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Crithidia dobrovolskii sp. n. (Kinetoplastida: Trypanosomatidae) from parasitoid fly *Lypha dubia* (Diptera: Tachinidae): morphology and phylogenetic position

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Summary

The article provides characteristics of a new parasite, *Crithidia dobrovolskii* sp.n., which was isolated from the tachinid fly captured in the Leningrad Region of Russia. The presented description of *Crithidia dobrovolskii* sp.n. is based upon light microscopic, ultrastructural, and molecular phylogenetic data. Molecular phylogenetic analyses of SSU rRNA gene and GAPDH gene sequences have demonstrated that the new species is most closely related to *Crithidia fasciculata*.

Key words: *Crithidia*, Trypanosomatidae, phylogeny, SSU rRNA, GAPDH, ultrastructure

Introduction

Flagellates belonging to the Trypanosomatidae family are widespread parasites of animals, plants and protists. Dixenous (i.e. "two-host") parasites from the genera *Trypanosoma* and *Leishmania*, the most well-known representatives of the group that are pathogens of humans and animals, have significant economic and medical importance. Until recently, monoxenous (i.e. "one-host") trypanosomatids, parasitizing mainly in representatives of two insect orders Diptera and Hemiptera, were overshadowed by dixenous species of the family. However, in recent years, interest in monoxenous trypanosomatids of insects has increased significantly. This fact is explained by the discovered high biodiversity of the group, a wide range of hosts (Podlipaev, 2001; Týč et al., 2013; Maslov et al., 2013), as well as the fact that it is monoxenous insect parasites that are now considered ancestral forms of all representatives of the family (Frolov, 2016). One of the most significant findings in the history of the family study was the discovery and description of the new genus *Paratrypanosoma*. Monoxenous flagellates *P. confusum*, found in the gut of culicid mosquitoes, are located at the base of the phylogenetic tree of trypanosomatids, occupying a position between free-living kinetoplastids and other species of the family Trypanosomatidae (Flegontov et al., 2013).

The results of numerous studies upon insect-parasitizing trypanosomatids obtained in the last decade entailed revision of many views on diversity, origin and entire system of the family Trypanosomatidae (Podlipaev, 2001; Teixeira et al., 2011;

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Flegontov et al., 2013; Kostygov et al., 2014; Votýpka et al., 2014). New species have been described and continue to be described (Yurchenko et al., 2014), as well as new taxa of a higher rank (genus and subfamily), for example, the genus *Jaenimonas* (Hamilton et al., 2015) and the genus *Kentonomas* (Votýpka et al., 2014), the subfamilies Phytomonadinae (Yurchenko et al., 2015) and Strigomonadinae (Teixeira et al., 2011).

Despite the growing interest in monoxenous parasites of flies, as representatives of one of the least studied groups of trypanosomatids, their fauna remains studied in a fragmented manner. At the same time, the geography of the research is extremely heterogeneous and tends to study the parasite fauna in the tropics and subtropics zones.

The present work was carried out in the context of investigation of the diversity of trypanosomatids parasitizing dipteran insects in the north of Eurasia. The article provides description of a new species *Crithidia dobrovolskii* sp.n. from the intestines of the fly *Lypha dubia* Fall. (Diptera: Tachinidae).

Material and methods

Field work, insect's dissection and cultivation of trypanosomatids

The collection of insects was carried out in June 2018 on a field near the village Vysokoklyuchevskoy of the Leningrad Region, Russia (59°47'N, 30°08'E). The insects were euthanized in chloroform and dissected in a drop of physiological saline after that the intestines were isolated. Fragments of the intestine and its contents were studied for infection. Intestinal fragments infected with trypanosomatids were placed in tubes with Brain Heart Infusion culture medium (BHI, Difco) supplemented with hemin (25 μg/ml), benzylpenicillin antibiotics at a rate of 500 units/ml and streptomycin 500 μg / ml. Subsequently, the culture of flagellates was purified from related organisms in M-shaped tubes (Podlipaev and Frolov, 1987).

