the farmer, lethargy, anoxia, and, eventually, death has been seen in infected shrimp, and these might be associated with the damage to the gills (respiratory organs). Fig. 2A shows the amoeba nucleus with an amphiphilic core surrounded by an irregular basophilic ring and the parasome with eosinophilic cytoplasm and vacuoles, indicating that the amoeba is closely related to the members of the order Dactylopodida, and maybe the genus Paramoeba, according to Sühnel et al. (2014).

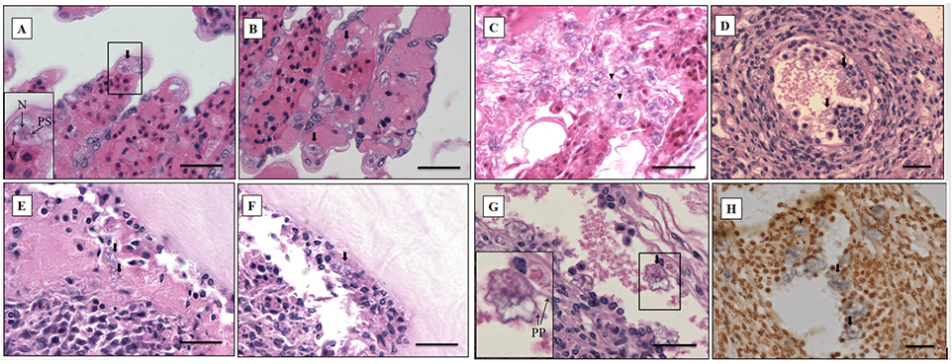



Fig. 2: The amoeba (Paramoeba sp.) infection was found within several tissues by histopathology examination and in situ hybridization. Mayer–Bennett hematoxylin–eosin–phloxine stain; (A), (B) and (C) the gill, (D) the antennal gland, (E) the subcuticular areas, (F) the cuticular epithelium, and (G) the perineurium. By in situ hybridization with a digoxigenin-labeled gene probe, dark blue

precipitate indicates the presence of the parasite (H) within the antennal gland. Flattened and irregular-shaped protozoan amoeba, trophozoite forms (diameter approximately 25 to 30 mm; black arrow), cyst-like forms (diameter less than 10 mm.; black arrowhead), and magnification of trophozoites showing granular endoplasm with (V) vacuoles, (N) nucleus, (PS) parasome, and ectoplasm with (PP) pseudopodia. Scale bars = 30 mm.

In addition, flattened and irregular-shaped trophozoite forms with pseudopodia radiation (approximately 25 to 30 μm in diameter) were predominantly observed. The presence of digitate pseudopodia, lack of cysts (less than 10 μm in diameter), and marine habitat also identify the amoeba as the genus Paramoeba, according to Kent et al. (1988). These amoebic parasites were also observed in other organs, including the antennal gland, lymphoid organ, cuticular epithelium, and the subcutis of appendages and connective tissue surrounding the ventral nerve cord of the tested samples, with severity grade G1 to G4 (Figs. 2D–G).

## PCR assay and sequence analysis

Strong amplicons (an amplicon is a piece of DNA or RNA that is the source and/or product of amplification or replication events) were detected in the tested shrimp (five representative samples), by the PCR assay targeting the SSU rRNA sequence (Fig. 3). Results showed that the nucleotide sequence was 100 percent identical to the sequences of *Paramoeba* sp. (*Neoparamoeba* sp.) from several marine crustacean and echinoderm species. And there was no cross-reaction with genomic DNA from *L. vannamei* shrimp and other shrimp (*P. monodon, P. indicus, L. stylirostris,* and *M. rosenbergii),* polychaetes, squid and *Artemia* spp., or to other shrimp parasites by the specificity test.

Fig. 3: PCR assay targeting the small subunit rRNA sequences. Strong amplifications (131 bp) with DNAs of gill tissues from Paramoeba sp.-infected shrimp (Lanes 1 to 5), specific-pathogen-free shrimp (Lane 6), a non-template control (Lane 7), and 1-kb Plus DNA Ladder (Lane M).

We also conducted a phylogenetic study to investigate the relationship between identified Paramoeba-like sp. from shrimp and other amoeba species. In the resulting phylogenetic tree, the SSU rRNA sequence from *Paramoeba*-like sp. was grouped together with sequences from the order Dactylopodida, especially the family Paramoebidae.

## In situ hybridization

The ISH method can also be used to determine the etiological agents of protozoan infection. For ISH, the *Paramoeba*-like gene probe was generated from the SSU rRNA sequence of the amoeba infecting shrimp, and this hybridized with the amoebic cells in the representative sample (Fig. 2H), corresponding to the histopathology results. The probe appears to be highly specific, and no reaction was seen in any of the tissues prepared from SPF shrimp (data not shown).

So far, the amoeboid protozoan parasite *Paramoeba* sp. has been reported in several marine crustaceans, including American lobster and crab, but not in cultured shrimp. This study is the first report of infection by the amoebic protozoan parasite *Paramoeba*-like sp., in the gill of *L. vannamei*

cultured in an anonymous shrimp hatchery in North America. It is most likely that the amoeba infection was due to stress factors, such as increased water temperature and/or high salinities, combined with high densities of animals in ponds, which provide an advantage to this protozoan naturally present in the marine environment. According to farmers, the salinity had dropped from 34 to 10 ppt due mechanical equipment issues, so it is likely that the resulting stress had triggered the parasite infestation in shrimp.

# Perspectives

Shrimp infected with the amoeba showed reduced appetite, lethargy, respiratory distress, eroded carapaces and blackened gills. Under the microscope, histopathological characteristics included the typical morphology of amoebic protozoa displaying granular endoplasm with vacuoles, nucleus, parasome and ectoplasm with pseudopodia. These features suggest that the parasite is related to the order Dactylopodida, probably a *Paramoeba* species and this was confirmed by the SSU rRNA sequence analysis, PCR assay and in situ hybridization (ISH).

In shrimp, the amoeba infection has resulted in significant mortality and associated economic losses. Therefore, the ISH and PCR diagnostic assays developed in this study can provide informative data for shrimp farmers and can be used as the initial screening methods for amoeboid protozoan in shrimp.

This study provides informative data to shrimp producers and helps farmers monitor for amoeba infections in shrimp farms. In the examined shrimp, the parasites have histological characteristics of *Paramoeba* sp., but no PCR bands were detected for the primers generated from *P. perurans*, *P. pemaquidensis*, or *P. branchiphila* spp. Therefore, we assume that it could be a new *Paramoeba* species infecting shrimp. Additional work is needed to diagnose the species and develop the species-specific diagnostic methods for amoeba infecting farmed shrimp.

*References available from first author or the original publication.*

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