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Parathelohania iranica sp. nov. (Microsporidia: Amblyosporidae) infecting malaria mosquito *Anopheles superpictus* (Diptera: Culicidae): Ultrastructure and molecular characterization

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ABSTRACT

Microsporidia are common pathogens of insects and sometimes are considered as a candidate in the biological control of mosquitoes. Recently a microsporidium infection was discovered in *Anopheles superpictus* (Diptera: Culicidae) larvae, in Iran. The responsible agent belonged to the genus *Parathelohania* (Microsporidia: Amblyosporidae). This study has been carried out to identify its identity at the species level.

Fresh infected larvae were collected from the type locality, Kiar district, in Chahar Mahal and Bakhtiari province, at the central western of Iran. Superficial and the internal ultrastructure of the recovered spores were explored by scanning and transmission electron microscopy, respectively. Molecular techniques were also employed to amplify parts of its ssu rDNA. The obtained data were compared with the available information of congener species and other closely related microsporidia to elucidate evolutionary relationship.

A small apical depression and two posterolateral ridges extending backward from a pear shaped anterior body mass were notable under scanning electron microscopy. Transmission electron microscopy revealed 2 broad and 3–4 narrow coils in the either side of spores, respectively. The sequence of a 1062 nucleotide fragment of ssu rDNA was determined by means of PCR technique.

This study indicates that the microsporidium infecting *An. superpictus* differs from other previously described species in the genus *Parathelohania*. It means that the microsporidium infecting *An. superpictus* is a new species and hereby it is called *Parathelohania iranica*. Further work is necessary to clarify its life cycle and probable value in the biological control of mosquitoes.

1. Introduction

Mosquitoes are commonly considered the most important disease vectors in the world (Mullen and Durden, 2009). While they transmit a wide range of pathogens to humans and livestock, they are parasitized in turn by a diverse group of microbial agents including microsporidia (Roberts and Castillo, 1980).

Microsporidia are a large group of intracellular, eukaryotic parasites closely related to fungi (Corradi and Keeling, 2009) and are among the most common and widely distributed parasites of mosquitoes in nature. To date, more than 179 species of microsporidia in 25 different genera are recognized as infective to at least 14 genera of mosquitoes (Andreadis, 2007; Andreadis et al., 2012, 2013).

Recently, a microsporidium infection in *Anopheles superpictus* Meigen larvae was reported from Iran (Omrani et al., 2016). To our knowledge, there is no other report on such an infection in this mosquito species. *Anopheles superpictus* is a malaria vector in the Middle East (Sinka et al., 2010) including Iran (Edrissian, 2006) and has a widespread distribution in the Palearctic region (Zahar, 1974).

Preliminary observations on the light microscopic features of the spores (i.e. bottle shape appearance and aggregation in clusters of 8) suggested a species belonging to the genus *Parathelohania* Codreanu (1966) (Codreanu, 1966; Hazard and Anthony, 1974; Omrani et al., 2016). *Parathelohania* accounts for a relatively large genus within the family Amblyosporidae (Becnel and Andreadis, 2014). The members of this genus are largely restricted to anopheline mosquitoes, have polymorphic spores and an obligatory intermediate copepod host in their life cycle (Andreadis, 2007).

Although light microscopy is a primary tool in the general recognition of microsporidia, species identification requires further visualization of the internal ultrastructure by transmission electron microscopy (Garcia, 2002). Additionally, molecular methods determining the

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nucleotide sequence of ssu rDNA provide confirmation on its unique identity and invaluable information on its phylogenetic relationship with the other known microsporidia species (Vossbrinck and Debrunner-Vossbrinck, 2005).

The present study provides taxonomic identification of this newly discovered microsporidium at the species level based on its ultrastructural and molecular characteristics.

2. Materials and methods

2.1. Field collection and host identification

The type locality was visited to collect fresh infected *An. superpictus* larvae. The larval habitat is located at the Helen Protected Area, bordering Kiar district in Chahar Mahal and Bakhtiari province, Iran. All visits were made during August to September 2013. Infected 3rd or 4th instar anopheline larvae were collected from a shallow leakage pool nearby a small spring. They were placed in a black pan and examined for the whitish discoloration of their thorax and/or abdominal segments as the common gross diagnostic sign of microsporidium infection (Andreadis, 2007). The species identity of the larvae was verified in the laboratory with the use of a valid local identification key (Azari-Hamidian and Harbach, 2010).

2.2. Electron microscopy

For transmission electron microscopy, the affected body segments of a typical infected larva were fixed in 2.5% (v/v) glutaraldehyde for 24 h at 4 °C. They were buffered, then, in 200 mM sodium cacodylate (pH 7) and postfixed in aqueous 1% (w/v) OsO_4 (pH 7.4) for 1 h at room temperature. After washing the specimens in sodium phosphate buffer (pH 7) they were dehydrated through a graded ethanol series and embedded in epoxy resin plus propylene oxide (1:1). Thin sections (~60–100 nm) were stained with 2% (w/v) uranyl acetate in 50% ethanol followed by 1% lead citrate and examined under CM-30 transmission electron microscope (Philips, United States) at an accelerating voltage of 150 kV.