Light microscopy

The cells morphology was studied using vital slides and dry smears. Dry smears made from fragments of infected intestine or a drop of culture were fixed for 10 minutes in 96% ethanol, after which Giemsa staining was performed for 25 minutes. To visualize DNA containing trypanosomatid structures, DAPI staining with fluorescent dye was used according to the methods described previously (Ganyukova et al., 2017). The slides were studied using a Leica DM 2500 microscope. Microphotographs were obtained using a 14 Mps USB camera UCM0S14000KPA (TOUPCAM). Flagellate cell sizes were measured using the Toup View software (version 3.7, 2013). Statistical data processing was carried out in the LibreOffice Calc program. The statistical significance of differences in the average values of morphological characteristics was evaluated using Student's t criterion (n=25) (significance level of $P<0.01$).

Transmission and scanning electron microscopy

For electron microscope studies, the flagellate culture, previously purified from concomitant organisms, was precipitated by centrifugation (3000 rpm), the supernatant was discarded, and the sediment was fixed with 1.5 % glutaraldehyde in 0.1 M cacodylate buffer (1 hour), after which the cells washed in a 0.1 M solution of cacodylate buffer, followed by postfixation with a 2 % solution of OsO_4 in cacodylate buffer for 30 minutes. Then the material was dehydrated in alcohols of increasing concentration and acetone and embedded in a mixture of Araldite and Epon. Sections were obtained on a Leica UC-6 ultramicrotome, stained with an aqueous solution of uranyl acetate and lead citrate and examined under a Morgagni 268-D microscope (FEI Company).

For scanning electron microscopy, the fixed and dehydrated material deposited on poly-L-lysine-coated coverslips was processed in an HCP-2 critical point dryer (Hitachi Ltd., Tokyo, Japan) and after coating with a 20 nm platinum layer in an IB-5 coating device Ion (Giko Co. Ltd., Tokyo, Japan) was examined under a Tescan Mira3 LMU microscope with accelerating voltage of 25.00 kV.

DNA isolation, amplification and sequencing

Genomic DNA was isolated from cell culture and alcohol-fixed insect tissues using the PureLink Genomic DNA Kit (Invitrogen). The host COI gene fragment was amplified using LCOI and HCOI primers according to the previously described protocols (Cywinska et al., 2010). Fragments of the 18S and GAPDH genes were amplified using specific primers S762-763 and M200-M201, respectively

(Kostygov et al., 2014). The amplification protocol and the composition of the reaction mixture are described earlier (Kostygov and Frolov, 2007). The resulting PCR fragments were isolated from the reaction mix and purified using a Cleanup Standard kit (Eurogen) and sequenced on a 3500 xL Applied Biosystems automated sequencer using Thermo Sequenase Cy5 Dye Terminator Kit.

Phylogenetic analyses

The alignment of SSU rRNA gene sequences of trypanosomatids, representing known phylogroups of these flagellates was prepared as described before using the software package MEGA (Molecular Evolutionary Genetic Analysis Version 5.05, Tamura et al., 2011, www.megasoftware.net) (Schwarz et al., 2014). The final file included 35 sequences of the 18S rRNA gene of trypanosomatids. The number of positions in the final file with indices was 2174. Phylogenetic analysis of the sequences was performed by the Bayesian algorithm in MrBayes 3.2.7 program using the GTR+G+I model with the following parameters: 2 runs, 4 Markov chains, 3 million generations, sampling every 100 generations, removal of the first 8 thousand trees when building consensus.

Results

We have discovered *C. dobrovolskii* infection in a fly rectum identified as *Lypha dubia* (Diptera: Tachinidae) on the basis of the COI gene sequence. The axenic culture of flagellates that is maintained on BHI culture medium with hemin is deposited in the cell culture collection of the ZIN RAS (Zoological Institute of the Russian Academy of Sciences) under the C23 code.

