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Protistology

Perezia nelsoni (Microsporidia) in *Agmasoma penaei*-infected Atlantic white shrimp *Litopenaeus setiferus* (Paenaidae, Decapoda) and phylogenetic analysis of *Perezia* spp. complex

Yuliya Sokolova[1,2] and John P. Hawke[3]

[1] *Department of Comparative Biological Sciences, School of Veterinary Medicine, Louisiana State University, Baton Rouge LA, USA*
[2] *Institute of Cytology, Russian Academy of Sciences, St. Petersburg, Russia*
[3] *Department of Pathobiological Sciences, School of Veterinary Medicine, Louisiana State University, Baton Rouge LA, USA*

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Summary

During the years 2012-2014 samples of Atlantic white shrimp *Litopenaeus setiferus* collected offshore near Plaquemines Parish, Louisiana (USA) were submitted to the Louisiana Aquatic Diagnostic Laboratory with "tumor like" lesions in the exoskeleton and internal organs. Light (LM) and electron microscopy (EM) examination indicated that 19 shrimp were infected with the microsporidium *Agmasoma penaei* (= *Thelohania penaei* Sprague, 1950, n. comb. Hazard and Oldacre, 1975). The Louisiana isolate of *A. penaei* was morphologically and genetically characterized previously (Sokolova et al., 2015). In 15 of 19 shrimp the infection was limited to subcutaneous and ovarian tissue, and in 4 shrimp musculature also seemed to be affected looking unusually whitish and/or opaque. According to our observations, *A. penaei* did not occur in muscle tissue, however, records on tissue tropism of *A. penaei* obtained by earlier authors, occasionally included skeletal muscles. Upon examination of skeletal muscles of these 4 shrimp by LM we found out that the tissue contained microsporidia-like individual spores of much smaller (2.0 × 1.1μm) size, than those of *A. penaei*. Electron microscopy and molecular analysis indicated that *L. setiferus* was co-infected by another microsporidium, structurally and genetically similar to *Perezia nelsoni* Sprague 1950 from the Mississippi coast of the Gulf of Mexico (Cheney et al., 2000; Canning et al., 2002; Genebank reference: "*Pleistophora* sp. LS" Accession # AJ252959). Persistent co-infection of white shrimp with two microsporidia with different tissue tropism reported herein, suggests that infection of muscles previously attributed to *A. penaei*, might be due to the overlooked *P. nelsoni*. The paper describes fine morphology and phylogenetic relations of *P. nelsoni* from a new location. The novel SSUrDNA sequence was deposited in Genebank, under accession number KX85642.

Key words: Microsporidia, Decapoda, *Perezia nelsoni, Agmasoma penaei, Litopenaeus setiferus*, Louisiana, electron microscopy, molecular taxonomy

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Introduction

More than 20 species of Microsporidia (Kingdom Opisthokonta; phylum Microsporidia) belonging to 17 genera have been reported from representatives of five sub-taxa of Decapoda (subphylum Crustacea, class Malacostraca, order Decapoda): in penaeid shrimps (suborder (s/o) Dendrobranchiata, family Penaeidae) representatives of 4 genera of Microsporidia have been found; in caridean shrimps (s/o Pleocyemata, infraorder (i/o) Caridea) - 3 genera; in lobsters and crayfish (s/o Pleocyemata, i/o Astacoidea) - 3 genera; in true crabs (s/o Pleocyemata , i/o Brachyura) - 5 genera, and in snow and hermit crabs (s/o Pleocyemata , i/o Anomura) - 2 genera (Canning et al., 2002; Stentiford et al., 2013, 2016). Microsporidiosis has been always considered the most conspicuous and harmful disease of decapods caused by eukaryotic microbes (Overstreet, 1973; Kelly, 1979; Johnson, 1995; Morado, 2011). New species are constantly being described, and actual biodiversity of microsporidia parasitizing decapods is believed to greatly exceed the number of the described species. This has been limited so far by descriptions from economically important species or accidental findings (Stentiford et al., 2016). Microsporidiosis in decapods is often linked to lowered commercial value of the catch, reduced host fecundity, elevated susceptibility to predators and to other diseases, and increased sensitivity to unfavorable environmental conditions (Hutton et al., 1959; Stentiford et al., 2013, 2016).

