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NOTE

Detection of a new microsporidium *Perezia* sp. in shrimps *Penaeus monodon* and *P. indicus* by histopathology, *in situ* hybridization and PCR

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ABSTRACT: Samples of microsporidia-infected shrimps exhibiting clinical signs of cotton shrimp disease were collected from Madagascar, Mozambique, and the Kingdom of Saudi Arabia from 2005 to 2014. The tails of the infected shrimps appeared opaque and whitish; subsequent histological examination revealed the presence of cytoplasmic inclusions and mature spores in tissues of the muscle, hepatopancreas, gills, heart, and lymphoid organ. PCR analysis targeting the small subunit rDNA (SSU rDNA) from infected samples resulted in the amplification of a 1.2 kbp SSU rDNA sequence fragment 94% identical to the corresponding region in the genome of the microsporidian *Perezia nelsoni*, which infects populations of *Penaeus setiferus* in the USA. Its SSU rDNA sequence was 100% identical among isolates from Madagascar and Saudi Arabia, indicating that shrimps from the Red Sea and Indian Ocean were infected with the same microsporidium, the novel *Perezia* sp. A 443 bp fragment of the SSU rDNA sequence was cloned, labeled with digoxigenin and subjected to an *in situ* hybridization assay with tissue sections of *Perezia* sp.-infected *Penaeus monodon* from Madagascar and Mozambique, and *P. indicus* from Saudi Arabia. The probe hybridized to the mature spores in the hepatopancreas and muscle from which the spores had been obtained for DNA isolation. This assay was specific, showing no reaction to another microsporidium, *Enterocytozoon hepatopenaei* (EHP), infecting the hepatopancreas of shrimp *P. stylirostris* cultured in SE Asian countries. We also developed an SSU rDNA-based PCR assay, specific for the novel *Perezia* sp. This PCR did not react to EHP, nor to genomic DNA of shrimp and other invertebrates.

KEY WORDS: Cotton shrimp disease · SSU rDNA · Hepatopancreas · Muscle · *Perezia nelsoni*

INTRODUCTION

Microsporidia are obligate intracellular parasites in a variety of hosts, from invertebrates to humans (Wittner & Weiss 1999). Research for microsporidia has been more focused on terrestrial hosts, but approximately 50% of the known microsporidia genera infect aquatic hosts, such as crustaceans and fish. The microsporidian infections of aquatic hosts are considered a potential health hazard as well as a financial risk in aquaculture (Lom & Dykova 2005, Abdel-Ghaffar et al. 2011).

One of the critical diseases that impact shrimp aquaculture is 'cotton shrimp disease' (CSD), and causative agents associated with this disease are microsporidians found in at least 5 genera including

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Pleistophora, *Thelohania*, *Perezia*, *Agmasoma*, and *Ameson* (Sprague & Couch 1971, Overstreet 1973, Lightner 1996, Sokolova et al. 2015). These microsporidian parasites mainly infect skeletal muscle, rendering the affected body regions white or opaque, which gives the common name to this disease (Sprague & Couch 1971, Lightner 1996). Lightly infected shrimp may look and behave normally, but heavy infections render the shrimp unmarketable or inedible (Overstreet 1973). In shrimp, the white-opaque appearance of muscle is primarily linked to microsporidian infections (van Banning 1985, Ramasamy et al. 2000, Panebianco et al. 2015). However, other causative agents such as dinoflagellates, bacteria, or viruses, may also be related to this disease (Lindqvist & Mikkola 1979, Cheng & Chen 1998, Stentiford & Neil 2000, Stentiford et al. 2000, Stentiford & Lightner 2011).

This study is focused on the microsporidium associated with CSD in shrimp collected from Madagascar, Mozambique and the Kingdom of Saudi Arabia. We investigated its histopathological characteristics, and determined its taxonomical classification by small subunit rDNA (SSU rDNA) sequencing. Also, specific diagnostic methods based on *in situ* hybridization (ISH) and PCR targeting the SSU rDNA sequence, were developed for this disease.

MATERIALS AND METHODS

Sampling and processing

A total of 298 shrimp samples exhibiting clinical signs of CSD were submitted from Madagascar, Mozambique and Saudi Arabia to the Aquaculture Pathology Laboratory at the University of Arizona from 2005 to 2014. The relevant information is summarized in Table 1.

