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SHORT COMMUNICATION

Infection of *Chorthippus loratus* (Orthoptera: Acrididae) with *Liebermannia* sp. (Microsporidia) in South-Western Russia

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ABSTRACT

Chorthippus loratus collected in Krasnodar Territory in 2017 was infected at 15% rate with a microsporidium possessing ovocylindrical binucleate spores, 2.6 × 1.2 μm in size. SSU RNA gene typing (Genbank accession # MH396491) showed its allocation to the genus *Liebermannia*. Degenerate primers based upon largest subunit RNA polymerase II (RPB1) sequences of closest relatives allowed amplifying the respective gene fragment of *Liebermannia* sp. (# MH396492). The present finding indicates worldwide distribution of the *Liebermannia* genus and parasitism in hosts with nonoverlapping geographic ranges (representing Neotropical versus Palearctic fauna), while previous observations were restricted to Acridoidea endemic for South America.

MICROSPORIDIA are parasitic unicellular eukaryotes widely occurring in animals. Over 1300 species belonging to more than 200 genera are described. And though insects, alongside with crustaceans and fish, harbor the vast majority of described species, known biodiversity of microsporidia from the order Orthoptera is comparatively low. In fact, as summarized by Sokolova et al. (2006) in a paper describing a new genus and a new species *Liebermannia patagonica* from *Tristira magellanica* (Acridoidea, Tristiridae), only 18 microsporidia species or undefined isolates were registered worldwide in orthopteran insects which is by an order of magnitude lower as compared to other insect orders such as Diptera, Coleoptera, or Lepidoptera. Since then, only three new species were described from Orthoptera, namely *Liebermannia covasacrae* (Sokolova et al. 2009) from *Covasacris pallidinota,* (Acridoidea, Acrididae), *Encephalitozoon romalae* (Lange et al. 2009) from *Romalea microptera* (Acridoidea, Romaleidae) and *Endoreticulatus poecilimonae* (Pilarska et al. 2015) from *Poecilimon thoracicus* (Tettigonoidea, Tettigoniidae), and one species, *Perezia dichroplusae* from *Dichroplus elongatus* (Acridoidea, Acrididae), was redefined as *Liebermannia dichroplusae* (Sokolova et al. 2007). In the present paper, we describe a case of infection of a grasshopper population with a microsporidium belonging to the genus *Liebermannia,* which is found for the first time in Eurasia.

MATERIALS AND METHODS

Adults of the grasshopper *Chorthippus loratus* ($N = 61$) were collected in reed beds alongside the river Anapka in the town of Anapa, Krasnodar Territory (44°54′N 37°23′E) in Russia. Insects perished during transportation to the lab and dry cadavers were stored at room temperature prior to examination. Whole body homogenates were prepared from individual specimens; unfixed smears of homogenates were examined in bright field using Carl Zeiss Imager M1. Smears in which spores had been observed were fixed with methanol and stained with diamidine phenylenindole (DAPI) to visualize nuclear apparatus as described elsewhere (Tokarev et al. 2007). Three positive samples each containing 1–3 mln spores (as estimated using a hemocytometer) were homogenized with plastic pestle and incubated in lysis buffer containing 2% CTAB, 0.2% β-mercaptoethanol and 0.3% proteinase K, followed by phenol-chloroform extraction of genomic DNA (Tokarev et al. 2010). The PCR was run with a standard protocol

using DreamTaq polymerase (Thermo Fisher Scientific) as a ready-to-use mixture (https://www.thermofisher.com/order/catalog/product/EP0701). The partial small subunit ribosomal RNA (SSU rRNA) gene was amplified using primers 18f and 1047r (Weiss and Vossbrinck 1999). The obtained SSU rRNA sequence was compared to entries available at Genbank using built-in BLAST utility (www.ncbi.nlm.nih.gov/Blast.cgi). After genus affiliation was established (see below), amino acid sequences of largest subunit RNA polymerase II (RPB1) available from Genbank were aligned in BioEdit (Hall 1999) for the four closest relatives known: *Vittaforma corneae* (# ELA41814), *Enterocytozoon bieneusi* (# EDQ31017), *Encephalitozoon cuniculi* (# KMV66392), and *Ordospora colligata* (# KHN70156). *Cystosporogenes operophtherae* also belonging to this group was not included as the available RPB1 sequence (# CAC33855) was shorter as compared to other taxa. The alignment (1625 aa long) was imported into Genefisher 2 sofware (Giegerich et al. 1996) available online (https://bibiserv.cebitec.uni-bielefeld.de/genefisher2) and degenerate primers Lros1373for (5′-GAyGArATGAAyyTnCAyATGC-3′) and Lros2519rev (5′-GTyTCnGCnGTyTTdATnGCnGTrTC-3′) were designed to amplify a fragment of ~1200 bp. The PCR products of SSU rRNA and RPB1 gene fragments were directly sequenced using ABI Prism Genetic Analyzer 3500. The obtained sequences were edited and aligned in BioEdit. Phylogenetic reconstructions were carried out with Maximum Likelihood method using Tamura-Nei model (Tamura and Nei 1993) with gamma distributed rates among sites (TN93+G) for SSU rRNA and an improved general amino acid replacement matrix (Le and Gascuel 2008) with gamma distributed rates among sites and a proportion of invariable sites (LG+G+I) for RPB1 in MEGA 7 software (Kumar et al. 2016).