The population of Atlantic white shrimp *Litopenaeus setiferus*, the major commercial shrimp in the Gulf of Mexico, has periodically suffered from massive epizootics of microsporidiosis ("cotton disease"), the most severe of which occurred in 1929 and resulted in an infection prevalence of 90%, mass mortality, loss of 99% of egg production, and an unprofitable shrimping industry for several years (Viosca, 1945; Gunter, 1967; Miglarese and Shealy, 1974; Muncy, 1984). In the last two years, there have been reports of microsporidian infections in the Gulf of Mexico, particularly in locations adjacent to the BP oil spill (Martin Bourgeois, Louisiana Department of Wildlife and Fisheries, unpublished observations). Historically the dominant species causing "cotton disease" in *L. setiferus* is *Agmasoma* (*Thelohania*) *penaei* Sprague (Sprague, 1950; Hazard and Oldacre, 1976), a globally distributed species, for which morphologic and phylogenetic relationships have been recently re-evaluated (Sokolova et al., 2015). *Agmasoma penaei* is presently considered as one of the most destructive pathogens of commercially important wild and farm-raised penaeid shrimp worldwide (Sprague and Cough, 1971; Sprague, 1977; Kelly, 1979; Flegel et al., 1992; Clotilde-Ba and Toguebaye, 1994, 2001; Vidal-Martínez et al., 2002; Toubiana et al., 2004; Laisutisan et al., 2009). According to our observations *A. penaei* was not found in the muscle tissue of shrimp we examined but was prevalent in gonads. However, records on tissue tropism of *A. penaei* obtained by earlier authors occasionally included skeletal muscles (Kelly, 1979; Clotilde-Ba and Toguebaye, 1994, 2001; Laisutisan et al., 2009). In our survey, skeletal muscles of several shrimp infected with *A. penaei*, looked somewhat whitish and/or opaque contrasting to a transparent appearance of the uninfected tissue. Upon microscopic examination, the affected muscle tissue contained microsporidia-like individual spores of smaller size (2.0 × 1.1 μm) than the ones of *A. penaei*. Light and electron microscopy, as well as molecular analysis suggested *L. setiferus* muscles were infected by another microsporidian species, structurally and genetically similar to *Perezia nelsoni* Sprague 1950 previously described from the Mississippi coast of the Gulf of Mexico (Cheney et al., 2000; Canning et al., 2002; Genebank reference: "*Pleistophora* sp. LS" Accession # AJ252959). Persistent co-infection of white shrimp with two microsporidia with the different tissue tropism reported herein, suggests that infection of muscle tissue attributed previously to *A. penaei*, might be due to the overlooked *P. nelsoni*. In this paper we describe the morphology and life cycle stages of *P. nelsoni* from a new location, and present its SSUrDNA sequence barcode. Phylogenetic analysis demonstrates *P. nelsoni* belongs to a cluster of closely related, presumably congeneric species, parasitizing penaeid shrimp worldwide.

Material and methods

Material

White shrimp, *Litopenaeus setiferus* Linnaeus 1767, were caught by commercial or recreational trawling in the Gulf of Mexico offshore from Plaquemines Parish, LA. An unusually high number of shrimp harboring macroscopic whitish "tumor like" lesions on the carapace and abdomen were

noticed by shrimpers and prompted them to contact the Louisiana Department of Wildlife and Fisheries (LDWF). More than 40 shrimp with these clinical signs were delivered to the Louisiana Aquatic Diagnostic Laboratory (LSU School of Veterinary Medicine, Baton Rouge, LA, USA) from May 2012 to November 2014. In all the cases, shrimp were caught alive, kept on ice and delivered within 24 hours after being caught. All delivered shrimp were females, with a size ranging from 100 to 120 mm for those caught in May, and 130 to 180 mm for those caught later in the year. The material examined in this study included 19 shrimp: 2 sampled on 05/16/2012 (case LADL12-047); 2 - on 05/22/2012 (case LADL12-053), 8 - on 10/2/2012 (case LADL12-113), 6 - on 10/11/12 (case LADL12-119), and 1 - sampled on 6/19/2014 (case LADL14-059). Clinical signs confirmed by light microscopy (LM) examination, indicated that all 19 shrimp were infected with the microsporidium *Agmasoma penaei*. In 4 shrimp (1 shrimp, case LADL12-047, 2, case LADL12-119, and 1, case LADL14-059) with unusually whitish and/or opaque skeletal muscles, individual microsporidia-like spores smaller than ones of A. *penaei*, were detected by LM. Muscles of the other 15 shrimp were transparent and did not contain infection as suggested by LM and histological analysis. Skeletal muscles containing small microsporidia-like spores, were subjected to electron microscopy (EM) analysis and were used for spore isolation for PCR.

Light and electron microscopy

One to three whole shrimp from each sampling were injected with 5 ml Davidson's fixative and placed in fresh Davidson's fixative at approximately 10× their volume for 24 to 48 h and then transferred to 70% ethanol (Bell and Lightner, 1988). Standard histological protocols, based on the procedures of Luna (Luna, 1968; Peterson et al., 2011), were employed by the Louisiana Animal Disease Diagnostic Laboratory, Histology Lab (Sokolova et al., 2015). Smears from gonads, hepatopancreas, thoracic and abdominal muscles, subcuticular lesions of the carapace, and intestines were either examined directly under the light microscope with phase contrast optics, or were fixed by absolute methanol, stained with Trichrome stain, Calcofluor and Giemsa, as previously described (Sokolova and Fuxa, 2008), and examined with bright field optics. Smears and sections were examined with a Zeiss Axioplan microscope equipped with Olympus DP73 digital camera. ImagePro7.0 software was used for spore measurements. For electron microscopy, small pieces of infected tissues were fixed in a mixture of 2% paraformaldehyde and 1.25% glutaraldehyde in 0.1M cacodylate buffer for 2 h, washed several times in the same buffer supplemented with 5% sucrose, and post fixed in 1% osmium tetroxide for 1 h, all procedures were performed at room temperature. Samples were then thoroughly washed in water and dehydrated in ascending ethanol series and propylene oxide, and embedded in Epon-Araldite. Blocks were sectioned with Ultratome Leica EM UC7. Thick (0.5–1 μm), sections were stained with the modified Methylene blue stain (Sokolova and Fuxa, 2008), examined and photographed under a Zeiss Axioplan microscope equipped with Olympus DP73 digital camera. Thin 70–80 nm sections were stained with uranyl acetate and lead citrate end examined in JEOL JEM 1011 microscope with the attached HAMAMATSU ORCA-HR digital camera. All reagents for LM were from SIGMA-ALDRICH (St. Louis, MO), and for EM - from EMS Chemicals (Fort Washington, PA).