Histopathology

The Davidson's alcohol-formalin-acetic acid (AFA)-fixed samples were processed, embedded in paraffin, and sectioned (4 µm thick) in accordance with standard methods (Lightner 1996). After staining with hematoxylin and eosin (H&E) or Giemsa, the sections were analyzed by light microscopy and the severity of the infection was graded according to a pre-established G-grading system (Lightner 1996): briefly, (G0) no signs of infection/infestation by pathogen; (G1) signs of infection/infestation by pathogen are present, but at levels below those needed for clinical disease; (G2–G3) moderate–high signs of infection/infestation by number and severity of pathogen-caused lesions;

Table 1. Cotton shrimp disease samples used in this study. Samples were analyzed by histopathological examination (for microsporidian presence) and PCR assays. G-grade system ranges from G0 (negative) to G4 (highest severity). PCR column indicates *Perezia* sp.-positive target organ (HP: hepatopancreas; LO: lymphoid organ). PL: post larvae; AFA: alcohol–formalin–acetic acid; pos.: positive; na: not available

Year	Life stage	Fixation/storage	Disease confirmation			
			G-grade	Target organ	No. pos./total sample	PCR
Madagascar (*P. monodon*)						
2005	Adult	Davison's AFA	G3–G4	Muscle	3/3	na
2006	Juveniles	Davison's AFA	G3–G4	Muscle/ HP	6/30	na
	Sub-adult	Frozen		Muscle/ HP		pos.
2009	Sub-adult	Davison's AFA	G3–G4	Muscle/ HP	4/19	na
2010	Adult	Davison's AFA	G2–G3	Muscle/ HP	3/5	na
2012	Sub-adult	Davison's AFA	G1–G3	Muscle/ HP/ heart	6/28	na
2013	Juveniles	95% ethanol	na	Muscle		pos.
2014	Juveniles	Davison's AFA	G3–G4	Muscle/ HP	35/35	na
		95% ethanol		Muscle/ HP		pos.
Mozambique (*P. monodon*)						
2005	Juveniles	Davison's AFA	G3–G4	Muscle/ HP/ gill/ heart/ LO	4/23	na
2006	PL	Davison's AFA	G3–G4	Muscle/ HP/ gill/ heart/ LO	2/50	na
2008	Adult	Davison's AFA	G3–G4	Muscle/ HP/ gill/ heart/ LO	4/4	na
Saudi Arabia (*P. indicus*)						
2010	Adult	Davison's AFA	G4	Muscle/ HP/ gill/ heart/ LO	3/3	na
2011	Adult	Davison's AFA	G2–G4	Muscle/ HP	30/93	na
		Frozen		Muscle		pos.
2012	Juveniles	Davison's AFA	G2–G4	Muscle/ HP	(5/5)	na

and (G4) severe lesions and advanced tissue destruction with high pathogen numbers.

SSU rDNA sequence analysis

For the SSU rDNA PCR, shrimp preserved in 95% ethanol (or frozen) were used. The muscle (or hepatopancreas) tissue was sampled from 4–5 shrimp and pooled into 1 sample. DNA extraction was performed using a Maxwell-16® Cell LEV DNA purification kit (Promega) and PCR was carried out with primers 18S-F (5′-CAA CAG GTT GAT TCT GCC TGA) and 18S-R (5′-TCT GAA ATA GTG ACG GGC GG) (Tang et al. 2015). The PCR products were sequenced at the University Arizona sequencing facility, and the obtained sequences were compared with similar sequences already in GenBank using BLAST (National Center for Biotechnology Information).

In situ hybridization (ISH)

After SSU rDNA PCR, purified PCR products (1.2 kbp) were cloned into the TOPO TA vector (Invitrogen) and designated as clone pMICRO-1. Then, the primers Cotton-443F (5′-TCA GGA AAC GGA GGC TAA AG) and Cotton-443R (5′-TTC AGA TTT CAC CCC TCG TC) were designed from the SSU rDNA sequence and used to generate a gene probe. The gene probe was labeled with digoxigenin-11-dUTP (Roche) in a PCR reaction as described by Mari et al. (1998). Following PCR, the digoxigenin-labeled DNA probe was precipitated with ethanol, re-suspended in water, and stored at –20°C.