RESULTS AND DISCUSSION

Out of 61 insects examined, nine were found infected with microsporidia spores, corresponding to 14.8 % ± 4.5 % (SE). Only dried cadavers were examined, so

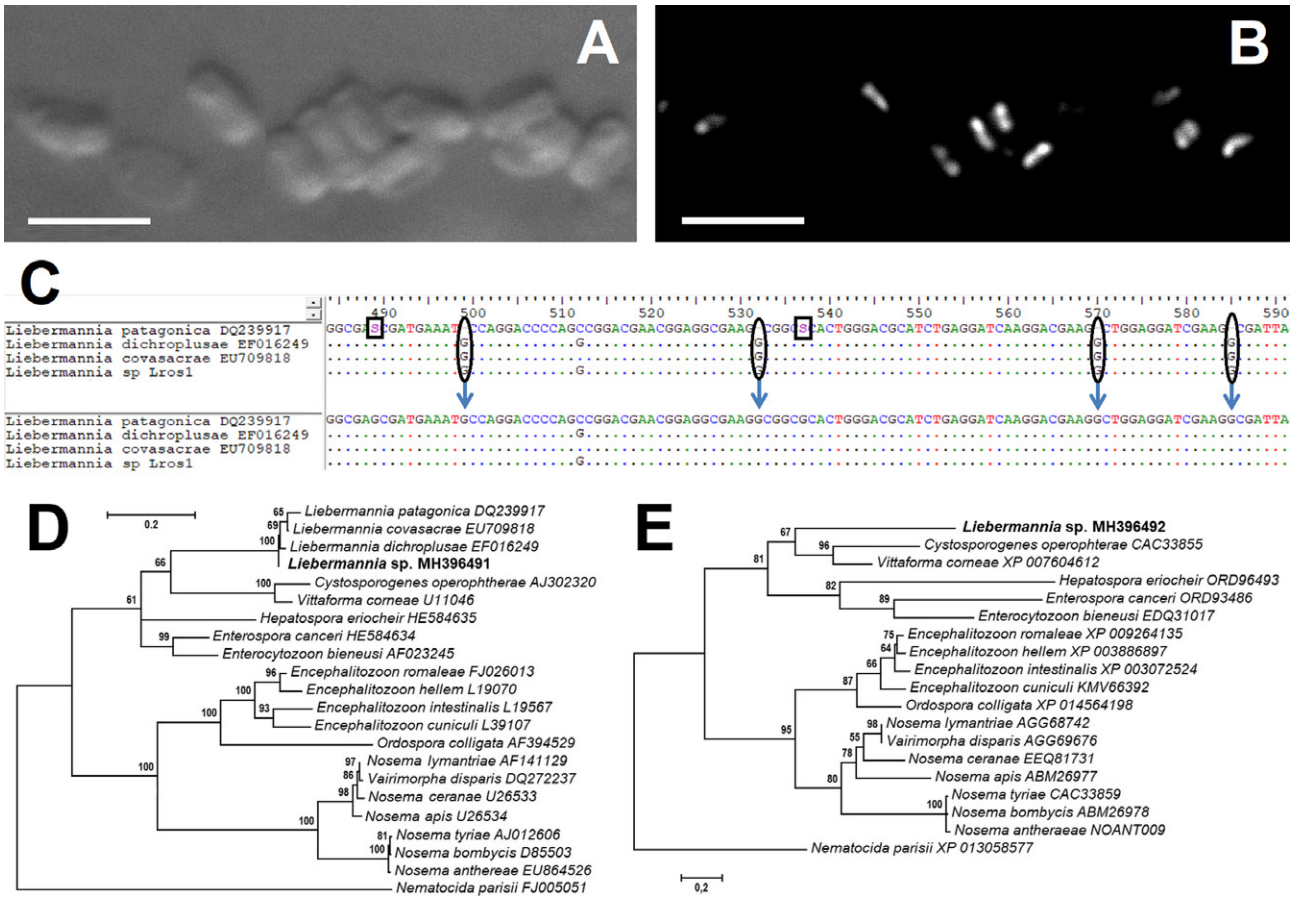

Figure 1 Light microscopic and molecular genetic analysis of *Liebermannia* sp. from *Chorthippus loratus*. (**A, B**) – bright field (A) and fluorescent microscopy of methanol-fixed DAPI-stained spores (B), scale bar = 4 μm; (**C**) – a fragment of alignment of small subunit rRNA gene of microsporidia of the genus *Liebermannia* before (upper rows) and after correction of sequencing artifacts (lower rows), rectangular frames indicate ambiguous sites, oval frames and arrows show erroneous indels in upper and corrected positions in lower rows; (**D, E**) – molecular phylogenetic position of *Liebermannia* sp. (in bold) as inferred from Maximum Likelihood analysis using alignments of 23 nucleotide SSU rRNA sequences, 1448 bp long (D) and 19 amino acid RPB1 sequences, 195 aa long (E), respectively, scale bar = 0.2 expected changes per site.

precise tissue localization of the parasites was not established. Spores were ovocylindrical, 2.2–3.1 (mean 2.6 ± 0.04) × 0.9–1.6 (mean 1.2 ± 0.04) μm (unfixed, number of examined spores = 23); spore index (length:width ratio) was equal to 2.1 ± 0.06 (Table S1). DAPI staining of methanol-fixed spores showed two nuclei within each spore examined (Fig. 1A, B).

SSU rRNA gene sequence (# MH396491) was identical in three samples examined, showing close affinity of the newly found microsporidium to the members of the genus *Liebermannia*. When an alignment of the respective sequences was scrutinized, a number of gaps and several incompletely recognized characters were found in *Liebermannia patagonica* (# DQ239917) supposed to be sequencing artifacts and corrected as exemplified in Fig. 1C. After this correction, the sequence similarity and genetic distances of three *Liebermannia* species from Genbank were 97.1–99.6% and 0.003–0.033, respectively. When the new microsporidium was compared to these three species, it showed sequence similarity of 97.4–98.4% and genetic distance of 0.015–0.022. Sequence similarity to the closest representatives of the other genera (*Vittaforma* and *Cystosporogenes*) was about 70% and genetic distance was above 0.330 (Table S2). It can be therefore