Spore purification and DNA isolation

Spores were extracted from tissues heavily infected with spores, previously stored at –20 °C. Briefly, thawed muscle samples were homogenized using a Teflon pestle in a 15 ml Wheaton tissue grinder, re-suspended in PBS and filtered through polycarbonate membrane filters with pore size 5 μm (Sterlitech, Kent, WA, USA) to remove larger debris and *Agmasoma penaei* sporophorous vesicles that heavily contaminated all tissues. The resulting spore suspension was then washed 2-3 times in 15 ml tubes with PBS by centrifugation at 3000g (Accu Spin Centrifuge, Fischer Scientific). The final pellet, still containing *A. penaei* spores was re-suspended in 5 ml of PBS, placed on 100% Percoll (Sigma, St. Louis, MO) in 15 ml tubes, and centrifuged at 600g (=2000 rpm) for 35 min. Due to less size and buoyant density, the smaller spores concentrated in a compact layer about 0.5 cm above the bottom of the tube, while larger and heavier *A. penaei* spores formed a pellet at the bottom of the tube. The layer with smaller spores was removed by syringe. Spores from the upper layer and from the bottom were washed several time by PBS, and their purity and integrity was inspected under phase contrast microscope using 10-15 μl aliquot of spore

suspension (Fig. 1, A, B). Finally, spores were transferred to 1.5 ml tubes, washed 3 additional times in PBS by centrifugation (3000g, Eppendorf centrifuge 5415C), and the final pellet was re-suspended in 150 ml of TAE buffer (0.04M Tris acetate, 0.01M EDTA).

To release the DNA content from the spores, 150 mg of 0.1 mm glass beads were added to each tube and shaken with a BulletBlenderTM24 bead-beater (Next Advance, Inc., Averill Park, NY) for 1 min at the maximal speed. The tube was then immediately placed on a hot plate at 95° for 3-5 min, and then placed on ice. The suspension was checked again under phase contrast optics for ruptured spores, and used directly or diluted 1:10 or 1:100 as a DNA template for PCR amplification (Vossbrinck et al., 2004).

PCR and sequencing

The SSU rDNA region was amplified by polymerase chain reaction (PCR) with 1-3 µl of the ruptured spore suspension as a DNA template, OneTaq® Quick-load® master mix (New England Biolabs, Inc., Ipswich, MA), and the following primers: V1 (5' – CAC CAG GTT GAT TCT GCC TGA C – 3') and 1492r (5' – GGT TAC CTT GTT ACG ACT T-3') (Vossbrinck et al., 2004). The PCR cycle included an initial denaturation step at 95 °C for 5 min, followed by 35 cycles with a denaturation at 95 °C for 30 sec, annealing at 45 °C for 60 sec and elongation at 72 °C for 120 sec, and a final extension at 72 °C for 10 min. Amplicons were loaded onto a 2% agarose gel, and bands of the expected size (1200 bp) were excised and purified using a QIAquick Gel Extraction Kit (QIAGEN, Germantown, MD). The purified PCR band was sequenced with the Applied BioSystems BigDye Terminator technology (version 3.1) and the resulting chromatogram was obtained by a Beckman Coulter Seq 8000 DNA. The primers for sequencing were V1, 530r (5' - CCG CGG C(T/G)G CTG GCA C – 3'), 530f (5' – GTG CCA GC (G/A) GCC GCG G), 1061f (5' – GGT GGT GCA TGG CCG – 3'), and 1492r (Weiss and Vossbrinck, 1999; Vossbrinck et al., 2004). These primers produced overlapping sequences that were assembled with Chromas Pro. 1.34 software (http:// www.technelysium. com.au/ ChromasPro.html). Direct PCR amplification and sequencing were performed at least twice for each DNA sample from a total of 2 samples of 2 shrimp, collected at different dates. The consensus sequence of the *Perezia nelsoni* LA isolate SSU was deposited in Genbank under Accession number KX856426.

Phylogenetic analysis

For the SSU rDNA-based phylogeny presented in this paper, 14 sequences were retrieved from Genbank. The selected sequences included primarily the closest matches in BLAST search (Table 1). The sequences were trimmed at the 5' end at the position 433 of the SSU gene to correspond to the shortest SSUrDNA sequence of the dataset ("Unidentified microsporidium from *Metapenaeus joineri*" Acc# AJ295328), and at about position #1200 of the SSU at the 3' end. The sequences were aligned with Muscle (MEGA 5.05), with default parameters (Edgar, 2004). The final dataset resulted in 703 informative positions. Pairwise genetic distances were calculated by the Kimura-2 parameter method with a gamma distribution 1 (Tamura et al., 2011). The alignment was subjected to phylogenetic reconstructions by maximum likelihood algorithm (ML) (MEGA 5.05, Tamura et al., 2011), using GTR-G model of nucleotide substitution (Nei and Kumar, 2000) as suggested by Modeltest, with 1000 bootstrap replications. *Larssonia obtusae* a microsporidium infecting *Daphnia*, was a source of the outgroup sequence.

