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Molecular revision of the genus *Wallaceina*

Alexei Yu. Kostygov[a], Anastasiia Grybchuk-Ieremenko[b], Marina N. Malysheva[a], Alexander O. Frolov[a], and Vyacheslav Yurchenko[b,c,1]

[a]Zoological Institute of the Russian Academy of Sciences, Universitetskaya nab. 1, St. Petersburg, 199034, Russia
[b]Life Science Research Centre, Faculty of Science, University of Ostrava, Chittussiho 10, 710 00 Ostrava, Czech Republic
[c]Biology Centre, Institute of Parasitology, Czech Academy of Sciences, Branišovská 31, 370 05 České Budějovice (Budweis), Czech Republic

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This work is focused on the molecular revision of the genus *Wallaceina* established in the very twilight of the classical morphotype-based approach to classification of the Trypanosomatidae. The genus was erected due to the presence of a unique variant of endomastigotes. In molecular phylogenetic studies four described species of *Wallaceina* were shown to be extremely close to each other and to some other undescribed isolates clustered within Leishmaniinae clade, while three recently included species formed a separate clade. Our results of morphological and molecular phylogenetic analyses demonstrated that all Leishmaniinae-bound wallaceinas are just different isolates of the same species that we rename back to *Crithidia brevicula* Frolov, Malysheva, 1989. To accommodate former *Wallaceina* spp. phylogenetically distant from the genus *Crithidia*, we propose a new generic name *Wallacemonas* Kostygov et Yurchenko, 2014.
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Key words: Trypanosomatidae; molecular phylogeny; Leishmaniinae; *Wallaceina*; *Wallacemonas*.

Introduction

Flagellates of the family Trypanosomatidae parasitize vertebrates (including humans), plants and invertebrates (Podlipaev 1990). Dixenous (= two hosts) parasites spend a part of their developmental cycle in an invertebrate vector and a part within a vertebrate (genera *Trypanosoma* and *Leishmania*) or plant (genus *Phytomonas*) host. The vast majority of monoxenous (= one host) Trypanosomatidae parasitize insects. Only recently the monoxenous species have started drawing a lot of research attention due to the extreme biological diversity, adaptability to various environmental conditions, pervasiveness, and impact on their insect hosts (Borghesan et al. 2013; Jirků et al. 2012; Maslov et al. 2013; Votýpka et al. 2013). Importantly, all Trypanosomatidae possess some peculiarities that are of special interest for general biology: kinetoplast, RNA editing, polycistronic transcription of most protein-coding genes, trans-splicing, and others (Lukeš et al. 2002; Michaeli 2011).

Traditionally, the genus-level classification of trypanosomatids was based on cell morphology (e.g. presence of specific cell morphotypes),

[1]Corresponding author; fax +420 596120478
e-mail vyacheslav.yurchenko@osu.cz (V. Yurchenko).

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and life cycle properties (e.g. monoxenous versus dixenous, and nature of the secondary host) (Vickerman 1976). The morphotypes were usually defined by the relative positioning of the kinetoplast, nucleus and the flagellar pocket, and the presence or absence of a single flagellum (Hoare and Wallace 1966). Introduction of the molecular methods to taxonomy quickly demonstrated that the traditional classification of the family is inconsistent with its phylogeny and some genera proved to be polyphyletic, while all three dixenous genera *Trypanosoma*, *Leishmania*, and *Phytomonas* remained monophyletic (Hamilton et al. 2004; Yurchenko et al. 2006). This led to negation of the traditional principles of trypanosomatid taxonomy and intensive revision of the family using a molecular phylogenetic approach (Jirků et al. 2012; Votýpka et al. 2010). Thus, the new genera *Sergeia*, *Paratrypanosoma* and *Blechomonas* were erected (Flegontov et al. 2013; Svobodová et al. 2007; Votýpka et al. 2013), the provisional nominal taxa *Angomonas* and *Strigomonas* were validated (Teixeira et al. 2011), and two traditional genera of monoxenous trypanosomatids, *Herpetomonas* and *Wallaceina*, were redefined (Borghesan et al. 2013; Yurchenko et al. 2014). However this process is far from being finished and there are still many groups within the family that should be revised.

This work is focused on the molecular revision of the genus *Wallaceina*. It was established in 1990, in the very twilight of the classical approach to classification of the Trypanosomatidae family. Primarily this taxon was described under the name *Proteomonas*, which turned out to be preoccupied by a cryptophyte alga (Podlipaev et al. 1990). It was subsequently renamed *Wallaceina* (Bulat et al. 1999). The genus was erected to adopt the newly described species *W. inconstans* along with the previously characterized *Crithidia brevicula* (Frolov and Malysheva 1989). All these species were morphologically different from representatives of the genus *Crithidia* since wallaceinas had endomastigotes, a morphotype not usually recognized in crithidias. Moreover the new genus had a completely unique character: in some endomastigotes the flagellum formed a loop around the nucleus. Motile cells of *Wallaceina* spp. are either typical promastigotes or choanomastigote-like cells with a continuum of intermediate forms.

The established taxonomy of this group is rather confusing. Over the years a few isolates attributed to several described species were shown to have phylogenetic affinity to *Wallaceina*. All these cases were proven to originate from mixed infections of wallaceinas with either *Blastocrithidia* spp. (*B. miridarum*, *B. gerricola* - isolates BM-33, and KV-1, respectively) or *Leptomonas peterhoffi* (isolate 101). Morphotypes predominant in the host were ultimately substituted by wallaceinas better adapted to cultivation (Kostygov et al. 2011; Malysheva and Frolov 2009).