The SSU rRNA and GAPDH gene sequences of the isolate we obtained have no analogues in existing databases containing trypanosomatid gene sequences. BLAST search against the sequences in the GenBank shows that the new species differs from *C. fasciculata* by three positions in the SSU rRNA gene and four positions of the gGAPDH.

In the phylogenetic tree, based on the analysis of the SSU rRNA gene, the new species is positioned within the infrafamily Crithidiatae (Kostygov and Yurchenko, 2017) and is closest to the type species of the genus *C. fasciculata*. These species form a common branch with high supports along with *C. otongatchiensis, C. brachyflagelli, Leptomonas tarcoles* and *L. acus* (Fig. 1). Summing it up, the sequence analysis demonstrated that the studied trypanosomatid *C. dobrovolskii* should represent a separate species.

Table 1. Morphometry of *Crithidia dobrovolskii* in the laboratory culture.

Parameters	Promastigotes	Paramastigotes
Cell length (μm)	7,74 ±0,86 (6,39-9,18)	7,58 ±0,92 (6,25-9,03)
Cell width (μm)	2,97 ±0,37 (2,47-3,72)	2,91 ±0,31 (2,39-3,63)
Free flagellum length (μm)	8,12 ±1,12 (6,33-10,19)	7,95 ±1,05 (6,28-10,10)
Nucleus length (μm)	2,61 ±0,32 (2,12-2,78)	2,55 ±0,37 (2,21-2,74)
Nucleus width (μm)	2,36 ±0,13 (2,12-2,56)	2,26 ±0,23 (2,13-2,43)
Anterior end to nucleus distance (μm)	2,45 ±0,49 (1,75-4,02)	2,73 ±0,40 (1,90-3,08)
Anterior end to kinetoplast distance (μm)	1,49 ±0,45 (0,68-2,13)	2,81 ±0,52 (1,95-3,19)

The SSU rRNA and GAPDH *C. doborvolskii* are deposited to the GenBank system under accession numbers MN809265 and MN807630 respectively.

Flagellates are presented in culture by two main morphotypes: promastigotes and paramastigotes (Fig. 2A). In promastigote cells kinetoplast is situated in the anterior third of the cell; the nucleus is displaced to its middle. The average length of such cells is 7.74±0.86 μm, the flagellum is well defined, and its length reaches an average of 8.12±1.12 μm. The nucleus of the paramastigote is situated in the middle of the cell or can be displaced to the posterior end. However, the kinetoplast is situated at the level of the nucleus to its posterior edge. The average length of promastigotes reaches 7.58±0.92 μm, the length of the flagellum — 7.95±1.05 μm. Other morphometric parameters are indicated in Table 1. Meanwhile, of all the analyzed characters, promastigotes and paramastigotes significantly differ only in the distance from the anterior end of their cells to the kinetoplast (at the accepted significance level $P < 0.01$).

Romanowsky-Giemsa staining of the smears (Fig. 2, A) and the use of the fluorescent DAPI staining (Fig. 2, B) showed that *C. doborvolskii* cells do not possess any DNA-containing structures besides the nucleus and kinetoplast inside their cytoplasm.

Ultrastructure of *C. dobrovolskii* is similar to the most of studied *Crithidia* species. Cells are of a slightly elongated shape (Fig. 2, C) having deep flagellar pocket. Glycosomes are present in the middle part