Results and discussion

Light microscopy

Fresh spores isolated from muscles measured 1.39 - 2.13 (Average 1.73 ± 0.040) × 0.70 -1.30 (Av 1.05 ± 0.030) µm, n=25. Spores were oval and were not assembled in groups (Fig. 1, A). The pattern of spore staining with Giemsa and Trichrome stain was typical for microsporidia (Fig. 1, C, D). On smears stained with Giemsa, chains of unicellular sporonts or sporoblasts connected with each other by narrow cytoplasmic bridges were seen (Fig. 1, C). Methanol-fixed and Trichrome or Giemsa stained spores measured 1.48 - 1.87µm (Average 1.7 ± 0.024) × 0.85 - 1.30 (Av 1.08 ± 0.026), n=20. On Luna-stained histological sections and thick sections of Epon-Araldite-embedded tissue stained with Methylene blue, spores were distributed along skeletal muscles, occasionally demonstrating extensive lesions packed with spores (Fig. 1, E) and prespore stages (Fig. 2, A). Tissues other than

Table 1. Microsporidia from crustaceans and fish included in the SSUrDNA-inferred phylogeny, with host species, localities, tissue tropism, and GenBank Accession numbers of relevant SSU rDNA sequences

Species	Host	Recorded Distribution	Env.*	Tissue tropism	GenBank Acc#
Agmasoma penaei LA isolate	*Litopenaeus setiferus* Decapoda, Penaeidae	Atlantic, Gulf of Mexico	MB	gonads	KF549987
Amezon michaelis	*Callinectes sapidus* Decapoda, Portunidae	Atlantic, Gulf of Mexico	MB	hepatopancreas, hemocytes, muscles	L15741
Anostracospora rigaudi	*Artemia* spp. Anostraca, Artemidae	France, Ukraine	S	intestine	JX915758
Glugea anomala	*Danio rerio,* Actinopterygii, Cyprinidae	worldwide	F	Various, hypodermis, gut	AF044391
Heterosporis anguillarum	*Anguilla japonica* Actinopterygii, Anguilidae	East China Sea	FM	muscles	AF387331
Larssonia obtusa	*Daphnia magna Cladocera, Daphniidae*	N. Europe	F	Fat body, hypodermis, ovaries	AF394527
Nadelspora canceri	*Metacarcinus magister* Decapoda, Canceridae	Atlantic	M	muscles	AY958070
Perezia nelsoni LA	***Litopenaeus setiferus* Decapoda, Penaeidae**	**Atlantic, Gulf of Mexico, LA**	**MB**	**muscles**	**KX856426**
Perezia sp.06-324	*Penaeus monodon, P. indicus* Decapoda, Penaeidae	Indian Ocean, Persian Gulf	M	Muscles, hepatopancreas	KP825331
Pleistophora sp. LS**	*Litopenaeus setiferus* Decapoda, Penaeidae	Atlantic, Gulf of Mexico,MI	MB	muscles	AJ252959
Pleistophora sp. PA***	*Penaeus aztecus* Decapoda, Penaeidae	Atlantic, Gulf of Mexico	MB	muscles	AJ252958
Spraguea lophii	*Lophius* spp. Actinopterygii, Lophiidae	worldwide	M	nervous system	AF033197
*Triwangia caridinae****	*Caridina formosae* Decapoda, Caridiidae	Taiwan	F	Hepatopancreas, intestibe, gills	JQ268567
"Unidentified microsporidian S1"	*Metapenaeus joineri* Decapoda, Penaeidae	Japan coast, Pacific	M	unknown	AJ29295328

*Environment: M– marine, F – fresh water, B – brackish waters, S –salt lakes; **identified as *Perezia nelsoni* (Canning et al., 2002); *** identified as *Tuzetia weidneri* by (Canning et al., 2002); ****Microsporidia sp. CHW-2012 in GenBank. The row with the target species, *Perezia nelsoni*, LA isolate, is bolded.

muscle, including hepatopancreas and intestine, were not infected. Hypertrophied ovaries were heavily infected by *Agmasoma penaei* (Sokolova et al., 2015).

Electron microscopy

Pre-sporogonic stages were rarely seen on thin sections. The earliest stages observed were large elongated merogonial plasmodia with multiple diplokarya enclosed in a thin membrane-like envelope (Fig. 2, B). The next stage could be defined as sporogonial plasmodia. It was surrounded by a thicker envelope and contained several individual nuclei. These elongated cells underwent plasmotomy (Fig. 2, D) to produce chains of uninuclear sporonts that eventually transformed into sporoblasts connected with each other by cytoplasmic bridges (Fig. 2, E). Sporoblasts contained randomly distributed membrane cisternae with precursors of polar tube and multilayered membrane structures, presumably future polaroplast membranes (Fig. 2, E, F). Mature spores were fairly well preserved. They were enclosed in 45-70 nm-thick envelopes with moderately thick (16-65 nm) uniform exospore. Spores exhibited a mushroom-shaped anchoring disc and relatively long (up to 2/3 of the spore length) and a slightly bent apical portion of the polar filament. The distal part of the polar filament curved into 6-8 isofilar coils arranged in two rows. The distinguishing