At present, this polyphyletic genus contains seven described species of monoxenous trypanosomatids: *Wallaceina brevicula* Frolov et Malysheva, 1989, *W. inconstans* Podlipaev, Frolov et Kolesnikov, 1990, *W. vicina* Malysheva et Frolov, 2004, *W. podlipaevi* Malysheva et Frolov, 2009 (all above are from the subfamily Leishmaniinae), and *W. collosoma* Wallace, Clark, Dyer et Collins, 1960, *W. rigida* Podlipaev, Malysheva et Kolesnikov, 1991, and *W. raviniae* Votýpka et Lukeš, 2014 from the non-Leishmaniinae clade. Since the phylogenetic position of the first four species had been evaluated using replicas of the corresponding type cultures, it was recently proposed that their affinity to Leishmaniinae subfamily could be artifactual (Yurchenko et al. 2014).

To clarify this rather confusing situation we performed molecular phylogenetic analysis of all the available isolates with phylogenetic affinity to Leishmaniinae-bound *Wallaceina* spp. We demonstrated that all of them are just different isolates of the same species that we are proposing to rename back to its original name *Crithidia brevicula* Frolov, Malysheva, 1989. Here we also propose to discontinue using the generic name *Wallaceina* and transfer the species recently attributed to it (*W. collosoma*, *W. rigida*, and *W. raviniae*) to the newly erected genus *Wallacemonas*.

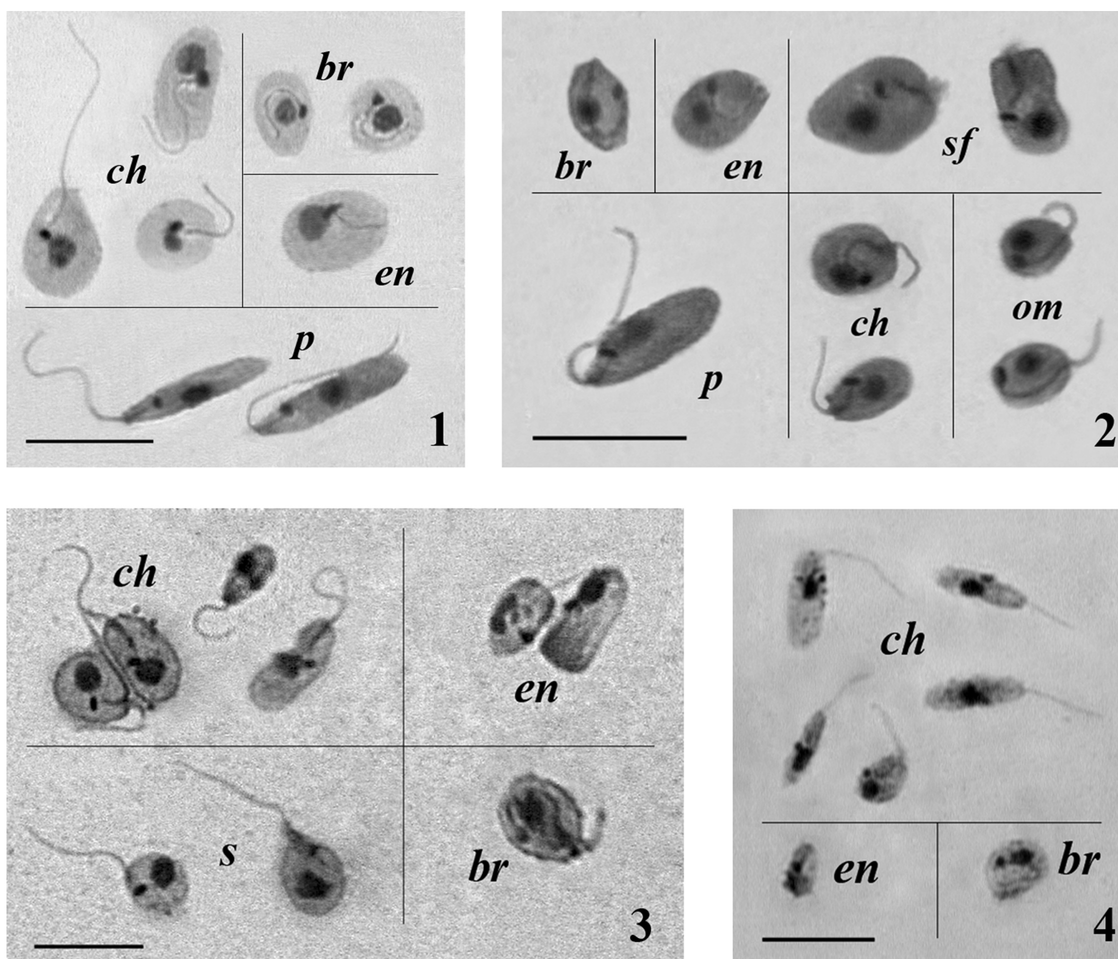
Results

All isolates used in the current study are original cultures established in the laboratory of Protozoology of the Zoological Institute, St. Petersburg, Russia. Their identity was confirmed by light microscopy.

Morphology of Cells in Laboratory Cultures

Formally described *W. brevicula* (isolate Nbr), *W. inconstans* (isolate ZK), *W. vicina* (isolate Wg), *W. podlipaevi* (isolate 101) were minutely studied in a series of articles published before (Frolov and Malysheva 1989; Malysheva and Frolov 2004, 2009; Podlipaev et al. 1990).

