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Diversity of Trypanosomatids (Kinetoplastea: Trypanosomatidae) Parasitizing Fleas (Insecta: Siphonaptera) and Description of a New Genus *Blechomonas* gen. n.

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To further investigate the diversity of Trypanosomatidae we have examined the species present within the flea (Siphonaptera) population in the Czech Republic. Out of 1549 fleas, 239 were found to be infected by trypanosomatids. Axenic cultures were established from 90 infected specimens and 29 of them were further characterized. Molecular phylogenetic analysis of the SL RNA, gGAPDH, and SSU rRNA genes revealed a striking diversity within this group and analyzed isolates were classified into 16 Typing units (TUs) of which 15 typified new species. In addition to one *Trypanosoma* species, two TUs grouped within the sub-family Leishmaniinae, two clustered together with *Herpetomonas*, wheras 11 TUs formed a novel clade branching off between *Trypanosoma* spp. and remaining trypanosomatids. We propose to recognize this clade as a new genus *Blechomonas* and a new subfamily Blechomonadinae,

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Abbreviations: gGAPDH, glycosomal glyceraldehyde-3-phosphate dehydrogenase; kDNA, kinetoplast DNA; SL, spliced leader RNA gene repeats; SSU, small subunit ribosomal RNA; TU, typing unit.

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and provide molecular and morphological description of 11 TUs representing this genus. Our finding of such an ancient host-specific group sheds new light at the origin of Trypanosomatidae and the roots of dixenous parasitism. The strict host restriction of *Blechomonas* to Siphonaptera with adult fleas' dependence on blood meal may reflect passing of parasites from larvae through pupae to adults and implies potential transmission to the warm-blooded vertebrates.

Key words: Trypanosomatidae; phylogeny; Siphonaptera; *Blechomonas*; host specificity; co-evolution.

Introduction

Monoxenous (=one host) trypanosomatids constitute a very large and diverse group of protists of the family Trypanosomatidae Doflein, 1901. Prevalence of these intestinal parasites exceeds 20% in some insect host populations (Maslov et al. 2007; Votýpka et al. 2010, 2012b) Also united in this family are dixenous (=two hosts) flagellates of the genera *Trypanosoma, Leishmania* and *Phytomonas*, causative agents of serious diseases in vertebrates including humans, and plants (Maslov et al. 2001). Dixenous parasites spend a considerable part of their life cycle in the insect host which is utilized as a vector for their transmission (Schaub 2006). For many years monoxenous species were contemplated as dull cousins of the dixenous trypanosomatids, but recently they started to attract attention due to the extensive biological diversity, virtual omnipresence and potential impact on their insect hosts (Maslov et al. 2013).

The enduring taxonomical system of Trypanosomatidae was established about 50 years ago based on morphology and differences in life cycle traits (Hoare 1966; Wallace 1966). However molecular techniques have unambiguously proven this system to be artificial (Borghesan et al. 2013; Merzlyak et al. 2001; Votýpka et al. 2010; Yurchenko et al. 2008). Recent analyses also demonstrated that for most trypanosomatid species morphological features cannot be used for taxonomical purposes as they widely vary within a given population or even differ in the invertebrate host and the respective axenic culture (Votýpka et al. 2012b; Yurchenko et al. 2006a; Zídková et al. 2010). Correspondingly, the taxonomy of Trypanosomatidae is being redefined using molecular data (Maslov et al. 2013).

The set of genetic markers routinely used for molecular phylogenetic reconstructions of kinetoplastid flagellates is based on the small subunit ribosomal RNA (SSU rRNA) (Hollar et al. 1998; Maslov et al. 1996), the glycosomal glyceraldehyde-3-phosphate dehydrogenase (gGAPDH) genes (Yurchenko et al. 2006a) and the spliced leader (SL) RNA gene repeats (Croan et al. 1997; Maslov et al. 2007; Westenberger et al. 2004; Yurchenko et al. 2000) but can also include other genetic elements (Yurchenko et al. 2000). The first two markers are instrumental in revealing the major subdivisions within the Trypanosomatidae but fail to resolve inter- and intra-species relationships among and within these groups (Noyes et al. 2002a). The SL RNA gene repeat combines conserved and variable sequences, including the hypervariable intergenic region useful as a high-resolution molecular marker for phylogenetic reconstruction. Due to the dearth of information resulting from its small size, the SL RNA gene is not suitable for addressing relationships between phylogenetically distant groups, but may serve for barcoding organisms at the level of species or populations. This nucleotide sequence-based approach allows substitution of a species with its operational proxy – a typing unit (TU), representing potential new species. Based on previous studies of different *Leishmania* and other Leishmaniinae species, the threshold of 90% sequence similarity was set up to distinguish individual TUs (Maslov et al. 2007; Votýpka et al. 2012a).