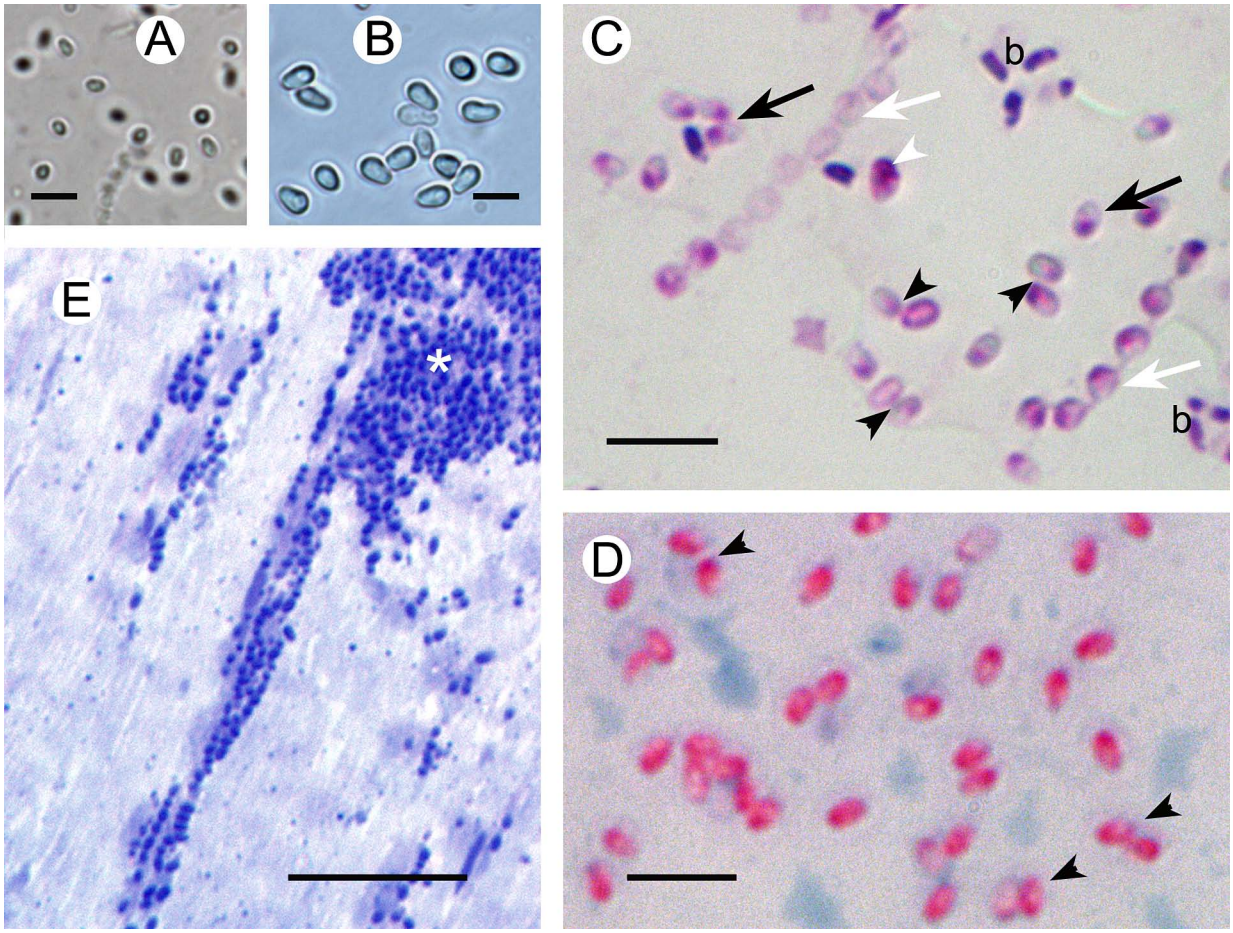

Fig. 1. Light microscopy of *Perezia nelsoni*. A – Live *P. nelsoni* spores purified by centrifugation in Percoll; B – purified *Agmasoma penaei* spores from the same shrimp; C – methanol-fixed and Giemsa stained smear of muscles infected with *P. nelsoni*; D – methanol-fixed, Trichrome-stained smear of *P. nelsoni*-infected skeletal muscles; E – thick section stained with Methylene blue: spores are distributed along skeletal muscles, or concentrated within extensive lesions (asterisk). *Abbreviations*: b – bacteria. *Black arrows* indicate spores, *white arrows* – chains of sporonts or sporoblasts; *black arrowheads* point to the pairs of spores originated presumably from one sporoblast by disporoblastic sporogony; *white arrowhead* points to the late sporoblast prior to the final division in two spores. Scale bars: A, B, C, D – 5 μm; E – 20 μm.

feature of the spores was the outer layer of polaroplast composed of tightly packed membranes. On sections this layer appeared as a prominent circular electron dense structure that embraced the rest of the polaroplast (Fig. 2, D-G). All stages developed without interfacial envelopes in direct contact with sarcoplasma. The above mentioned ultrastructural features of the microsporidium corresponded well to the description of the Mississippi isolate of *L. setiferus* (Canning et al., 2002), and to earlier publications describing the ultrastructure of *P. nelsoni* infecting other penaeids (Loubes et al., 1977; Clotilde-Ba and Toguebaye, 1996). Thus, host species, location, type of pathology, size, type of development, as well as fine morphology of spores and pre-spore stages allowed identification of the microsporidium as *Perezia* (=*Nosema*, =*Ameson*) *nelsoni* Sprague, 1950 (Vivares and Sprague, 1979; Canning et al., 2002). *Perezia nelsoni* or a morphologically similar muscle-infecting microsporidium has been recorded from several species of penaeid shrimps, namely *Farfantepenaeus duorarum, F. notialis, Penaeus semisulcatus, P. esculentus, Fenneropenaeus merguiensis*, and *L. setiferus* from various locations including coastal waters of southern USA, South Africa, Mediterranian and Australia (Sprague, 1950; Overstreet, 1973; Loubes et al., 1977; Owens and Glazebrook, 1988).

