AKADÉMIAI KIADÓ

Acta Veterinaria Hungarica

69 (2021) 1, 38–42

DOI:
10.1556/004.2021.00012

SHORT COMMUNICATION

Genetic diversity of *Loma acerinae* (Microsporidia: Glugeida) from different fish hosts and localities – Short communication

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Received: 25 November 2020 • Accepted: 18 March 2021
Published online: 15 April 2021

ABSTRACT

Loma acerinae is a xenoma-forming fish microsporidium described from common ruffe *Gymnocephalus cernua* (Perciformes: Percidae) and also found in Ponto-Caspian gobies (Gobiiformes: Gobiidae). This casts doubt on the strict host specificity of this parasite. The largest subunit RNA polymerase II (*rpb1*) was used as a genetic marker of the parasite isolated from six host species of Perciformes (*G. cernua* from the Baltic Sea), Atheriniformes (*Atherina boyeri* from the Azov Sea) and Gobiiformes (*Neogobius* spp. and *Zosterisessor ophiocephalus* from the Black Sea and *Ponticola kessleri* from the Caspian Sea basin). Two major *rpb1* haplogroups were found with 98.5% identity between the groups. Notably, Haplogroup I was associated with *Neogobius* spp. samples ($n = 6$) only, whereas Haplogroup II included the samples from other host species ($n = 7$). These findings confirm the broad distribution and host range of *L. acerinae*, but also indicate that certain patterns of host-driven intraspecific polymorphism may exist. Furthermore, the study revealed low similarity between the ribosomal RNA gene sequences of *L. acerinae* and the type species, *Loma morhua* (as well as other species of the genus). This suggests loose genetic association within the genus, and may raise the need for the taxonomic revision of *L. acerinae*.

KEYWORDS

microsporidia, fish parasites, *rpb1*, genetic polymorphism, host specificity

Loma acerinae is a xenoma-forming fish microsporidium that was originally described from the intestine of the common ruffe *Gymnocephalus cernua* (Perciformes: Percidae) (Lom and Pekkarinen, 1999). A recent morphological and molecular genetic study has confirmed that the microsporidium species found in Ponto-Caspian gobies (Gobiiformes: Gobiidae) and big-scale sand smelt *Atherina boyeri* (synonym *Atherina mochon pontica*) (Atheriniformes: Atherinidae) was *L. acerinae* (Ovcharenko et al., 2017). Due to the wide host range of *L. acerinae* including phylogenetically distant hosts, and the divergence in the sampling sites of gobies and the sand smelt (the Black Sea) vs. those of common ruffe (the Czech Republic and Finland), it is of great interest to elucidate the intraspecific genetic polymorphism of the parasite.

In the present paper, we used small subunit rRNA gene (SSU rDNA) and the largest subunit RNA polymerase II (*rpb1*) as genetic markers to compare *L. acerinae* samples. Thirteen samples of *L. acerinae* isolated from six species of fish hosts were examined: *G. cernua* (number of examined samples $n = 2$) from Finnish Bay of the Baltic Sea (60°06′N

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Fig. 1. Sampling sites of fishes infected with *Loma acerinae* in Europe. 1 – Finnish Bay, Baltic Sea; 2 – Karkinit Bay, Black Sea; 3 – Sivash Bay, Azov Sea; 3 – Volga River Delta, Caspian Sea basin

29°55′E), monkey goby *Neogobius fluviatilis* (n = 4), round goby *Neogobius melanostomus* (n = 2) and grass goby *Zosterisessor ophiocephalus* (n = 3) from Karkinit Bay of the Black Sea (45°52′N 33°28′E), *A. boyeri* (n = 1) from Sivash Bay of the Azov Sea (45°25′N 35°10′E) and bighead goby *Ponticola kessleri* (n = 1) from the Volga River Delta in the Caspian Sea (46°10′N 49°13′E) (Fig. 1).