Representative histological sections (3 *Penaeus monodon* from Madagascar, 3 *P. indicus* from Saudi Arabia, and 2 *P. monodon* from Mozambique) were used for ISH. After deparaffinization, hydration, Proteinase K digestion, and post-fixation, sections were overlaid with 500 µl of hybridization solution (4× saline sodium nitrate, 50% formamide, 1× Denhardt's solution, 5% dextran sulfate, 0.5 µg ml^{-1} salmon sperm DNA) containing the probe (0.25 µg ml^{-1}). Slides were placed on a heated surface at 90°C for 10 min and hybridized overnight at 42°C. Final detection was performed with anti-digoxigenin antibody conjugated to alkaline phosphatase (Roche) that was visualized using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

To test the specificity of the ISH assay, we used sections of pathogen-free *P. vannamei*; polychaetes (unknown species), *P. vannamei* and *P. monodon* infected by an unidentified *Agmasoma*-like microsporidium (confirmed by Carlos R. Pantoja by histopathology; authors' unpubl. data); and *P. stylirostris* infected by *Enterocytozoon hepatopenaei* (confirmed by PCR; PCR product corresponded to GenBank accession no. KP759285).

Cotton shrimp disease (CSD) PCR method

For the CSD-specific PCR assay, the primers (Cotton-443F/R) were used. The PCR reaction contained 200 µM of each dNTP, 0.2 µM of each primer, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 2.5 U Taq polymerase, and 1 µl of extracted DNA (10 to 100 ng $µl^{-1}$). Amplifications were performed with the following cycling parameters: initial denaturation at 94°C for 3 min; followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s; and a final extension at 72°C for 5 min.

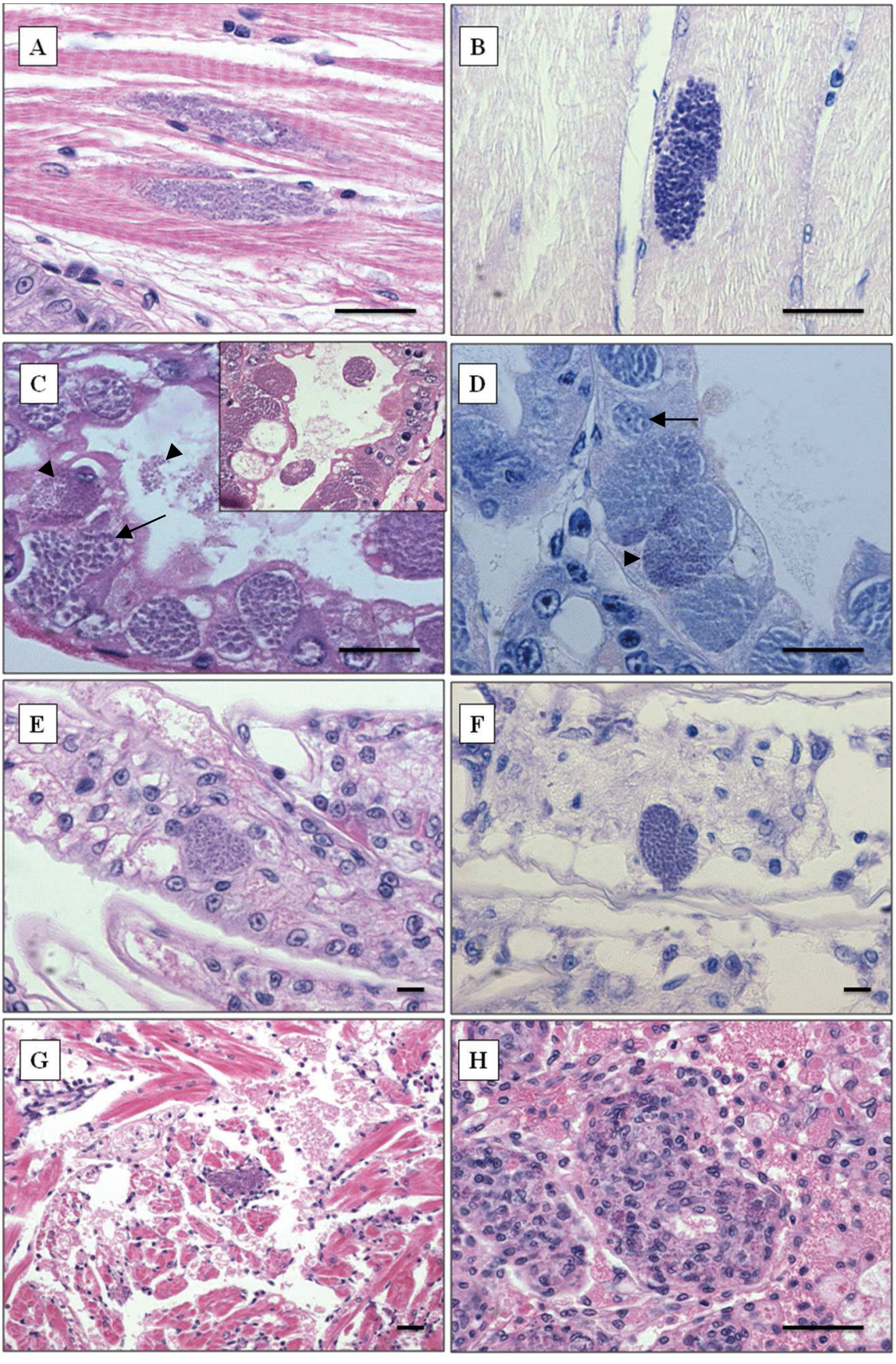
To test the specificity of the PCR assay, DNA from the microsporidian *Enterocytozoon hepatopenaei* (EHP, a parasite infecting the hepatopancreas of shrimp *P. stylirostris*) was used. Also, DNA from *P. vannamei*, *P. monodon*, *P. indicus*, *P. stylirostri, Macrobrachium rosenbergii*, polychaetes (unknown species), squid (unknown species) and brine shrimp *Artemia* spp. were used as templates.