The following morphotypes were documented for *Wallaceina* spp.: promastigotes, choanomastigotes, and endomastigotes. A subset of



Figures 1 – 4. Morphology of the cultured cells of the studied isolates on Giemsa-stained slides. **1** - "F" series, **2** - CL8; **3** - KV-1, and **4** - BM-33. ch — choanomastigotes; br — brochomastigotes; en — common endomastigotes; p — promastigotes; sf — cells with shortened flagella; s — spheromastigotes; om — opisthomorphs. Scale 10 μm.

endomastigotes had a specific trait – a loop-like flagellum surrounding the nucleus. This morphotype was considered to be genus specific for *Wallaceina* and was included in its diagnosis. Meanwhile, it did not have any name and was usually voluminously described. Here we propose to name these cells brochomastigotes. The term brochomastigote is derived from the Greek βρόχοζ meaning a loop (see Fig. 1 – 4, br). For the purpose of morphometric measurements presented below, brochomastigotes were not separated from common endomastigotes. Pro- and choanomastigotes were also measured together since there is a continuum of shapes between these two morphotypes.

Isolates F2, F5, F6, F7, and F8 were obtained from the same host, *Nabis flavomarginatus* in 1986. The cells of all five strains were morphologically indistinguishable. All cultures contained the same continuum of morphotypes including typical choanomastigotes with anterior or posterior kinetoplast-to-nucleus position and variable length of flagellum, promastigotes and endomastigotes, including brochomastigotes (Fig. 1). The detailed morphometrical characteristics of these isolates are presented in Table 2.

Isolate CL8 included many rounded or oval endomastigotes with variable position of kinetoplast and flagella bending inside the cell body, as well as choano- and promastigotes (Fig. 2). In some choanomastigotes we were able to see shortened and widened flagella similar to those described earlier in the isolate Nbr (Frolov and Malysheva 1990). Opisthomorphs were also detected and had the same size and shape as choanomastigotes. The culture also contained oval brochomastigotes. The

Table 1. Cultures of trypanosomatids used in this work.

Isolate	All known taxonomic names	Host	Place and year of isolation
KV-1	*Blastocrithidia gerricola*[*]	*Gerris lacustris*	Kaliningrad region, 1981
BM-33	*Blastocrithidia miridarum*[*]	*Leptopterna dolabrata*	Pskov region, 1985
CL8	*Leptomonas* sp.	*Nabis limbatus*	Leningrad region, 1984
F2	*Leptomonas* sp.	*Nabis flavomarginatus*	Northern Karelia, 1986
F5	*Leptomonas* sp.	*Nabis flavomarginatus*	Northern Karelia, 1986
F6	*Leptomonas* sp.	*Nabis flavomarginatus*	Northern Karelia, 1986
F7	*Leptomonas* sp.	*Nabis flavomarginatus*	Northern Karelia, 1986
F8	*Leptomonas* sp.	*Nabis flavomarginatus*	Northern Karelia, 1986
Nbr	*Wallaceina brevicula*[+] *Crithidia brevicula* *Crithidia allae* *Proteomonas brevicula* *Proteomonas allae*	*Nabis brevis*	Pskov region, 1986
ZK	*Wallaceina inconstans*[+] *Proteomonas inconstans*	*Calocoris sexguttatus*	Pskov region, 1986
101	*Wallaceina podlipaevi*[+] *Leptomonas peterhoffi*[*]	*Nabis flavomarginatus*	Leningrad region, 1980
Wg	*Wallaceina vicina*[+]	*Limnoporus rufoscutellatus*	Leningrad region, 2000

[*] cultures originated from mixed infections.
[+] the last valid name associated with a culture.

detailed morphometrical characteristics of this isolate are presented in Table 2.

Isolate KV-1 was represented by choanomastigotes of various shapes (rounded, oval, barley-like, etc.), endomastigotes and brochomastigotes. Some of the rounded cells had a narrow and short flagellar pocket resembling spheromastigotes of *T. cruzi* (Rondinelli et al. 1988; Fig. 3). The detailed morphometrical characteristics of this isolate are presented in Table 2.

Isolate BM-33 consisted of choanomastigotes of various shapes (rounded, oval, barley-like etc.) with wide flagellar pocket and a kinetoplast situated in close proximity to the nucleus (Fig 4). Other

Table 2. Morphometric characteristics of studied isolates.