Adult fishes were caught by a fishing net, transported to the laboratory in cooled plastic bags, chilled on ice, then euthanised by quick and accurate decapitation (Jenkins et al., 2014). Then the fishes were dissected and their inner organs were examined visually for the presence of parasite xenomas (Lom and Dykova, 1992). Xenomas were excised and fixed with 95% ethanol for molecular genetic analysis. For DNA extraction, the ethanol was removed, the tissue sample was dried until remnant ethanol evaporated, then 100 µL of lysis buffer (2% CTAB, 1.4 M NaCl, 100 mM EDTA, 100 mM Tris-Cl, pH 8.0) (VWR Life Science AMRESCO) was added. The samples were homogenised with a plastic pestle adapted for a 1.5-mL microcentrifuge tube (SSIbio), followed by incubation at 65 °C for 2 h in 500 µL lysis buffer as above with the addition of 0.2% of β-mercaptoethanol (VWR Life Science AMRESCO). Genomic DNA was extracted by a phenol/chloroform extraction method, isopropanol sedimentation and 70% ethanol washing (Malysh et al., 2019).

To amplify a fragment of SSU rDNA, the universal SSU rDNA primers 18f (5′-GTTGATTCTGCCTGACG-3′) and 1047r (5′-AACGGCCATGCACAC-3′) were used (Weiss and Vossbrinck, 1999). For *rpb1*, novel degenerate primers lgtvRPB1F (5′-CCKGARTGGATGATCATMAGTG-3′) and lgtvRPB1R (5′-ATCRAARTCVGCRTTGTACG-3′) were designed on the basis of the respective sequences of *L. acerinae* (Genbank accession # AJ278951), *Glugea anomala* (# AJ278952), *Trachipleistophora hominis* (# AJ278945), and *Vavraia culicis* (#AJ278956), aligned in BioEdit software version 7.2.5 (Hall, 1999). The primer sequences were selected manually and checked for specificity using Primer-BLAST online utility (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and for compatibility using PerlPrimer software version 1.1.21 (Marshall, 2004).

The PCR was applied using a protocol including the initial denaturation at 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 1 min, the annealing at 54 °C for 1 min, the elongation at 72 °C for 1 min, and the final elongation step at 72 °C for 5 min (Malysh et al., 2020). The PCR mixture contained 1 × DreamTaq Green PCR Mastermix (Thermo Fisher Scientific) and 10 nM of primers. The amplicons were visualised using electrophoresis in 1% agarose gels. Then gel fragments containing the specific amplicons were excised, melted with 3M guanidine isothiocyanate (Thermo Fisher Scientific), absorbed using silicon dioxide (Sigma-Aldrich) and eluted with deionised molecular grade water (Vogelstein and Gillespie, 1979). The purified DNA fragments were sequenced in both directions by Sanger dideoxy method using BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Inc.) with respective forward and reverse primers as above, and detected on ABI Prism 3500 Genetic Analyzer. The raw reads with no ambiguous sites (double peaks etc.) were aligned using Clustal W multiple alignments in BioEdit. The sequence identities and genetic distances were calculated using BioEdit. Phylogenetic reconstruction was performed in MEGA 7 (Kumar et al., 2016). The maximum likelihood method using the Tamura 3-parameter model with a proportion of invariable sites and gamma distribution of rate categories was applied. *G. anomala* was used as an outgroup. The *rpb1* sequence of *L. acerinae* available in Genbank (#AJ278951) was excluded from the analysis due to uncertainties in the nucleotide sequence and respective amino acid translation.

Partial sequences of SSU rDNA (950 bp in length) were obtained for 13 samples of microsporidia from Percidae, Gobiidae, and Atherinidae, showing 100% identity to each other. When compared to Genbank entries, the sequences showed 100% identity to *L. acerinae* from *G. cernua* (#AF356224) and big-scale sand smelt *A. boyeri* (#KT934810) and 99.6% identity to another isolate from *G. cernua* (#AJ252951). However, SSU rDNA sequence identity of *L. acerinae* ranged between 85.1 and 91.6% when compared to *Loma morhua* (#KX084449) from Atlantic cod *Gadus morhua* (Gadiformes: Gadidae), and other species attributed to the genus *Loma,* as well as representatives of other fish-infecting microsporidia from the genera *Glugea*, *Dasyatispora, Heterosporis, Ichtyosporidium* and *Pleistophora* (Table 1).