RESULTS AND DISCUSSION

Histopathology examination

In shrimp and other crustaceans, *Perezia* spp. destroy the muscles by causing depolymerization of contractile apparatus and gradual replacement of musculature by masses of parasite developmental stages and spores. At the late stages of infection, parasites may invade other tissues and organs, such as cardiomyocytes, epithelial and connective tissue cells of the antennal glands in the same shrimp (Canning et al. 2002, Ryazanova & Eliseikina 2010).

In this study, several tissues of the same shrimp were affected by this microsporidium. Histopathology examination revealed numerous microsporidian spores in samples collected from Madagascar, Mozambique and Saudi Arabia. The infection affected primarily hepatopancreas and muscle, at the severity level corresponding to grades ranging from G1 to G4. Mature spores were seen among skeletal muscle fibers (Fig. 1A,B), and pre-spore stages and spores infected epithelium of hepatopancreas tubules


A
B
C
D
E
F
G
H

(Fig. 1C,D). In severe infections, spores were also observed within epithelial cells of gill filament (Fig. 1E,F), heart muscle fibers (Fig. 1G), and parenchymal cells of the lymphoid organ (Fig. 1H). Spores spreading all over the organism at the advanced stages of infection might have derived from muscle infection: muscles gradually deteriorated and spores contaminated surrounding tissues. In addition, most cases (90%) of CSD were accompanied by infections of gill fouling disease caused by *Zoothamnium* sp. or *Epistylis* sp., which indicated that animals suffering microsporidiosis were probably weak and susceptible to secondary infections/infestations.

SSU rDNA sequence of the new microsporidium causing CSD

We performed PCR targeting SSU rDNA sequence from the shrimp exhibiting CSD, collected in Madagascar, and a 1.2 kbp fragment was obtained and sequenced. The nucleotide sequence of SSU rDNA was deposited in Genbank (accession no. KP825331).

According to the BLAST results, the nucleotide sequence (accession no. KP825331) had a 94% identity to the SSU rDNA sequence of *Pleistophora* sp. (accession no. AJ252959) infecting *Penaeus setiferus*, and 93% identity to that of an unidentified microsporidium from *Metapenaeus joineri* (accession no. AJ295328). In 2002, *Pleistophora* sp. from *P. setiferus* was re-identified as *Perezia nelsoni* by morphological examinations (Canning et al. 2002). Based on sequence information, we therefore consider the newly identified microsporidium to belong to the genus *Perezia*, and will refer to it as *Perezia* sp. hereafter.