concluded that the microsporidium from *C. loratus* represents a separate, yet to be described species of the genus *Liebermannia*. Dimensional characteristics of this microsporidium were most similar to those of *Liebermannia covasacrae* with spores 2.6 × 1.4 μm in size, spore index = 1.9, while spores of *L. patagonica* were slightly larger, 2.9 × 1.2 μm, spore index = 2.4. *Liebermannia dichroplusiae,* unlike the other species of this genus, possessed uninucleate spores, 3.5 × 1.5 μm in size, and spore index = 2.3 (Table S1).

As no genotyping information was available for *Liebermannia* species besides SSU rRNA gene sequence, RPB1 gene sequences of other related genera were used to design degenerate primers which successfully amplified the respective locus of genomic DNA of the new microsporidium (# MH396492) from one sample. The phylogenetic reconstruction using both SSU rRNA (Fig. 1D) and RPB1 (Fig. 1E) showed position of *Liebermannia* sp. in one phylogenetic lineage with a human pathogen *Vittaforma corneae* and an insect pathogen *Cystosporogenes operophtherae*, which in turn was in a sister position to the lineage containing a human pathogen *Enterocytozoon bieneusi*. The whole group belongs to the Clade 4 (Vossbrinck et al. 2014), previously known as Clade IV (Vossbrinck and Debrunner-Vossbrinck 2005) of the Microsporidia Tree of Life. Availability of an RPB1 sequence for a representative of the genus *Liebermannia* will facilitate parasite identification in future research as this molecular marker is more polymorphic as compared to SSU rRNA and is useful for discrimination of isolates within and between species of microsporidia (Tokarev et al. 2018).

Noteworthy, the three species of the genus *Liebermannia* were all detected in Acridoidea species sampled in Argentina and endemic to South America. The present finding of another (though undescribed) species of *Liebermannia* in European part of Russia in a host endemic to Eurasia indicates worldwide distribution of this genus and parasitism in hosts with nonoverlapping geographic ranges, representing Neotropical versus Palearctic fauna.

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REFERENCES

Giegerich, R., Meyer, F. & Schleiermacher, C. 1996. GeneFisher–software support for the detection of postulated genes. *Proc. Int. Conf. Intell. Syst. Mol. Biol.*, 4:68–77.