The PCR assay using the primers designed for the amplification of the *rpb1* gene fragment was successful, and the obtained 686-bp-long sequences showed 98.6–100% sequence identity among the samples examined (Table 2). In particular, *rpb1* sequence of *L. acerinae* from *G. cernua* (# MN335641) was 100% identical to those from *A. boyeri*

Table 1. Nucleotide sequence identity and genetic distance of small subunit rRNA gene (SSU rDNA) of *Loma acerinae* isolates and related microsporidium species

#	Name of species and specimen	Genbank accession #	Sequence identity (%, left-lower cells) and genetic distance (right-upper cells)*													
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	*Loma acerinae*	AF356224	ID	0.004	0.000	0.000	0.000	0.000	0.103	0.077	0.160	0.157	0.155	0.113	0.126	0.106	0.122
2	*Loma acerinae*	AJ252951	99.6	ID	0.004	0.004	0.004	0.004	0.100	0.079	0.157	0.153	0.152	0.112	0.127	0.105	0.124
3	*Loma acerinae*, GB4	KT934810	100	99.6	ID	0.000	0.000	0.000	0.103	0.077	0.160	0.157	0.155	0.113	0.126	0.106	0.122
4	*Loma acerinae*, GB8	KT964890	100	99.6	100	ID	0.000	0.000	0.103	0.077	0.160	0.157	0.155	0.113	0.126	0.106	0.122
5	*Loma acerinae*, GB6	KT964889	100	99.6	100	100	ID	0.000	0.103	0.077	0.160	0.157	0.155	0.113	0.126	0.106	0.122
6	*Loma acerinae*, BNM7	KT964891	100	99.6	100	100	100	ID	0.103	0.077	0.160	0.157	0.155	0.113	0.126	0.106	0.122
7	*Glugea anomala*	AF044391	89.9	90.1	89.9	89.9	89.9	89.9	ID	0.104	0.143	0.148	0.144	0.110	0.141	0.115	0.124
8	*Loma psittaca*	FJ843104	91.6	91.5	91.6	91.6	91.6	91.6	89.0	ID	0.173	0.174	0.173	0.120	0.139	0.132	0.139
9	*Loma morhua*	KX084449	85.1	85.3	85.1	85.1	85.1	85.1	86.2	83.6	ID	0.015	0.011	0.161	0.121	0.155	0.184
10	*Loma embiotocia*	HQ157529	85.3	85.6	85.3	85.3	85.3	85.3	85.9	83.5	98.5	ID	0.007	0.163	0.121	0.159	0.184
11	*Loma salmonae*	HM626203	85.5	85.7	85.5	85.5	85.5	85.5	86.1	83.6	98.9	99.3	ID	0.160	0.121	0.155	0.182
12	*Heterosporis anguillarum*	AB623036	88.3	88.3	88.3	88.3	88.3	88.3	88.4	87.2	84.4	84.2	84.4	ID	0.138	0.039	0.069
13	*Ichthyosporidium weissii*	JQ062988	87.6	87.5	87.6	87.6	87.6	87.6	86.6	86.2	88.2	88.2	88.2	86.1	ID	0.138	0.155
14	*Dasyatispora levantinae*	GU183263	87.4	87.6	87.4	87.4	87.4	87.4	86.4	85.5	83.5	83.1	83.5	94.4	84.5	ID	0.081
15	*Pleistophora mulleri*	AJ438985	87.0	86.8	87.0	87.0	87.0	87.0	86.5	85.6	82.6	82.6	82.7	91.4	84.0	89.7	ID

*ID – identical entries.

(# MN335645) and *Z. ophiocephalus* (#MN335642), whereas the DNA sequence obtained from *P. kessleri* (#MN335646) contained one nucleotide alteration (R) at position 485. The samples from *N. fluviatilis* (#MN335643) possessed 8 variable nucleotides at positions 120 (C/T = Y), 198 (C/A = M), 339 (Y), 354 (C/G = S), 393 (R), 471 (T/G = K), 598 (Y) and 672 (Y). Additionally, the samples from *N. melanostomus* (#MN335644) had nucleotide variation (Y) at position 378. As a result, 99.8–100% sequence identities were detected within the haplogroups, whereas the identity was 98.5–98.8% between the groups (Table 2). Notably, Haplogroup I was associated with *Neogobius* spp. samples (n = 6) only, and Haplogroup II included the parasite samples (n = 7) from the other host species (Fig. 2).