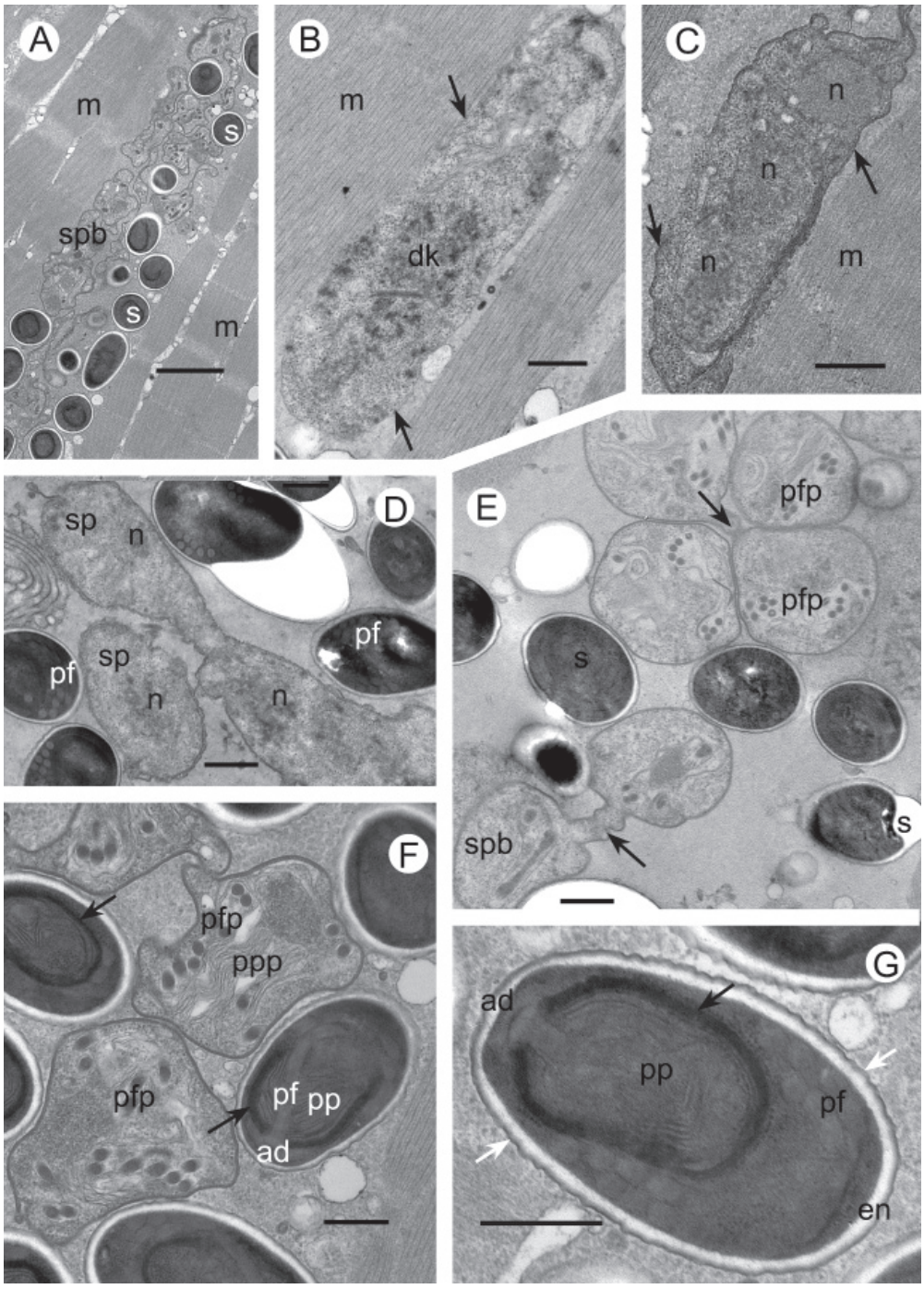

Fig. 2. Electron microscopy of *Perezia nelsoni*. A – Spores and sporonts in skeletal muscles of the shrimp *Litopenaeus setiferus* at lower magnification; B – multinucleate merogonial plasmodium with one diplokaryon and thin envelope (*arrows*); C – sporogonial plasmodium with isolated nuclei and thickened envelope (arrows), D – spores and dividing sporogonial plasmodium in the infected myocyte, E – sporoblasts and mature spores. Arrows indicate cytoplasmic bridges connecting individual sporoblasts; F – mature spores and sporoblasts at higher magnification (*arrow* points to external electron dense region of the polaroplast composed of tightly packed membranes); G – a mature spore at higher magnification (*black arrow* points to electron dense regions of the polaroplast, a characteristic feature of *P. nelsoni*, *white arrow* indicates slightly undulating exospore). *Abbreviations*: ad – anchoring disc, dk – diplokaryon, en – endospore, n – individual nucleus, m – myocytes, pf – polar filaments, pfc – polar filaments precursor, pp – polaroplarst, ppp – polaroplast precursor, s – spores, sp – sporonts, spb – sporoblsats. Scale bars: A –2 μm, B-G – 500 nm.

SMALL SUBUNIT RDNA SEQUENCE OF *PEREZIA NELSONI* FROM LOUISIANA COAST AND PHYLOGENETIC RELATIONSHIPS OF *PEREZIA* SPP.