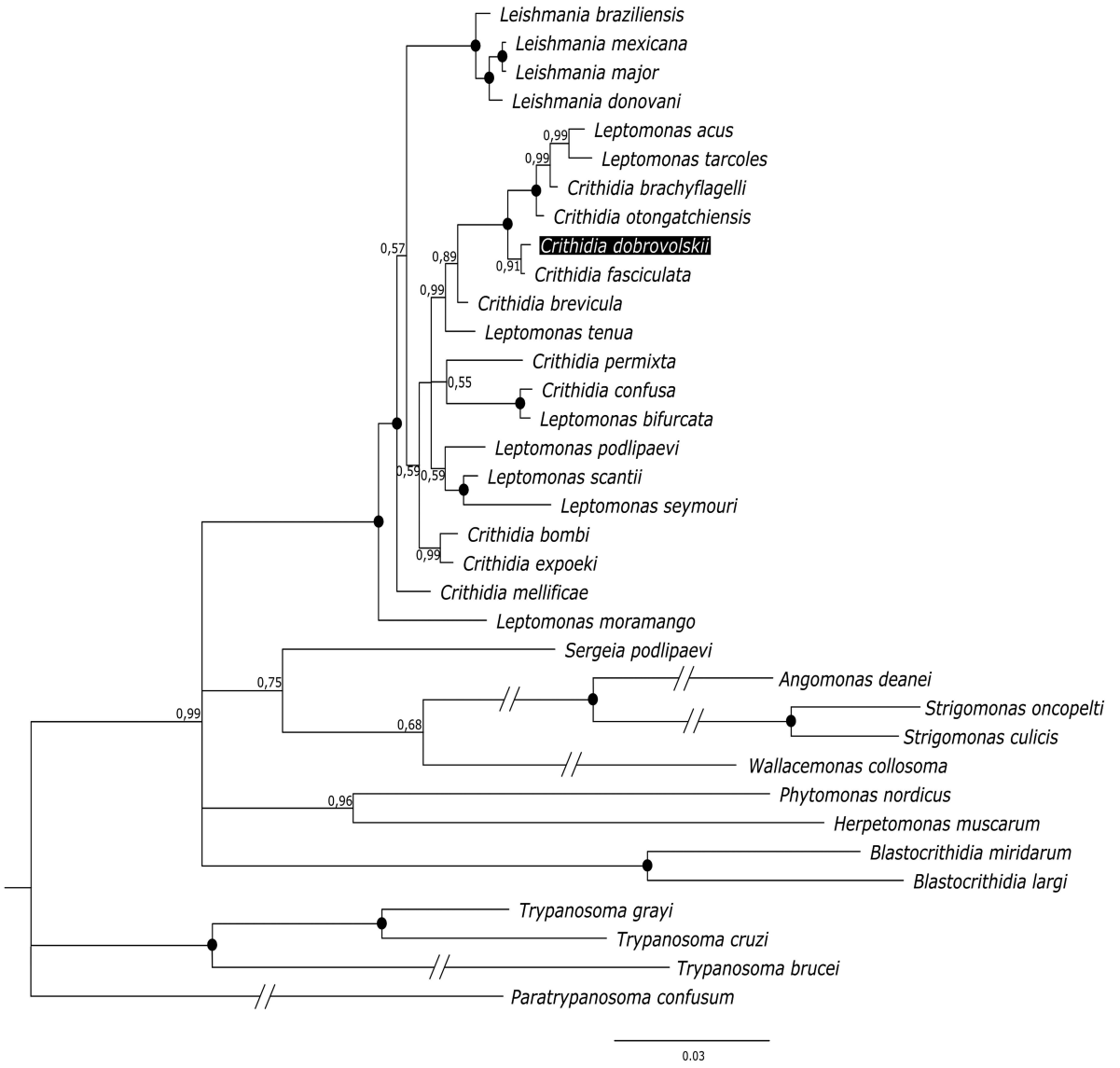