Isolate	Length, μm	Width, μm	N - A, μm	K - A, μm	Free flagellum, μm
F2 – F8	10.3 ± 2.2	3.9 ± 0.9	4.0 ± 0.9	3.1 ± 0.9	8.5 ± 3.7
Choano- / promastigotes, N = 50	(5.9 – 15.3)	(2.1 – 6.2)	(2.2 – 6.1)	(1.4 – 4.9)	(2.0 – 16.5)
F2 – F8	7.5 ± 1.6	4.1 ± 1.2	3.8 ± 1.1	3.3 ± 1.2	N.A.
Endomastigotes, N = 50	(2.9 – 12.8)	(2.0 – 6.6)	(0.5 – 6.3)	(1.4 – 7.1)	
CL8	7.6 ± 1.7	4.3 ± 1.2	3.0 ± 0.7	2.5 ± 0.9	6.4 ± 3.5
Choano- / promastigotes, N = 50	(5.1 – 10.6)	(2.7 – 6.7)	(1.7 – 4.8)	(1.3 – 4.2)	(1.2 – 14.8)
CL8	6.6 ± 1.2	4.4 ± 0.9	3.3 ± 1.0	3.2 ± 1.1	N.A.
Endomastigotes, N = 30	(4.6 – 9.3)	(2.5 – 6.4)	(1.6 – 5.4)	(0.8 – 5.4)	
KV-1	10.0 ± 2.3	5.2 ± 1.1	3.9 ± 0.9	3.3 ± 1.4	7.3 ± 4.5
Choano- / promastigotes, N = 50	(6.6 – 15.2)	(2.8 – 7.5)	(2.2 – 5.9)	(0.6 – 6.6)	(1.4 – 18.0)
KV-1	9.1 ± 1.7	5.1 ± 1.6	4.2 ± 1.3	2.9 ± 1.3	N.A.
Endomastigotes, N = 30	(5.7 – 12.8)	(2.6 – 8.3)	(1.0 – 6.3)	(0.6 – 6.3)	
BM-33	7.6 ± 1.9	3.1 ± 0.7	3.9 ± 1.2	3.5 ± 1.0	5.8 ± 3.0
Choano- / promastigotes, N = 50	(3.9 – 12.9)	(1.9 – 5.2)	(1.0 – 5.6)	(0.8 – 5.5)	(1.0 – 13.0)
BM-33	7.2 ± 1.7	3.4 ± 0.7	3.7 ± 1.3	3.5 ± 1.0	N.A.
Endomastigotes, N = 30	(3.0 – 10.2)	(2.3 – 4.9)	(1.1 – 6.4)	(1.2 – 5.4)	

N - A is a distance between the nucleus and the anterior end of the cell.
K – A is a distance between the kinetoplast and the anterior end of the cell.

morphotypes present were typical endomastigotes and brochomastigotes. The detailed morphometrical characteristics of this isolate are presented in Table 2.

Combined together, our data did not reveal any isolate-specific morphological trait. The phylogenetic relationships between these isolates and described species of *Wallaceina* as well as their genetic diversity were analyzed using molecular markers.

Phylogenetic Analyses

Although 18S rRNA gene sequences for some isolates under study (KV1, Nbr, ZK, 101, F6) were already deposited in GenBank (accession numbers AF153036, AF153039, AF153042, AF153044, and AF153045, respectively), they had some minor differences at the 5' and 3' ends apparently resulting from poor quality reads. Therefore we decided to re-sequence them along with other *Wallaceina* isolates available. Our results demonstrated 100% identity of sequences of all 12 studied isolates (GenBank accession numbers KJ443344 – KJ443355) and thus we used only one of them in the subsequent phylogenetic analysis. The alignment of the 18S rRNA gene sequences was combined with that of another popular phylogenetic marker, gGAPDH gene, which is known to be less conservative.

Both maximum likelihood and Bayesian trees inferred using the concatenated dataset were identical in topology (Fig. 5). Individual trees reconstructed using 18S rRNA and gGAPDH genes were similar in topology but had lower resolution (Supplementary Material Figs S1 and S2). The clade of the Leishmaniinae subfamily as well as all those corresponding to genera outside this subfamily had substantial support. In this respect our results did not differ from the previously published phylogenetic trees. Meanwhile the resolution within the Leishmaniinae group, to which all the isolates investigated belonged, proved to be much better compared to other studies. This was apparently due to the use of the better fit model. The tree was rooted with the sequence of *Paratrypanosoma confusum* (Flegontov et al. 2013). Our isolates (labeled as *Crithidia brevicula*, see Discussion) appeared as a sister lineage to the clade containing *Leptomonas tenua*, *Crithidia permixta*, and *C. pragensis*. This position was supported by high posterior probability and moderate bootstrap percentages. Altogether they formed a sister group to another clade that included the type species of the genus *Crithidia*, *C. fasciculata*.

To estimate the intra- and inter-isolate genetic diversity of wallaceinas (i.e. *Crithidia brevicula*, see Discussion) we sequenced a number of molecular clones of gGAPDH and SL RNA (GenBank accession numbers KJ443306 - KJ443343, and KJ474901 - KJ474937, respectively) genes as well as those that originated from the ITS1-5.8S-ITS2 fragments (GenBank accession numbers KJ474866 - KJ474900) and used these sequences in neighbor-joining analyses (exemplified in Fig. 6). In all the cases we couldn't detect any explicit isolate-specific grouping. On the contrary, individual clones of different isolates appeared to be intermingled.

In the case of the SL RNA gene repeats (Fig. 6 and Supplementary Material Fig. S3 at higher resolution) minimum intra- and inter-isolate identity were 92.7% and 93%, respectively. These numbers are within the limits of intraspecific diversity for this genetic marker (>90%) usually adopted in biodiversity studies (Westenberger et al. 2004). So we concluded that all the isolates investigated here belong to the same species of family Trypanosomatidae.

On the gGAPDH gene dendrogram all clonal sequences of wallaceinas (i.e. *Crithidia brevicula*, see Discussion) formed a well-supported group (Supplementary Material Fig. S4). Interestingly, though the clonal sequences of gGAPDH were shuffled, the distances between them proved to exceed some interspecific distances for this gene in their relatives. This indicates an accelerated evolutionary rate of the gGAPDH gene in this group. Four clones belonging to three isolates formed a small cluster separated from the rest of the clones. The sequence identity of the members of this group with those of other clones was 97.4 - 98.5% in nucleotides and 96.2 - 97.6% in amino acids compared to 98.3 - 100% and 97.9 - 100%, respectively within the main group. The sequences of this small cluster had a series of shared substitutions distinguishing them from the rest of the clones. They did not originate from pseudogenes as no frame shift mutations or stop codons within these sequences were detected.