The CSD caused by *Perezia* sp. seems to be spread over several shrimp farming countries including Madagascar, Mozambique, Saudi Arabia (the present study), the USA (Cheney et al. 2000), and Japan (Bell et al. 2001), but it has not been sufficiently studied. In this study, extracted genomic DNAs from the Madagascar and Saudi Arabia samples were subjected to PCR with specific primers targeting SSU rDNA. The sequences of the obtained 1200 bp-long amplicons were 100% identical for both samples. Coincidentally, white spot syndrome virus (WSSV) detected in these same 3 countries (Madagascar, Mozambique and Saudi Arabia) also showed identical genomic patterns (Tang et al. 2013).

Perezia sp.-specific ISH

The probe strongly reacted to the both skeletal muscle and hepatopancreas (Fig. 2A–C), and also other organs such as gill, heart, and lymphoid organ (data not shown), corresponding to the histopathology results. The probe (443 bp) designed from the SSU rDNA sequence of *Perezia* sp. appeared to be specific to shrimps with symptoms of CSD from Madagascar, Mozambique and Saudi Arabia. At the beginning, we misidentified *Perezia* sp. infection as EHP infection by normal histopathology examination. However, this ISH probe did not react to *P. stylirostris* infected with *E. hepatopenaei* (Fig. 2D). No reaction was seen in any of the tissues prepared from pathogen-free *P. vannamei.* Also *P. vannamei* and *P. monodon*, as well as polychaetes infected with an unidentified *Agmasoma*-like microsporidium, did not react to ISH probes (data not shown).

Perezia sp.-specific PCR

For diagnostic purposes, we designed a pair of PCR primers (443F/R) targeting *Perezia* sp. DNA, and a 443 bp amplicon was generated as shown in Fig. 3. This PCR reaction is specific to the microsporidium *Perezia* sp. that causes CSD and thus may be useful for monitoring this parasite. These primers did not react to the microsporidian DNA from *E. hepatopenaei*-infected shrimps (data not shown). Also, there was no cross-reaction to genomic DNA from shrimp (*P. vannamei*, *P. monodon, P. indicus, P. stylirostris* and *Macrobrachium rosenbergii*), polychaetes, squid and *Artemia* spp. (data not shown).

We did not analyze Mozambique samples by PCR, but the probe reacted intensely to ISH by depositing the reaction products in the target cells in Mozambique samples.

In this study, we describe muscle and hepatopancreas-infecting microsporidia causing CSD in shrimps from Madagascar, Mozambique and Saudi Arabia in the Red Sea–Indian Ocean region.

Fig. 1. Representative lesions characteristic of the disease caused by the microsporidian found in penaeid shrimp from Madagascar, Mozambique and Saudi Arabia. (A,B) Mature spores accumulated in between skeletal muscle fibers; (C,D) pre-spores (arrows) and spore stages (arrowheads) within hepatopancreas tubule epithelial cells (inset shows sloughing cells); (E,F) intracytoplasmic accumulations of mature spores within gill filament epithelial cells; (G) mature spores within heart muscle fibers; (H) parenchymal cells of lymphoid organ tubules. Stain: (A,C,E,G,H) Mayer-Bennett hematoxylin/eosin-phloxine; (B,D,F) Giemsa. Scale bars = 30 µm