The amplified SSUrDNA sequence of the LA isolate of *Perezia nelsoni* was 1244 nucleotides long and revealed 99% SSUrDNA similarity to the sequence of "*Pleistophora* sp. LS" deposited in GenBank under Acc#AJ252959 by Cheney et al. (2000). This "*Pleistophora* sp. LS" was isolated from the same species *L. setiferus* from the Mississippi coastal waters of the Gulf of Mexico, and was subsequently identified as *P. nelsoni* by Canning et al. (2002). The pairwise evolutionary divergence of sequences belonging to the Louisiana and Mississippi isolates of *P. nelsoni* was as low as 0.001 (Table 2), which together with morphological identity suggests that these isolates represented one species. The second closest match in the BLAST search, the microsporidium *Perezia* sp. 06-324, was described recently from *Penaeus monodon* and *P. indicus* from the Indian Ocean (Madagacar and Mozambique coasts) and Persian Gulf (Saudi Arabi) correspondingly (Han et al., 2016). The latter *Perezia* sp. revealed similar general morphology to *P. nelsoni* and an identical pattern of muscle infection, but unlike *P. nelsoni*, it also infected the hepatopancreas. Evolutionary distance between *Perezia* sp. and *P. nelsoni* is 0.020 (Table 2), and judging from sequence analysis, geographical distribution, host range, and tissue tropism, *Perezia* sp. 06-324 is a closely related but separate species. Its formal description is pending until ultrastructural data become available. A similar evolutionary distance (0.016) separates *P. nelsoni* and "Undescribed microsporidium from *Metapenaeus joineri*" (GenBankAcc # AJ29295328), collected off the Pacific Coast of Japan. No morphological data are available for this sequence, but it may be assumed that it belongs to another species of the genus *Perezia*, yet to be described. Phylogenetic analysis placed these four sequences, all parasites of penaeid shrimps, into one well supported clade (Fig. 3). This clade forms dichotomy with the group of muscle-infecting microsporidia from decapod crustaceans represented in our phylogeny by *Ameson michaelis* and *Nadelspora canceri*. Two other parasites of penaeids, *Tuzetia weidneri* and *Agmasoma penaei*, belong to two unrelated clades, embracing species infecting fish (*T. weidneri* clade), and those parasitizing inhabitants of brackish coastal waters and inland salt basins (*A. penaei* clade), as it was previously demonstrated and discussed (Sokolova et al., 2015).

Conclusions and general remarks on the genus *Perezia*

Our research (Sokolova et al., 2015; herein) brought us to the conclusion that the two most abundant and vastly distributed microsporidian parasites of penaeids with worldwide distribution, *Agamasoma penaei* and *Perezia nelsoni*, are both represented by clines of species that have evolved and diversified along with their hosts, shrimps of the family Penaeidae of the infraorder Dendrobranchiata, a basal lineage of Decapoda. Interestingly, the phylogenetic analysis suggests quite different evolutionary history of these two microsporidia. *Agmasoma penaei* belong to the Clade 4 of microsporidia of terrestrial origin ("Terresporidia", Vossbrinck and Debrunner-Vossbrinck, 2005) and its ancestors parasitized probably inhabitants of brackish and inland saltwater basins like *Arthemia* spp. (Sokolova et al., 2015). *Perezia* spp. fits in the Clade 5 of microsporidia of marine origin ("Marinosporidia", Vossbrinck and Debrunner-Vossbrinck, 2005), together with fish parasites of genera *Spraguea, Heteorsporis* and *Glugea* (Fig. 3). *Agmasoma penaei* and *P. nelsoni* are characterized by completely different life cycles, morphology, and tissue tropism. These microsporidia occupy in fact two different niches within the same host, muscles (primarily) in the case of *P. nelsoni*, and gonads in the case of *A. penaei*, which allows their occasional (or habitual?) co-existence within one organism, the phenomenon, described in this paper.

The type species of the genus *Perezia lankestriae* Léger and Dubosque, 1909, a rare hyperparasitic microsporidium, was described from gregarines *Lankesteria ascidiae* Lankester, 1872 (Apicomplexa, Lecudiniidae), gut symbionts of marine ascidians *Ciona intestinalis* Linneus, 1767. After its initial description *P. lankestriae* has been recollected and re-described once in 1977 (Ormières et al.,1977), and no molecular data are available for this organism. The ultrastructural description of *P. lankestriae* is incomplete: images of mature spores are lacking in the Ormier's et al. paper. In addition, *P. lankestriae* fine morphology does not match the one of *P. nelsoni*: (i) *P. lankestriae* spores do not demonstrate characteristic electron dense external region of the polaroplast; (ii) its polar filament is much longer, making13-14 coils, (iii) *P. lankestriae* demonstrates polysporous sporogony (Fig. 17 in Ormières et al., 1977), unlike disporoblastic *P. nelsoni*. No molecular data are available for *P. lankestriae*. In other words,

Table 2. Distance matrix: pairwise evolutionary divergence between sequences estimated by Kimura-2-parameter model