The sequences of ITS1-5.8S-ITS2 region for Leishmaniinae available from GenBank, were represented mainly by those of *Leishmania* spp. that proved to be quite distant from our group. A reliable alignment with outgroup sequences was not possible and therefore we analyzed these sequences for wallaceinas only. All the molecular clones' sequences were intermingled (data not shown). Moreover, they were 97.3% or more identical, which roughly corresponds to the differences observed

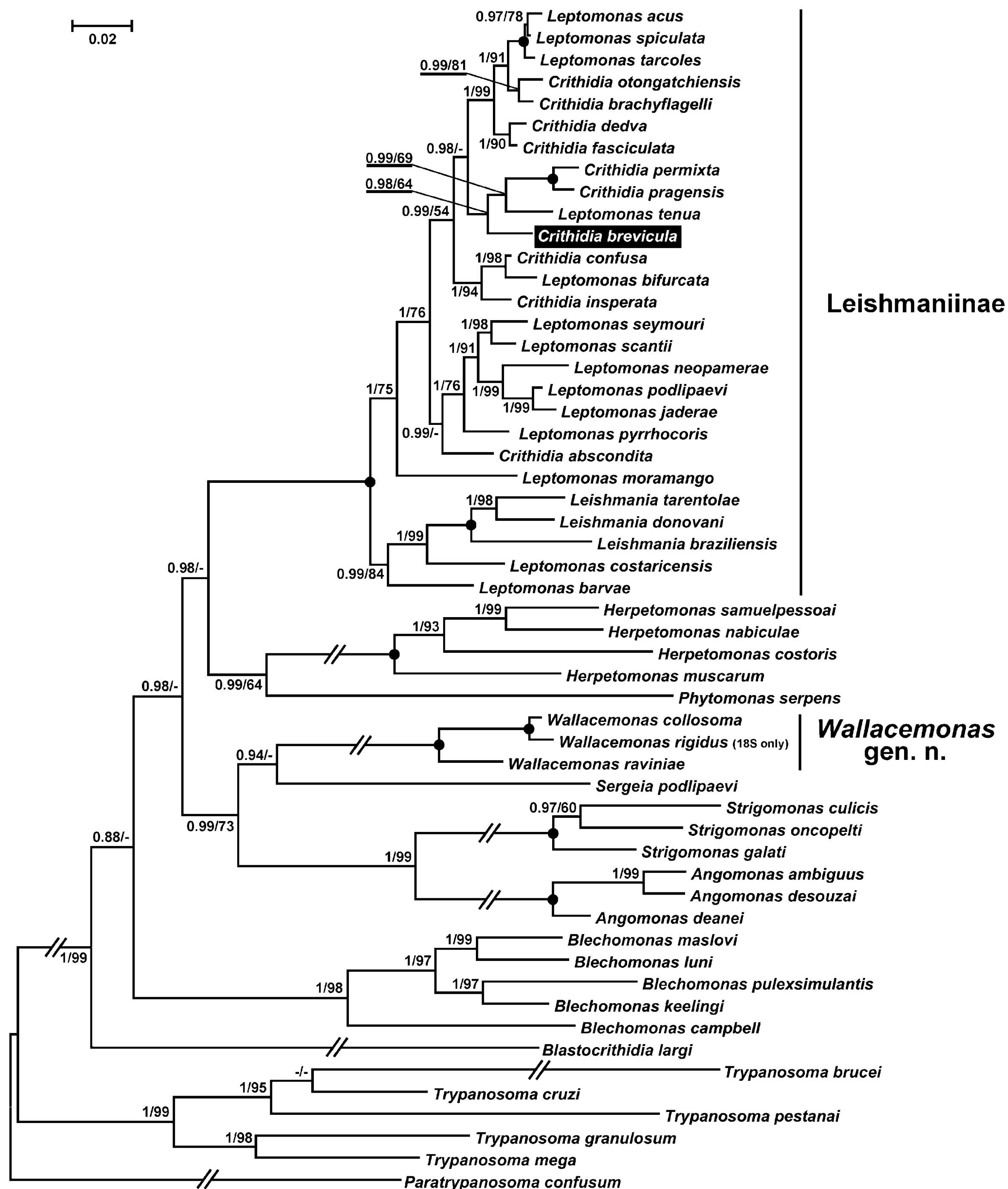


Figure 5. Phylogenetic tree of isolates studied in this work inferred by maximum likelihood method using 18S rRNA + gGAPDH concatenated set. The length of all branches is proportional to the number of substitutions per site. Double-crossed branches are at 50% of their original lengths. Numbers at nodes indicate posterior probability and bootstrap percentage, correspondingly. Values less than 0.5 and 50% are replaced with dashes. Nodes having 1.0 posterior probability and 100% bootstrap support are marked with black circles. The tree is rooted with *Paratrypanosoma confusum*. The bar represents number of substitutions per site. All studied isolates are represented as one taxon, *Crithidia brevicula* (highlighted).