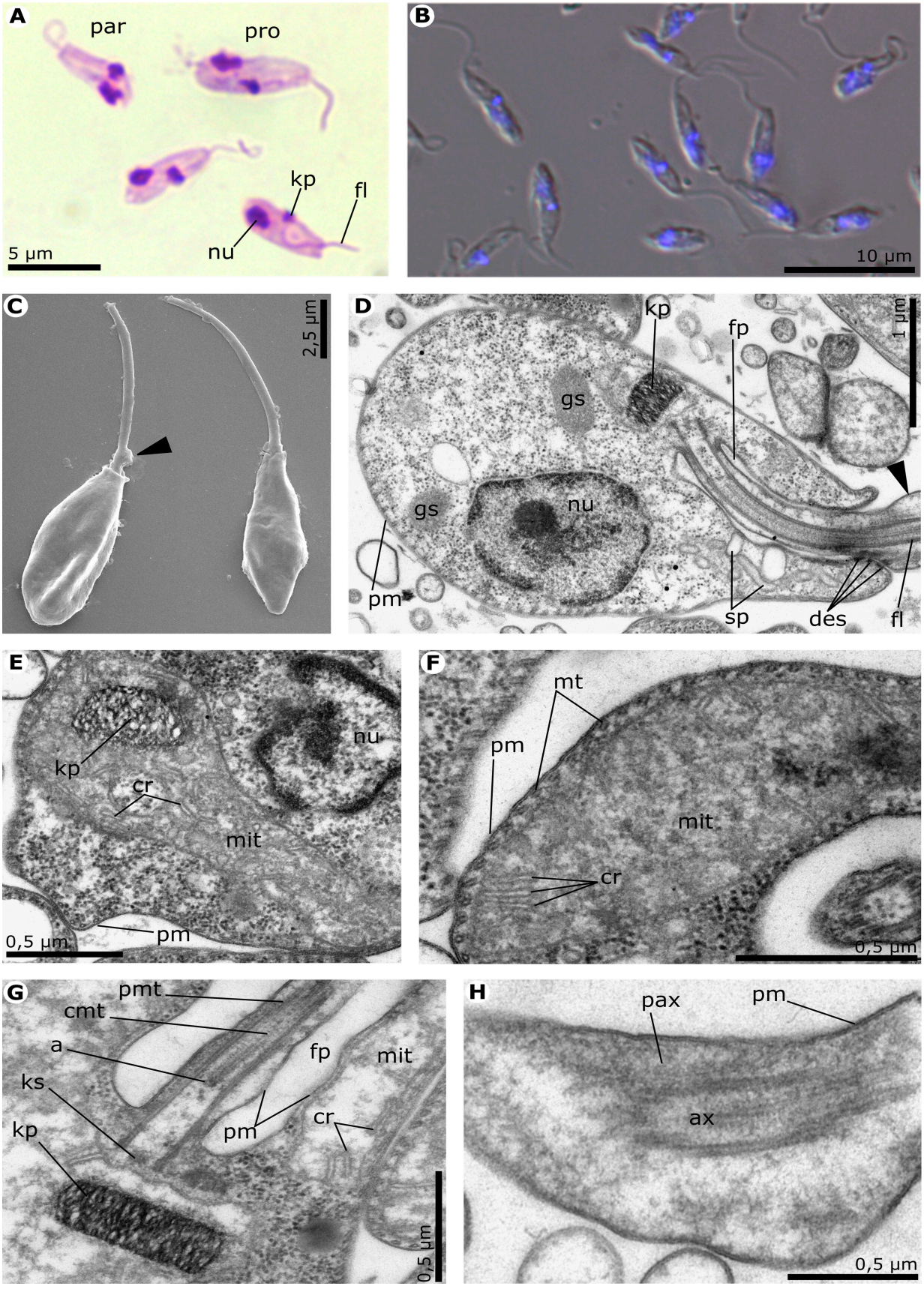