The type host of *L. acerinae* is *G. cernua* collected from Czechoslovakia (Jirovec, 1930), whereas *L. acerinae* was also reported from the same host in Finland (Lom and Pekkarinen, 1999). In the present study, two samples of the microsporidium from this host species were collected in Finnish Bay, adjacent to Finland and belonging to the same water basin (Baltic Sea) and the same geographic area (Northern Europe). Thus, all examined samples of *L. acerinae* are of European origin.

Previous studies have shown that SSU rDNA was a reliable molecular genetic marker for the species identification of Microsporidia (Weiss and Vossbrinck, 1999; Issi et al., 2010; Tokarev et al., 2010; Sokolova et al., 2016; Issi, 2020), although closely related species may be very similar both in ultrastructure and in rDNA sequences (Malysh et al., 2013). The present results strengthen the findings of Ovcharenko et al. (2017). The identical SSU rDNA sequences in microsporidia samples obtained from distant locations indicate that this haplotype is common for *L. acerinae,* though a limited level of variation exists, as seen for another isolate from *G. cernua* (#AJ252951).

When comparing the SSU rDNA of *L. acerinae* to *L. morhua* and other species allocated to the genus *Loma,* the DNA sequence identity does not exceed 92%. Since L. *morhua* is the type species for the genus *Loma,* the DNA sequence-based comparisons suggest that *L. acerinae* does not fit to this genus, therefore taxonomic redefinition may be needed.

For a more robust identification and intraspecific differentiation of microsporidium samples, the *rpb1* gene is widely exploited as a reliable genetic marker (Cheney et al., 2001; Grushevaya et al., 2018; Tokarev et al., 2018a,b; Tokarev et al., 2020). A high level of sequence identity of this protein-coding gene obtained for all examined samples of *L. acerinae* was consistent with the morphological findings that they all belong to the same species. Interestingly, *L. acerinae* from *G. cernua, A. boyeri* and *P. kessleri* and *Z. ophiocephalus* possessed almost identical *rpb1* sequences belonging to the same haplogroup (with a single nucleotide alteration in the isolate from *P. kessleri*). On the other hand, the *Neogobius*-associated samples were clearly distinct, belonging to another haplogroup. These findings confirm the broad distribution and host range of *L. acerinae*, but also indicate that certain patterns of host-driven intraspecific

Table 2. Nucleotide sequence identity and genetic distance of RNA-polymerase II largest subunit gene (*rpb1*) of *Loma acerinae* isolated from different hosts and localities. Haplogroup I: #8–13; Haplogroup II: #1–7

#	Host*	Locality	Genbank accession #	Sequence identity (%, left-lower cells) and genetic distance (right-upper cells)**												
				1	2	3	4	5	6	7	8	9	10	11	12	13
1	*Gymnocephalus cernuus* 1	Baltic Sea	MN335641	ID	0.000	0.002	0.000	0.000	0.000	0.000	0.012	0.012	0.012	0.012	0.013	0.013
2	*G. cernuus* 2		–	100	ID	0.002	0.000	0.000	0.000	0.000	0.012	0.012	0.012	0.012	0.013	0.013
3	*Ponticola kessleri*	Caspian Sea	MN335646	99.8	99.8	ID	0.002	0.002	0.002	0.002	0.013	0.013	0.013	0.013	0.015	0.015
4	*Atherina boyeri*	Azov Sea	MN335645	100	100	99.8	ID	0.000	0.000	0.000	0.012	0.012	0.012	0.012	0.013	0.013
5	*Zosterisessor ophiocephalus* 1	Black Sea	MN335642	100	100	99.8	100	ID	0.000	0.000	0.012	0.012	0.012	0.012	0.013	0.013
6	*Z. ophiocephalus* 2		–	100	100	99.8	100	100	ID	0.000	0.012	0.012	0.012	0.012	0.013	0.013
7	*Z. ophiocephalus* 3		–	100	100	99.8	100	100	100	ID	0.012	0.012	0.012	0.012	0.013	0.013
8	*Neogobius fluviatilis* 1		MN335643	98.8	98.8	98.6	98.8	98.8	98.8	98.8	ID	0.000	0.000	0.000	0.002	0.002
9	*N. fluviatilis* 2		–	98.8	98.8	98.6	98.8	98.8	98.8	98.8	100	ID	0.000	0.000	0.002	0.002
10	*N. fluviatilis* 3		–	98.8	98.8	98.6	98.8	98.8	98.8	98.8	100	100	ID	0.000	0.002	0.002
11	*N. fluviatilis* 4		–	98.8	98.8	98.6	98.8	98.8	98.8	98.8	100	100	100	ID	0.002	0.002
12	*N. melanostomus* 1		MN335644	98.6	98.6	98.5	98.6	98.6	98.6	98.6	99.8	99.8	99.8	99.8	ID	0.000
13	*N. melanostomus* 2		–	98.6	98.6	98.5	98.6	98.6	98.6	98.6	99.8	99.8	99.8	99.8	100	ID