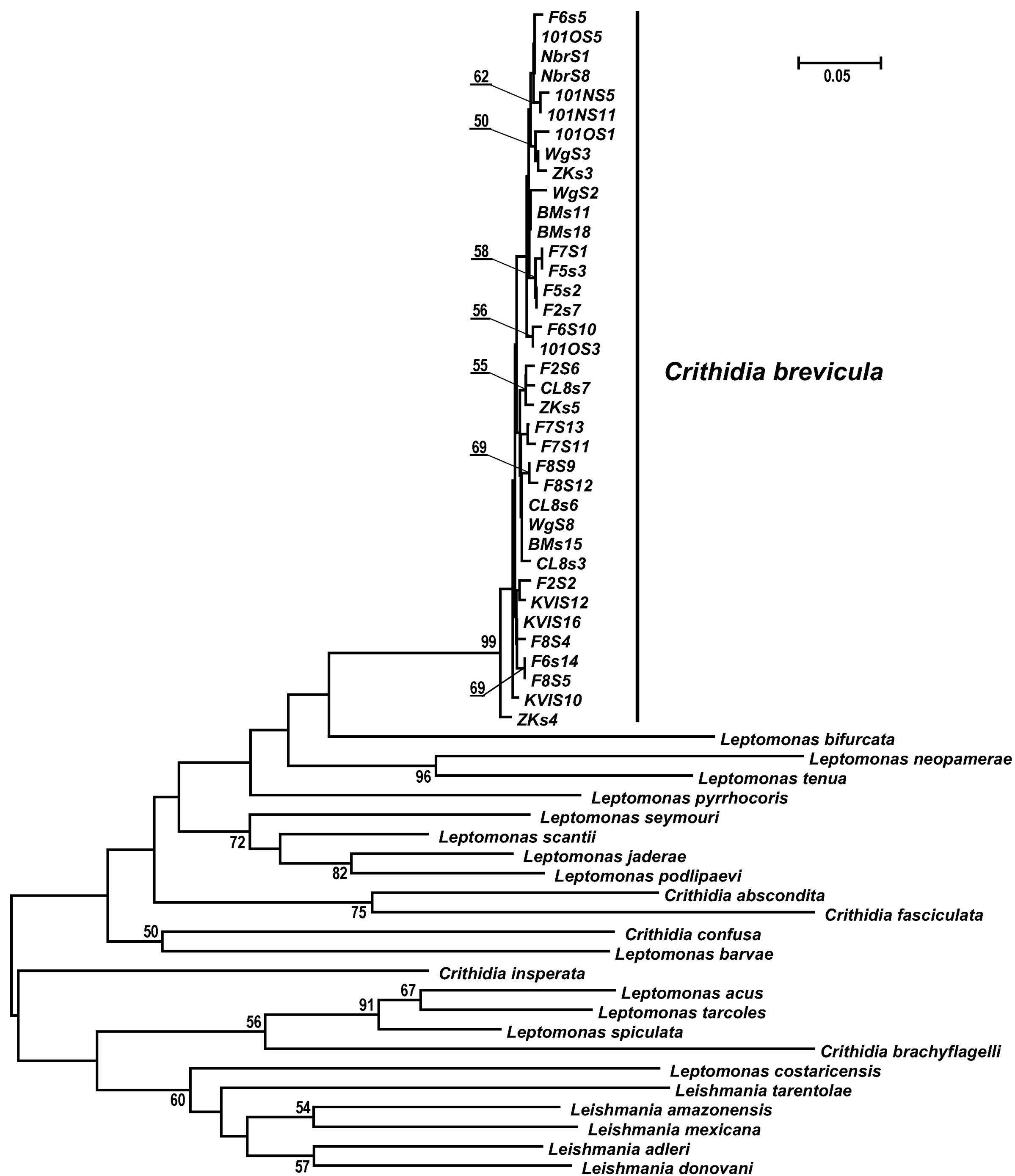


Figure 6. Neighbor-joining dendrogram of SL RNA gene sequences of Leishmaniinae. The most conserved region (from “-100” position upstream of the exon to the 3′ end of the intron, excluding the amplification primer sequences) was used. The bar represents number of substitutions per site. Numbers at nodes indicate bootstrap percentage. Values less than 50% are omitted.

between very closely related and hardly distinguishable *Leishmania* species of the subgenus *Viannia* (*L. guyanensis, L. panamensis*, and *L. braziliensis* (de Almeida et al. 2011)) further supporting our conclusion that all the isolates analyzed in this work represent just a single species of Trypanosomatidae.

Discussion

All 12 isolates studied here were previously ascribed to different species of Trypanosomatidae. However our results convincingly demonstrate that they cannot be distinguished from each other using even highly variable molecular markers, such as SL RNA and ITS sequences. Minor dissimilarities in cell sizes and relative abundance of different morphotypes observed between the investigated cultures can be easily explained by intra-specific variability and inevitable differences in cultivation conditions. Thus there is no obvious disparity between morphological and molecular data concerning similarity of all twelve isolates of wallaceinas. We conclude that all these isolates represent a single species.

Morphologically this species resembles representatives of the genus *Crithidia*. The only difference concerns the presence of brochomastigotes but this feature (that previously served as a generic diagnostic feature) now appears as a species-specific trait. Phylogenetically this parasite is also very close to crithidias including the type species of the genus, *C. fasciculata*.

There are several reasons why so many different names were attributed to these isolates before. 1) According to the traditional “one host – one parasite” concept, strains isolated from different hosts were considered to be different species. This can be exemplified by *W. brevicula*, *W. inconstans*, *W. vicina*, and *W. podlipaevi.* 2) Mixed infections are widespread in trypanosomatids. This was not recognized at the time of description of *Blastocrithidia gerricola*, *B. miridarum*, and *Leptomonas peterhoffi.* Meanwhile, it was known from the very beginning that cells in cultures were different from those predominant in the host (Podlipaev 1985; Podlipaev and Frolov 1987). In vitro conditions favored parasites present as minor fractions in the original infections – wallaceinas, in these cases. This is evident from the fact that culture forms were not documented in the life cycles of the described species (Frolov 1987; Frolov et al. 1997). 3) Some cultures were provisionally labeled as *Leptomonas* spp. based on the presence of promastigotes (isolates F2, F5, F6, F7, F8, and CL8). However, promastigotes are considered to be the least specific morphotype as they are inherent in most trypanosomatid genera.