N	*Species*	1	2	3	4	5	6	7	8	9	10	11	12	13
1	*P.nelsoni* LA													
2	*P. nelsoni* MI*	0.001												
3	*Perezia* sp.**	0.020	0.022											
4	*Microsporidium MJ*	0.016	0.017	0.031										
5	*A. michaelis*	0.351	0.353	0.346	0.352									
6	*N.canceri*	0.314	0.316	0.310	0.320	0.083								
7	*T. weidneri****	0.227	0.225	0.221	0.233	0.397	0.365							
8	*G. anomala*	0.228	0.230	0.226	0.234	0.370	0.342	0.069						
9	*H. anguillarum*	0.216	0.218	0.214	0.219	0.372	0.343	0.066	0.055					
10	*T. caridinae*	0.265	0.267	0.259	0.269	0.341	0.330	0.205	0.199	0.181				
11	*S. lophii*	0.226	0.228	0.225	0.238	0.375	0.347	0.156	0.157	0.139	0.126			
12	*A. penaei*	0.458	0.455	0.467	0.479	0.522	0.487	0.446	0.437	0.434	0.452	0.455		
13	*A. riguadi*	0.401	0.403	0.393	0.409	0.476	0.441	0.405	0.402	0.381	0.362	0.363	0.319	
14	*L. obtusa*	0.380	0.383	0.386	0.400	0.521	0.468	0.335	0.342	0.332	0.357	0.326	0.412	0.423

* "*Pleistophora* LS" in GenBank; ** *Perezia* sp.06-324 in GenBank; ***"*Pleistophoea* PA" in GenBank.

relatedness of *P. lankesriae* to *P. nelsoni* is doubtful, and sooner or later the creation of another genera for *P. nelsoni* and related species from penaeids, might be needed. Notable that all species parasitizing terrestrial arthropods that had been previously assigned to *Perezia*, were subsequently moved to other genera (Sokolova et al., 2007; Boyko, 2012). In addition, the validity of the very generic name *Perezia* for Microsporidia has been debated (Boyko, 2012).

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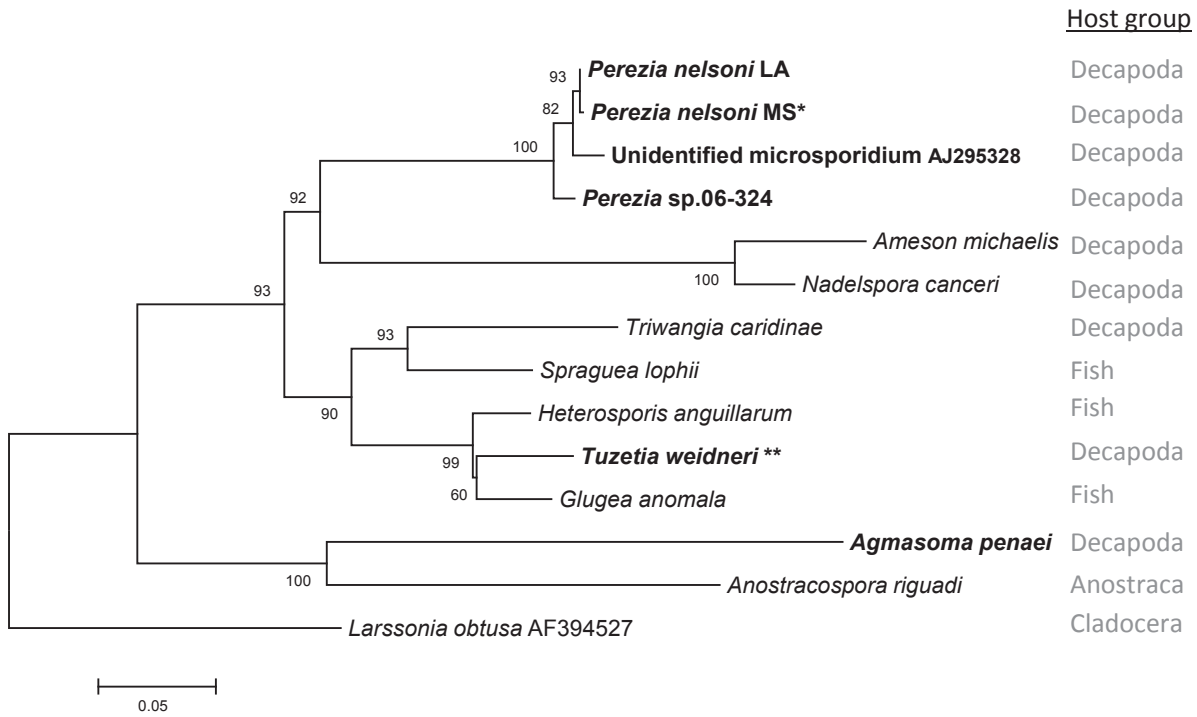

Fig. 3. Small subunit rDNA-inferred Maximum likelihood phylogenetic analysis: original tree with the highest log likelihood and the branch length proportional to the number of substitutions per site, based on 702 informative positions for 14 microsporidian sequences. *Larssonia obtusa* was an outgroup. Scale bar: 0.05 substitutions per site. Bootstrap support for brunches is indicated at nodes. Names of species parasitizing penaeid shrimps (Decapoda, Dendrobranchiata, Penaeidae) are printed in bold. **Pleistophora* LS in Genbank; ***Pleistophora* PA in Genebank. For GenBank accession numbers see Table 1.

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Address for correspondence: Yuliya Sokolova. Microscopy Center, Department of Comparative Biological Sciences, School of Veterinary Medicine, Louisiana State University, 1909 Skip Bertman Drive, Baton Rouge LA, 70803; e-mail: *yysokolova@gmail.com*