Fig. 1. Bayesian phylogenetic tree reconstructed using 18S ribosomal RNA gene sequences. Numbers at nodes indicate posterior probabilities. Nodes having 1.0 posterior probability support are marked with black circles. Double-crossed branches are at 50% of their original lengths. The tree is rooted with the sequence of *Paratrypanosoma confusum*. The scale bar denotes the number of substitutions per site. The species under study (*Crithidia dobrovolskii*) is highlighted. Accession numbers of the sequences retrieved from GenBank are available are available upon request.

of the cell located at the nucleus level (Fig. 2, D). Spongiome localized in the front part of the cell beside the flagellar pocket (Fig. 2, D). Outside flagellates are covered with plasmalemma that lines the flagellar pocket and passes to the flagellum. Desmosome-like contacts between the flagellar membranes and flagellar pocket are distinguishable at the flagellar pocket exit (Fig. 2, D). Directly outside the flagellar pocket, the undulipodium slightly widens (Fig. 2, C, D). The paraxial rod is well developed (Fig. 2, H). Kinetosomes are located at the front edge of the kinetoplast. The axial granule is well defined in the transition zone of flagellum (Fig. 2, G). There is a layer of longitudinally oriented regular submembrane microtubules directly beneath the plasmalemma (Fig. 2, F). Mitochondrion is weakly branched; particular branches can form saccular bulgings with many plate-like cristae (Fig. 2, E, F, G). The kinetoplast is wide (Fig. 2, E), ribbons of DNA rings are parallel to the longitudinal axis of cell (Fig. 2, D, E, G).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Discussion

At the beginning of the last century Louis Leger described flagellates *Crithidia fasciculata* from the gut of mosquitoes *Anopheles maculipennis* in France, placing this species in a new genus *Crithidia* (Leger, 1902). In accordance with Leger's drawings and the accompanying description these trypanosomatids were represented by two cell morphotypes: short barleycorn-shaped cells with a wide flagellar pocket, and elongated cells with a distinct undulating membrane (Leger, 1902). Only by the middle of the twentieth century did it become clear that the description made by Leger was mistakenly based on the study of mixed invasion of mosquitoes by parasites of at least two different trypanosomatid genera (Laird, 1959; Hoare and Wallace, 1966). Henceforth, the genus name *Crithidia* was assigned to the small flagellates with a terminally opened wide flagellar pocket, and trypanosomatids with an undulating membrane were placed in the genus *Blastocrithidia* (Laird, 1959; Wallace, 1966; Podlipaev, 1990). It is noteworthy that in the pre-molecular period the majority of small trypanosomatids with a morphotype called "choanomastigote" were attributed to the genus *Crithidia*. Moreover, the definition of this term did not have clear boundaries and was interpreted by researchers quite widely and arbitrarily. By 1990, the genus *Crithidia* included 28 species of flagellates from the Old and New Worlds. Out of these, 13 species were described from the intestines of Hemiptera, 10 species from Diptera, single species were also described from the intestines of Hymenoptera and Trichoptera insects (Podlipaev, 1990). However, the widespread use of molecular biology and genetics methods in the studies of trypanosomatids over the past two decades has led to the fact that the ideas about the biological diversity of trypanosomatids and the principles of their systematics have radically changed. Most of trypanosomatid genera turned out to be polyphyletic in their previous composition (Merzlyak et al., 2001; Maslov et al., 2013), and the morphological features used in the diagnoses of various trypanosomatid taxa were seriously compromised (Yurchenko et al., 2008). Flagellates of the genus *Crithidia* did not escape this fate, some of which turned out to be species of other genera and even other subfamilies of trypanosomatids, such as *Angomonas* (*Crithidia*) *deanei* and *Strigomonas* (*Crithidia*) *oncopelti* (Teixeira et al., 2011). Another part on the contrary was transferred to the genus *Crithidia* from other taxa (Kostygov et al., 2014). At present, the composition of the genus *Crithidia* is not obvious, since a significant part of the species described in the pre-molecular era and traditionally included in its composition is still not verified by molecular genetic methods. Although in the infrafamily Crithidiatae (Kostygov and Yurchenko, 2017) phylogenetic relations are resolved very weakly, the only reliable method that allows us to determine whether a flagellate belongs to their particular genus is molecular barcoding. It was this consideration that guided us in identifying the new species.