*Numbers indicate microsporidium sequences from individual fish samples.
**ID – identical entries

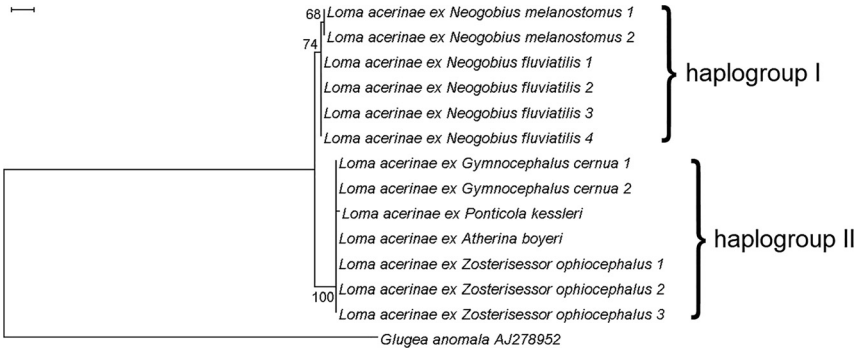

Fig. 2. Phylogenetic reconstruction of *Loma acerinae* samples based on the 686-bp-long alignment of the largest subunit RNA polymerase II gene (*rpb1*). Values at branches indicate bootstrap support. *Glugea anomala* was used as an outgroup. Scale bar = 0.01 expected changes per site

polymorphism of the parasite may exist. Further studies are needed to elucidate the biological meaning of the genetic divergence between *Neogobius*-associated samples and those from other host species.

ACKNOWLEDGEMENTS

The research was supported by the Ministry of Science and Higher Education of the Russian Federation, projects ## AAAA-A18-118020890074-2 & AAAA-A16-116080510096-7. The research was performed using the equipment of the Core Centrum 'Genomic Technologies, Proteomics and Cell Biology' at the All-Russian Institute of Agricultural Microbiology and the Core Centrum 'Innovative Plant Protection Technologies' at the All-Russian Institute of Plant Protection (St. Petersburg, Russia).

REFERENCES

Cheney, S. A., Lafranchi-Tristem, N. J., Bourges, D. and Canning, E. U. (2001): Relationships of microsporidian genera, with emphasis on the polysporous genera, revealed by sequences of the largest subunit of RNA polymerase II (RPB1). J. Eukaryot. Microbiol. **48,** 111–117.

Grushevaya, I. V., Ignatieva, A. N., Malysh, S. M., Senderskiy, I. V., Zubarev, I. V. and Kononchuk, A. G. (2018): Spore dimorphism in *Nosema pyrausta* (Microsporidia, Nosematidae): from morphological evidence to molecular genetic verification. Acta Protozool. **57,** 49–52.

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

Issi, I. V. (2020): Development of microsporidiology in Russia [in Russian]. Plant Prot. News **103**, 161–176.

Issi, I. V., Tokarev, Y. S., Voronin, V. N., Seliverstova, E. V., Pavlova, O. A., and Dolgikh, V. V. (2010): Ultrastructure and molecular phylogeny of *Mrazekia macrocyclopis* sp.n. (Microsporidia, Mrazekiidae), a microsporidian parasite of *Macrocyclops albidus* (Jur.) (Crustacea, Copepoda). Acta Protozool. **49,** 75–84.

Jenkins, J. A., Bart, Jr., H. L., Bowker, J. D., Bowser, P. R., MacMillan, J. R., Nickum, J. G., Rose, J. D., Sorenson, P. W., Whitledge, G. W., Rachlin, J. W., Warkentine B. E. and Bart, H. L. (2014): Guidelines for Use of Fishes in Research. American Fisheries Society, Bethesda, MD.