In summary, the former numerous species of *Wallaceina* represent just one species within the genus *Crithidia*. Therefore, the original name given to these flagellates (*C. brevicula*) must return. The former genus *Wallaceina* must be abolished. Three species recently added to the genus *Wallaceina* (*W. collosoma, W. rigida*, and *W. raviniae*) phylogenetically distant from Leishmaniinae, must be reassigned to a new genus. We propose a name *Wallacemonas* for this taxon.

Taxonomic Summary

Class **Kinetoplastea** Honigberg, 1963 emend. Vickerman, 1976
Subclass **Metakinetoplastina** Vickerman, 2004
Order **Trypanosomatida** Kent, 1880
Family **Trypanosomatidae** Doflein, 1901
Genus ***Crithidia*** Léger, 1904
Crithidia brevicula Frolov, Malysheva, 1989

Synonyms: *Crithidia allae* Frolov et Malysheva, 1989; *Wallaceina brevicula* (Frolov et Malysheva, 1989); *Wallaceina inconstans* (Podlipaev, Frolov et Kolesnikov, 1990); *Wallaceina vicina* Malysheva et Frolov, 2004; *Leptomonas peterhoffi* Podlipaev, 1985 (in part); *Wallaceina podlipaevi* Malysheva et Frolov, 2009; *Blastocrithidia gerricola* Podlipaev, 1985 (in part); *Blastocrithidia miridarum* Podlipaev et Frolov, 1987 (in part); *Leptomonas* sp. F2; *Leptomonas* sp. F5; *Leptomonas* sp. F6; *Leptomonas* sp. F7; *Leptomonas* sp. F8, *Leptomonas* sp. CL8.

Generic assignment: The morphotypes of the organism in culture are choanomastigotes, stumpy promastigotes, and endomastigotes (including brochomastigotes). Based on the existing taxonomic system and phylogenetic affinity to *Crithidia* spp. on the SSU-gGAPDH tree, this species is assigned to the genus *Crithidia* Léger, 1904. This is done with the realization that the genus is non-monophyletic and therefore should be revised in the future.

Type host: *Nabis brevis* Scholtz, 1847 (Heteroptera: Nabidae)

Terra typica: village Lyady, Pskov region, Russia

Hapantotype: smear #145, kept in the laboratory of Protozoology of the Zoological Institute, St. Petersburg, Russia.

Reference culture: isolate Nbr preserved in the collection of living cultures of the laboratory of Protozoology of the Zoological Institute of the Russian Academy of Sciences, St. Petersburg, Russia

Diagnosis: cells are represented by choanomastigotes with variable position of kinetoplast, stumpy promastigotes, oval or rounded common endomastigotes and brochomastigotes. Pro- and choanomastigotes measure between 3.9 to 15.3 μm in length, 1.4 and 4.6 μm in width with their nuclei ranging between 1.9 and 7.5 μm. Endomastigotes are between 2.9 and 12.8 μm long, 1.2 to 4.6 μm wide with their nuclei varying between

1.4 and 8.3 μm. This species can be identified by the unique sequences of 18S rRNA, gGAPDH, SL RNA genes and ITS region with Genbank accession numbers KJ443350, KJ443335 - KJ443337, KJ474931 - KJ474932, and KJ474892 - KJ474894, respectively.

Remark: This description formally invalidates the genus ***Wallaceina*** Podlipaev, Frolov, Kolesnikov, 1990 (Synonym: *Proteomonas* Podlipaev, Frolov, Kolesnikov, 1990, preoccupied) as a junior subjective synonym of *Crithidia* Léger, 1904. Sequences reported in GenBank entries AF322391, AY547459, AJ401379, AF153036, EU084897, EU084896, AY547483, AY547462, EU079128, EU088285, EU088284, EU088283, AF322390, AF153039, AY547460, AY547478, AY029073, AY547461, AF153042, EU088279, EU088278, EU088277, AF316620, AJ401357, Z32854, AJ401378, AF153045, EU088282, EU088281, EU088280, EU076608, AJ401356, AJ401382, AF153044, AF124056, AF121798, JN944133, AF375664, DQ140170 belong to *Crithidia brevicula*.

***Wallacemonas* gen. n.** Kostygov et Yurchenko, 2014

Type species: *Leptomonas collosoma* Wallace, Clark, Dyer et Collins, 1960, now *Wallacemonas collosoma* (Wallace, Clark, Dyer et Collins, 1960) Kostygov et Yurchenko, 2014 comb. n.

Diagnosis: a monophyletic group of trypanosomatids as determined by sequences of 18S rRNA and gGAPDH genes. This group is characterized by almost complete reduction of the editing domain of ND8 mitochondrial gene (Gerasimov et al. 2012), absence of undulating membrane and cytoplasmic bacterial endosymbionts.

Etymology: the name is formed from the surname of F.G. Wallace, a prominent protistologist who contributed much to our knowledge of trypanosomatids, and Greek word *monas* – monad in third declension (*monas*), feminine. The word *monas* is included in many generic names of flagellates.

Genus composition, described species (new combinations):

(1) *Wallacemonas collosoma* (Wallace, Clark, Dyer et Collins, 1960) Kostygov et Yurchenko, 2014, comb. n. This species was isolated from *Gerris dissortis* (Heteroptera) in Minneapolis, USA. Synonyms: *Leptomonas collosoma*, *Wallaceina collosoma*. This is the type species for the genus.