According to our results *C. dobrovolskii* nests within the subclade of infrafamily Crithidiatae with the high level of statistical support. In the phylogenetic tree *C. dobrovolskii* forms a relatively isolated branch within the infrafamily, which also includes *C. fasciculata, C. otognatiensis, C. brachyflagelli*, *L. tarcoles* and *L. acus*. The closest described relative of *C. dobrovolskii* is the type species of the genus *C. fasciculata*. The sequence identity of these species is 99.86% for the SSU gene and 99.55% for gGAPDH.

A high percentage of identity is easily explained by the conservatism of these molecular markers. 18S rRNA gene is the golden standard for the studies of trypanosomatid diversity, but today there is no generally accepted threshold of species identity for these markers. Nevertheless, *Blastocrithidia papi* and

Fig. 2. Light (A, B), scanning (C), and transmission (D– H) electron microscopy of *Crithidia dobrovolskii* sp. n. (isolate C23). A – Giemsa-stained cells in culture; B – combination of DIC and fluorescence microscopy (DAPI-stained) of cells in culture; C – scanning electron microscopy demonstrates cells in culture (the *arrow* indicates the expanded part of the base of the flagellum); D– longitudinal section of paramastigote (the *arrow* indicates the expanded part of the base of the flagellum); E – cross section of kinetoplast; F – cross section on the level of flagellar pocket filled with large mitochondria; G – longitudinal sections in the base of the flagellum pocket; H – longitudinal sections through the free part of flagellum. *Abbreviations:* a – axial granule; ax – axoneme; cmt - central microtubules of axoneme; cr – plate-like cristae; des – desmosome-like contacts; fl – flagellum; fp – flagellar pocket; gs – glycosome; kp – kinetoplast; ks – kinetosome; mit – mitochondrion; mt – microtubule; nu – nucleus; par – paramastigote; pax – paraxial rod; pm - plasmatic membrane; pmt – peripheral microtubules of axoneme; pro – promastigote; sp – spongiome.

B. largi also differ only in two nucleotide positions of 18S rRNA gene, but demonstrating 20 % differences in SL RNA gene. Status of species difference is confirmed by their morphological features (Frolov et al., 2017). Therefore, we consider that the differences in the presented sequences are sufficient to define a new species.

Usually the term "choanomastigotes" is used for describing morphotypes in species that are positioned in a clade including *C. fasciculata*. However, as mentioned above, this term has a rather broad interpretation and usually characterizes small rounded cells with a wide flagellar pocket. Choanomastigots of *C. fasiculata* have a length of 6 to 8 μm (Hoare and Wallace, 1966). Cells of *L. tarcoles, C. brachyflagelli* and *C. otongatchiensis* also have small sizes, their length varies from 8 to 9 μm (Yurchenko et al., 2008; Jirků et al., 2012; Yurchenko et al., 2014). Thus, *C. dobrovolskii* has the size that fits into the size inherent in "good crithidia". However, another representative of the cluster, *L. acus*, differs significantly from these species in considerably larger size: the average length of large promastigotes is 21.7 μm and it ranges from 8.4 to 40.4 μm (Yurchenko et al., 2008). This fact once again indicates the unreliability of morphological criteria in the description of suprаspecific trypanosomatids taxa.

Species within the considered clade possess the characteristic features inherent in all trypanosomatid cells, such as electron-dense kinetoplast, oval nucleus, single flagellum, a single peripherally located reticulated mitochondrion, conspicuous paraxonemal rod and a complete corset of regularly spaced subpellicular microtubules. However, at the ultrastructural level, features characteristic for the individual strains were also found. Mitochondrion of *C. dobrovolskii* is very large, forms short but extensive outgrowths with many distinct cristae. A similar developed saccular mitochondrion can be observed in *C. otongatchiensis* cells. This fact may indicate that cells of these two species may need more ATP per cell to maintain their metabolism (Yurchenko et al., 2014).