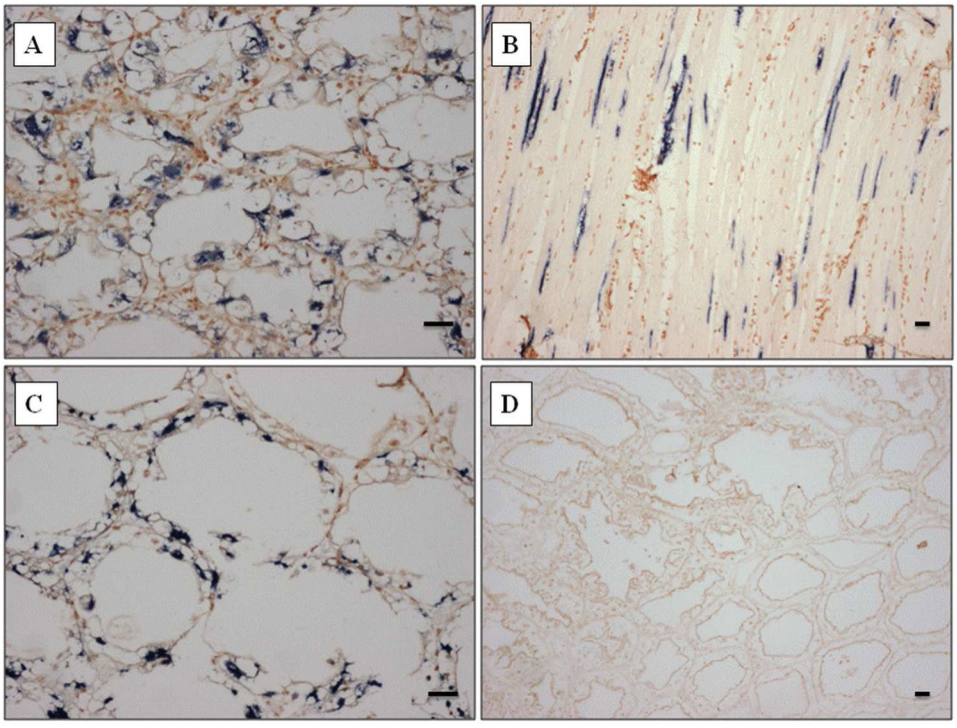


Fig. 2. *In situ* hybridization assay (ISH) with a digoxigen-labeled gene probe specific for detection of the microsporidian causing cotton shrimp disease. Dark blue precipitate indicates the presence of the parasite (A) within hepatopancreas tubule epithelial cells of *Penaeus indicus* from Saudi Arabia, (B) among skeletal muscle fibers of *P. monodon* from Mozambique and (C) within hepatopancreas tubule epithelial cells of *P. monodon* from Madagascar. (D) Portion of the hepatopancreas from *P. stylirostris* from Brunei infected by *Enterocytozoon hepatopenaei* showing representative results, emphasizing the specificity of the probe as demonstrated by the absence of dark blue precipitate. Stain: Bismark-Brown counterstain. Scale bars = 30 µm

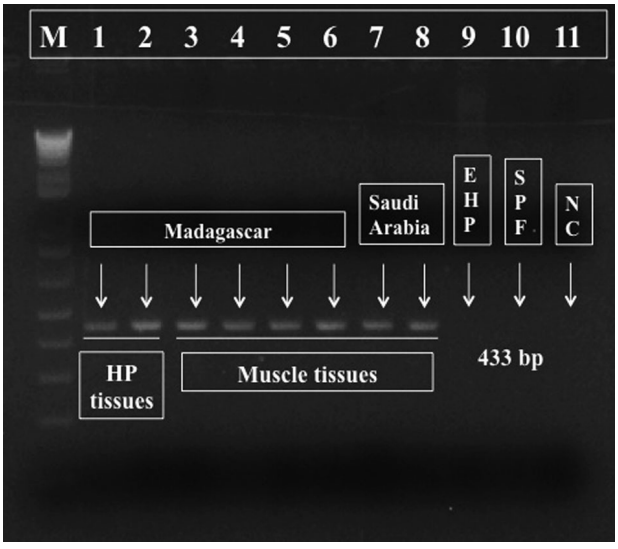


Fig. 3. *Perezia* sp.-specific PCR results. Lanes 1–2: *Penaeus monodon* hepatopancreas from Madagascar; Lanes 3–6: *P. monodon* muscle from Madagascar; Lanes 7–8: *P. indicus* muscle from Saudi Arabia; Lane 9: *Enterocytozoon hepatopenae* (EHP); Lane 10: specific pathogen free (SPF) control; Lane 11: negative control (NC)

Microsporidian infections were confirmed by histopathological analysis and the parasites were identified as *Perezia* sp. Also, we developed ISH and PCR assays capable of detecting this parasite. These methods might help shrimp producers to diagnose and manage CSD caused by the microsporidium *Perezia* sp.

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