(2) *Wallacemonas rigidus* (Podlipaev, Malysheva et Kolesnikov, 1991) Kostygov et Yurchenko, 2014, comb. n. This species was isolated from *Salda littoralis* (Heteroptera) at the White Sea coast, North-West Russia. Synonyms: *Leptomonas rigidus*, *Wallaceina rigida.*

(3) *Wallacemonas raviniae* (Votýpka et Lukeš, 2014) Kostygov et Yurchenko, 2014, comb. n. This species was isolated from *Ravinia* sp. (Diptera) in the vicinity of Otongatchi, western edge of the Cloud forest, Ecuador. Synonym: *Wallaceina raviniae*.

Remarks: Since the genus *Wallaceina* is now formally invalidated, all its species must be renamed. To accommodate three species which are phylogenetically distant from the genus *Crithidia* we propose a new generic name *Wallacemonas* in honor of F.G Wallace.

Wallacemonas sp. Wsd, isolated by S. Podlipaev from *Salda littoralis* (Heteroptera), White Sea coast, North-West Russia, and available in culture, represents another member of this genus.

Methods

Isolates cultivation and DNA extraction: Twelve isolates from the collection of living cultures of the Zoological Institute of the Russian Academy of Sciences used in this work are listed in the Table 1 along with the information about host species, geographic origin and year of isolation. They were cultivated in BHI medium supplemented with antibiotics and 10 μg/ml of hemin at 25 °C. The cultures were used to prepare dry smears (stained with Giemsa) for subsequent morphological examination and to isolate DNA using SDS-Proteinase K lysis and phenol-chloroform extraction as described previously (Kostygov and Frolov 2007).

PCR, cloning and sequencing: The 18S rRNA gene was amplified from 10-100 ng of total genomic DNA using primers S762 (5′-catatgcttgtttcaaggac-3′) and S763 (5′-gacttttgcttcctctawtg-3′) (Maslov et al. 1996) or UMx (5′-atctggttgatcctgccagtagtca-3′) and LMx (5′-ctacagctacc-ttgttacgacttttgc-3′) and then sequenced directly as described elsewhere (Kostygov et al. 2011).

Genes for glycosomal glyceraldehyde-3-phosphate dehydrogenase (gGAPDH), SL-RNA and the fragment ITS1-5.8S-ITS2 were amplified with the following primer pairs: M200 (5′-atggctccvvtcaargtwggmat-3′) and M201 (5′-takccccactcgttrtcrtacca-3′), M167 (5′-gggaagcttctgattgg-ttactwta-3′) and M168 (5′-gggaattcaataaagtacagaaactg-3′), IAMWE (5′-gctgtaggtgaacctgcagctgga-3′) and IRBAB (5′-gcgggtagtcctgccacactcag-3′), respectively (Dollet et al. 2012; Maslov et al. 2010; Westenberger et al. 2004). PCR products were cloned using InsTA PCR Cloning Kit (Thermofisher Scientific, Waltham, USA) and both strands of 2-3 clones were sequenced.

Phylogenetic analyses: To infer phylogeny of the Trypanosomatidae family we used concatenated dataset of 18S rRNA and gGAPDH genes. Sequences of both genes were aligned separately using Muscle 3.8.3.1 (Edgar 2004), the resulting alignments were refined manually using the BioEdit sequence alignment editor and ambiguously aligned positions 18S rRNA dataset were removed. After that, both alignments were concatenated resulting in a dataset with 54 taxa and 3,100 (2,008+1,092) nucleotide positions.

Maximum likelihood inference of phylogeny and model testing were performed with the use of Treefinder (v. 03.2011). Comparison of different partitioning schemes using AIC and BIC showed substantial advantage of separating model parameters for each of the two genes and for all three codon positions of gGAPDH gene. The selected substitution models were as following: J2 (= TIM2) + GI (18S rRNA), J3(= TIM1) + G (gGAPDH 1st pos.), GTR + G (gGAPDH 2nd and 3rd pos.) with 5 gamma categories in each case. Statistical support of bipartitions was assessed with the use of bootstrap resampling (1,000 replicas). Trees for individual genes were also inferred with the same set of parameters.

Bayesian inference of phylogeny was accomplished in MrBayes 3.2.2 with analysis run for 5 million generations, sampling every 1,000 generation and other parameters of MCMC set as default (Ronquist et al. 2012). We also compared different partitioning schemes and Bayes factors showed significant advantage of the same scheme as in ML analysis with unlinked branch lengths for each gene. The substitutions models used were GTR + GI for 18S rRNA gene and GTR + G for each of the three codon positions of the gGAPDH gene with 5 gamma categories in each case.

In order to explore diversity of haplotypes of the isolates under study, we also built alignments of 3 markers with intraspecific variation. For the SL alignment the whole exon and intron were taken together with the upstream region (-100 to -1 nt). Other molecular markers (gGAPDH and ITS1-5.8s-ITS2 fragments) were taken as they are after removal of amplification primers. The alignments of all these three markers were made

using ClustalW algorithm in Bioedit and subjected to cluster analysis using neighbor-joining algorithm with K2P model in MEGA6 (Tamura et al. 2013).

The accession numbers of sequences determined in the course of this work, as well as those retrieved from the GenBank, along with the alignments used and trees reconstructed were deposited to TreeBASE (16022, www.treebase.org).

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Appendix A. Supplementary Data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.protis.2014.07.001.

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