For scanning electron microscopy no specific preparation was made. The freshly dried spores were coated with gold and viewed under XL30 scanning electron microscope (Philips, United States) at an accelerating voltage of 20 kV.

2.3. Molecular investigations

Ten milliliters of the water containing 2–3 ruptured dead larvae was filtered through a 10 μm nylon mesh to collect their infecting spores. The collected material was washed with tap water and centrifuged at 12,000 rpm for 10 min and the aliquot was discarded. 150 μl of TAE buffer (containing Tris-Acetate 0.04 M and EDTA 0.001 M) plus 150 mg 0.1 N hydrochloric acid washed fine ground glass (300–500 μm) were added to the precipitate in a clean 1.5 ml microtube. The microtube was shaken vigorously in a high speed vortex machine for 50 s and then incubated for 3 min at 95 °C on a hot plate. The adequacy of the concentration of extracted DNA was verified by Nanodrop® micro-spectrophotometer.

The NCBI GenBank was checked for species specific ssu rDNA sequences in the genus *Parathelohania*. In this respect, the relevant sequences of four species i.e. *P. anophelis* (AN: AF027682.1), *P. obesa* (AN: AY090065.1), *P. divulgata* (AN: JF826420.1) and *P. tomski* (AN: JF826421.1) were aligned by Chromas 2.3 software and GTGGATGCTCGCCTTAAAGAC and CCAATCTAGCAGCACGGATTC were designed as the forward and reverse primers, respectively.

PCR reaction was performed in 25 μl final volume containing 2.4 μl of 1X PCR buffer, 1 μl 100 mM $MgCl_2$, 0.5 μl 10 mM dNTP, 0.1 μl 3 unit per microliter of Taq polymerase (Kowsar Biotech Company, Iran) and 0.5 μl of each primers. Amplification was carried out in Corbett thermal cycler (CG1-96, Australia) under condition, 95 °C for 5 min followed by 5 cycles of 95–59–72 °C for 90 s, 30 cycles of 95–59–72 °C for 60 s, and final extension of 72 °C for 6 min. Eight microliters of PCR product was electrophoresed on 1% of agarose gel and visualized after ethidium bromide staining. PCR product was sequenced using ABI 3730 sequencer (Applied Biosystems, Macrogen, South Korea). The result was compared with the data available for other known *Parathelohania* species to calculate the percentage of similarity using Clustal Omega Software. The evolutionary history for *Parathelohania* species and selected closely related microsporidia as outgroups i.e. *Novothelohania ovalae* (JF837528), *Takaokaspora nipponicus* (KF110990) and *Senoma globulifera* (DQ641245) was inferred using the UPGMA method. The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analyzed. The evolutionary distances were computed using the Maximum Composite Likelihood method. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA6 software.

3. Results and discussion

The scanning electron micrographs showed that there is a small apical depression over the bulged and pear shaped body mass of spores (Fig. 1). Two posterolateral ridges are extended from the latter towards the rear where they coalesce to form a complex structure. Comparison with the corresponding transmission electron micrographs shows that this structure is an expanded thin floppy layer asymmetrically stretched out from the periphery of the posterior projection of spore body mass.

The fine superficial structure of spores resemble *P. africana*, *P. anophelis*, *P. obesa* and *P. octolagenella*, the only other members of *Parathelohania* for which published scanning electron micrographs are available. Apart from coarse markings on the pear-shaped body mass of spores, the presence of a shrunken posterior part is notable. However, the actual spatial structure of this part is not clear and more information is needed to elucidate its real configuration and the functional role in such a typical *Parathelohania* species.

Under transmission electron microscope, spore aggregates were detected within a well-defined sporophorous vesicle (Fig. 2). The bilayer wall of spores was such that the granular outer layer (exospore) was absent on the anterior end of the spore and the hyperlucent inner layer (endospore) was prominently bulged at the posterior end. In addition to the anchoring disk, lamellar polaroplast, polar filament and the posterior vacuole, 2 broad and 3–4 narrow coils were recognizable in the either side of spores. This polar filament configuration differs from all other *Parathelohania* species with known coil ultrastructure

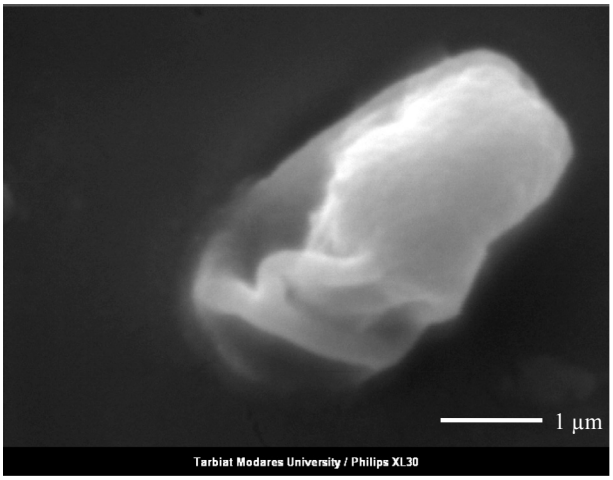

Fig. 1. The scanning electron micrograph of a typical spore of *P. iranica* sp. nov. in *An. superpictus* larva.