Jirovec, O. (1930): Über eine neue Microsporidienart (*Glugea acerinae* n.sp.) aus *Acerina cernua*. Archiv für Protistenkunde **72**, 198–213.

Kumar, S., Stecher, G. and Tamura, K. (2016): MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol. Biol. Evol. **33,** 1870–1874.

Lom, J. and Dyková, I. (eds) (1992): Protozoan parasites of fishes. In: Developments in Aquaculture and Fisheries Science, **Vol. 26**. Elsevier, Amsterdam. p. 315.

Lom, J. and Pekkarinen, M. (1999): Ultrastructural observations on *Loma acerinae* (Jírovec, 1930) comb. nov. (phylum Microsporidia). Acta Protozool. **38,** 61–74.

Malysh, J. M., Kononchuk, A. G. and Frolov, A. N. (2019): Detection of microsporidia infecting beet webworm *Loxostege sticticalis* (Pyraloidea: crambidae) in European and Asian parts of Russia in 2006–2008. Plant Prot. News **100,** 45–51.

Malysh, J. M., Malysh, S. M., Kireeva, D. S., Kononchuk, A. G. and Demenkova, M. A. (2020): Detection of *Wolbachia* in larvae of *Loxostege sticticalis* (Pyraloidea: crambidae) in European and Asian parts of Russia. Plant Prot. News **103,** 49–52.

Malysh, J. M., Tokarev, Y. S., Sitnicova, N. V., Martemyanov, V. V., Frolov, A. N. and Issi, I. V. (2013): *Tubulinosema loxostegi* sp.n. (microsporidia: Tubulinosematidae) from the beet webworm *Loxostege sticticalis* L. (Lepidoptera: crambidae) in Western Siberia. Acta Protozool. **52,** 299–308.

Marshall, O. J. (2004): PerlPrimer: cross-platform, graphical primer design for standard, bisulphite and real-time PCR. Bioinformatics **20,** 2471–2472.

Ovcharenko, M., Wróblewski, P., Kvach, Y. and Drobiniak, O. (2017): Study of *Loma acerinae* (microsporidia) detected from three Ponto-caspian gobies (Gobiidae) in Ukraine. Parasitol. Res. **116,** 1453–1462.

Sokolova, Y. Y., Senderskiy, I. V. and Tokarev Y. S. (2016): Microsporidia *Alfvenia sibirica* sp. n. and *Agglomerata cladocera* (Pfeiffer) 1895, from Siberian microcrustaceans and phylogenetic relationships within the 'Aquatic outgroup' lineage of fresh water microsporidia. J. Invertebr. Pathol. **136,** 81–91.

Tokarev, Y. S., Huang, W. F., Solter, L. F., Malysh, J. M., Becnel, J. J. and Vossbrinck, C. R. (2020): A formal redefinition of the genera *Nosema* and *Vairimorpha* (Microsporidia: Nosematidae) and reassignment of species based on molecular phylogenetics. J. Invertebr. Pathol. **169,** 107279.

Tokarev, Y. S., Peat, K. M., Malysh, J. M. and Senderskiy, I. V. (2018a): 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., Vasilieva, A. A. and Issi, I. V. (2018b): Molecular and morphological characterization of *Anncaliia azovica* sp. n. (Microsporidia) infecting *Niphargogammarus intermedius* (Crustacea, Amphipoda) from the Azov Sea. J. Eukaryot. Microbiol. **65,** 296–307.

Tokarev, Y. S., Voronin, V. N., Seliverstova, E. V., Pavlova, O. A. and Issi I. V. (2010): Life cycle, ultrastructure and molecular phylogeny of *Crispospora chironomi* g.n. sp.n. (Microsporidia: Terresporidia), a microsporidian parasite of *Chironomus plumosus* L. (Diptera: chironomidae). Parasitol. Res. **107,** 1381–1389.

Vogelstein, B. and Gillespie, D. (1979): Preparative and analytical purification of DNA from agarose. Proc. Natl Acad. Sci. USA **76,** 615–619.

Weiss, L. H. and Vossbrinck, C. R. (1999): Molecular biology, molecular phylogeny, and molecular diagnostic approaches to the microsporidia. In: Wittner, M. and Weiss, L. M. (eds) The Microsporidia and Microsporidiosis. ASM Press, Washington. pp. 129–171.