Hall, T. A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp. Ser.*, 41:95–98.

Kumar, S., Stecher, G. & Tamura, K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.*, 33:1870–1874.

Lange, C. E. 1987. A New Species of *Perezia* (Microsporida: Pereziidae) from the Argentine Grasshopper *Dichroplus elongatus* (Orthoptera: Acrididae). *J. Protozool.*, 34(1):34–39.

Lange, C. E., Johny, S., Baker, M. D., Whitman, D. W. & Solter, L. F. 2009. A new *Encephalitozoon* species (Microsporidia) isolated from the lubber grasshopper, *Romalea microptera* (Beauvois) (Orthoptera: Romaleidae). *J. Parasitol.*, 95:976–986.

Le, S. Q. & Gascuel, O. 2008. An improved general amino acid replacement matrix. *Mol. Biol. Evol.*, 25:1307–1320.

Pilarska, D. K., Radek, R., Huang, W. F., Takov, D. I., Linde, A. & Solter, L. F. 2015. Review of the genus *Endoreticulatus* (Microsporidia, Encephalitozoonidae) with description of a new species isolated from the grasshopper *Poecilimon thoracicus* (Orthoptera: Tettigoniidae) and transfer of *Microsporidium itiiti* Malone to the genus. *J. Invertebr. Pathol.*, 124:23–30.

Sokolova, Y. Y., Lange, C. E. & Fuxa, J. R. 2006. Development, ultrastructure, natural occurrence, and molecular characterization of *Liebermannia patagonica* n. g., n. sp., a microsporidian parasite of the grasshopper *Tristira magellanica* (Orthoptera: Tristiridae). *J. Invertebr. Pathol.*, 91:168–182.

Sokolova, Y. Y., Lange, C. E. & Fuxa, J. R. 2007. Establishment of *Liebermannia dichroplusae* n. comb. on the basis of molecular characterization of *Perezia dichroplusae* Lange, 1987 (Microsporidia). *J. Eukaryot. Microbiol.*, 54:223–230.

Sokolova, Y. Y., Lange, C. E., Mariottini, Y. & Fuxa, J. R. 2009. Morphology and taxonomy of the microsporidium *Liebermannia covasacrae* n. sp. from the grasshopper *Covasacris pallidinota* (Orthoptera, Acrididae). *J. Invertebr. Pathol.*, 101:34–42.

Tamura, K. & Nei, M. 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol. Biol. Evol.*, 10(3):512–526.

Tokarev, Y. S., Peat, K. M., Malysh, J. M. & Senderskiy, I. V. 2018. Discovery of a novel microsporidium in laboratory colonies of Mediterranean cricket *Gryllus bimaculatus* (Orthoptera: Gryllidae): *Microsporidium grylli* sp. nov. *Parasitol. Res.*, 117:2823–2829.

Tokarev, Y. S., Sokolova, Y. Y. & Entzeroth, R. 2007. Microsporidia-insect host interactions: teratoid sporogony at the sites of host tissue melanization. *J. Invertebr. Pathol.*, 94:70–73.

Tokarev, Y. S., Voronin, V. N., Seliverstova, E. V., Dolgikh, V. V., Pavlova, O. A., Ignatieva, A. N. & Issi, I. V. 2010. Ultrastructure

and molecular phylogeny of *Anisofilariata chironomi* sp.n. g.n. (Microsporidia: Terresporidia), a microsporidian parasite of *Chironomus plumosus* L. (Diptera: Chironomidae). *Parasitol. Res.*, 107:39–46.

Vossbrinck, C. R. & Debrunner-Vossbrinck, B. A. 2005. Molecular phylogeny of the Microsporidia: ecological, ultrastructural and taxonomic considerations. *Folia Parasitol.*, 52:131–142.

Vossbrinck, C. R., Debrunner-Vossbrinck, B. A. & Weiss, L. M. 2014. Molecular phylogeny of the Microsporidia. *In*: Weiss, L. M. & Becnel, J. J. (ed.), Microsporidia: Pathogens of Opportunity. Wiley-Blackwell, Hoboken, NJ. p. 203–220.

Weiss, L. M. & Vossbrinck, C. R. 1999. Molecular biology, molecular phylogeny, and molecular diagnostic approaches to the Microsporidia. *In*: Wittner, M. & Weiss, L. M. (ed.), The Microsporidia and Microsporidiosis. American Society of Microbiology, Washington, DC. p. 129–171.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Spore morphology, host taxonomy, and geographic range of the genus *Liebermannia.*

Table S2. SSU rRNA gene sequence similarity/genetic distance of microsporidia of the genus *Liebermannia* and allied species.