Within the considered phylogenetic branch there is no uniformity with respect to insect hosts of flagellates. The type species *C. fasciculata* is a widespread intestine parasite of the larvae and imagoes of the family Culicidae (Diptera: Nematocera) and is characterized by transphase transmission (Clark et al., 1964; McGhee and Cosgrove, 1980), however, is not noted outside this group of mosquitoes. *L. tarcoles, L. acus*, and *C. brachyflagelli* were originally described from various plant bugs of the family Miridae (Yurchenko et al., 2008; Jirků et al., 2012). *C. otognatiensis* was isolated from the midgut of the nectar-consuming fly *Eristalis* sp. (Diptera: Brachycera: Syrphidae), localizing inside the peritrophic matrix (Yurchenko et al., 2014). However, it is noteworthy that the isolate G15 (TU83) from the hindgut of *Rhynocoris rapax* (Heteroptera: Reduviidae) (Votýpka et al., 2012) is a close relative of *C. otongatchiensis*.

The new species *C. dobrovolskii* described in the article was isolated from the intestine of the imago of the parasitoid fly *Lupha dubia* (Diptera: Brachycera: Tachinidae). Adult flies of the family Tachinides are nectar-consuming, larvae develop in the caterpillars of the families Geometridae and Tortricidae (Coppel 1947; Cheng, 1949; O'Hara, 2002). Today, this is the first case of trypanosomatid infection discovered in this family of flies.

Critidia have wide hostal radiation: they were found in the heteropterans of the family Miridae and Nematocera of the family Culicidae, as well as in two families of flies: the Syrphidae and the Tachinidae. Moreover, probably many of them may have inherent wide hostal specificity. Thus, another species of monoxenous trypanosomatids, *C. brevicula*, close to the branch including *C. fasiculata,* exhibits wide hostal radiation, parasitizing the bugs of the families Miridae, Gerridae, and Nabidae (Malysheva and Frolov, 1989; Podlipaev, 1990; Kostygov et al., 2014), and also in mosquitoes of the family Culicidae (Svobodova et al., 2015) and several species of flies (Ganyukova et al., 2018).

It is likely that further studies of the biology of these flagellates will allow us to expand our modern understanding of the composition, evolution, distribution strategies, and hostal radiation of the group.

Taxonomic summary

Class Kinetoplastea (Honigberg, 1963) Vickerman, 1976

Subclass Metakinetoplastina Vickerman, 2004

Order Trypanosomatida (Kent, 1880) Hollande, 1952

Family Trypanosomatidae (Doflein, 1901) Grobben, 1905

Genus *Crithidia* Leger, 1902

Crithidia dobrovolskii Ganyukova et Frolov sp. n.

Diagnosis: cells are represented by ob-long promastigotes and paramastigotes with wide flagellar

pockets ("choanomastigotes"). The sizes of motile cells vary within the following ranges: 6.39–9.18 μm length, 2.39–3.72 μm width, 6.28–10.19 μm length of the free part of the flagellum. This species can be identified by the 18S rRNA (MN809265) and GAPDH (MN807630) gene sequences.

Type host: *Lypha dubia* (Diptera: Tachinidae).

Site: rectum.

Type locality: field near Vysokokluchevskoi village, Leningrad region, Russia (59°47'N, 30°08'E).

Type material: Xenotype, hapanotype and axenic culture (C23) are deposited in the research collection of the laboratory of Protozoology of the Zoological Institute of the Russian Academy of Sciences.

Etymology: the name *Crithidia dobrovolskii* is given in honor of the recently passed away outstanding Soviet and Russian zoologist, parasitologist, pedagogue and teacher of the article authors, Andrej A. Dobrovolskij.

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