The SL-based approach has been tested in a series of studies addressing diversity and host-parasite relationships of insect Trypanosomatidae in true bugs – Heteroptera (Maslov et al. 2010, 2013; Votýpka et al. 2010, 2012b; Yurchenko et al. 2006b) and flies – Diptera (Týč et al. 2013). As a result of these studies, many new TUs of trypanosomatids were described reflecting considerable diversity of these organisms. One of the major factors defining biodiversity is the number of available biological niches. The previously widely adopted "one host – one parasite" paradigm (Wallace 1966) is no longer applied for species description because many trypanosomatid species exhibit wide host specificity (Maslov et al. 2013; Votýpka et al. 2012b). Nevertheless, it is safe to postulate that host specificity still plays an important role in expounding biodiversity, as the parasite must evolve a molecular fit to its host to establish a stable infection. Prominent examples of high

host specificity can be found among *Leishmania* spp. which are confined to specific phlebotomine sand fly species (Sacks 2001; Volf and Myskova 2007). Others, such as *Leptomonas pulexsimulantis* can develop only in fleas of the genus *Pulex* but not *Ctenocephalides* (Beard et al. 1990), while *Leptomonas pyrhocorris* was found parasitizing members of one family of the true bugs (Pyrhocorridae) exclusively (Votýpka et al. 2012b). However, many monoxenous species are able to parasitize true bugs as well dipteran flies belonging to Hemimetabola and Holometabola (Týč et al. 2013).

In addition to the questions of host specificity, one of the fundamental and not sufficiently investigated questions concerns the actual distribution of trypanosomatids among host taxa. A vast majority (over 80%) of documented monoxenous trypanosomatid species was found in just two insect orders, Heteroptera and Diptera (Podlipaev 1990; Wallace 1966). Other orders of insects, for example Siphonaptera and Lepidoptera, were only occasionally reported as hosts for monoxenous flagellates (Guttman 1963; McGhee and Cosgrove 1980; Molyneux et al. 1981; Podlipaev 1990). In addition to typical monoxenous species morphologically similar to representatives of the genera *Leptomonas* and *Blastocrithidia* (McGhee and Cosgrove 1980), dixenous parasites classified into the genus *Trypanosoma* were also described in fleas from rats (Minchin and Thomson 1910; Nuttall 1908), wood mice, bank voles (Noyes et al. 2002b) and badgers (Lizundia et al. 2011). In the latter case a host-specific flea *Paraceras melis* was found to be a vector of *T. pestanai*. In a study of endosymbionts of fleas collected from dogs and cats in Florida, non-*Trypanosoma* trypanosomatids were found in approximately 5% of all cases and a new species, *Leptomonas pulexsimulantis*, was described from a dog's flea, *Pulex simulans* (Beard et al. 1989). As of today, no molecular phylogenetic data concerning this species are available but a parasite similar to *L. pulexsimulantis* by multi-locus enzyme and minicircle kinetoplast DNA analyses, was described as a possible cause of opportunistic infection in HIV-positive patients (Pacheco et al. 1998), although the exact relationship between these two species has not been resolved with high confidence.

In this study, a comprehensive analysis of trypanosomatids from fleas (Siphonaptera) collected in several localities in the Czech Republic was performed. We assessed the genetic diversity of new isolates by RAPD analysis and DNA barcoding of their SL RNA gene repeats, and inferred their phylogenetic positions using the SSU rRNA and gGAPDH genes. Our data indicate that only a small fraction of trypanosomatids parasitizing fleas belong to the established genera *Trypanosoma* and *Herpetomonas*, and subfamily Leishmaniinae, whereas the majority of isolates form a separate branch supported by high bootstrap values. Here we propose to recognize these isolates as a new genus, *Blechomonas* gen. n. (Kinetoplastea: Trypanosomatidae) of a newly establish subfamily, Blechomonadinae subfam. n., and provide a molecular description of eleven new species, supplemented by a morphological and ultrastructural study of several selected isolates.

Results

Isolation of Trypanosomatids from Fleas

The following hosts of the several mammalian orders were examined for the presence of fleas in several localities in the Czech Republic between 2005 and 2009: **rodents** (Rodentia): yellow-necked and wood mouse (*Apodemus flavicollis* and *A. sylvaticus*, respectively), bank vole (*Myodes glareolus*), common vole (*Microtus arvalis*), edible dormouse (*Glis glis*), and Eurasian red squirrel (*Sciurus vulgaris*); **insectivores** (Insectivora): hedgehogs (*Erinaceus concolor* and *E. europaeus*), Eurasian shrew (*Sorex araneus*), and European mole (*Talpa europaea*); **bats** (Chiroptera): common noctule (*Nyctalus noctula*); and **carnivores** (Carnivora): dog (*Canis lupus* f. *domesticus*), cat (*Felis silvestris* f. *catus*), Eurasian badger (*Meles meles*), and red fox (*Vulpes vulpes*). For 102 fleas collected from rodent nests the particular host species could not be determined.

Each of 1549 collected fleas was identified as a species belonging to the genera *Amphipsylla, Archeopsylla, Ctenocephallides, Ctenophthalmus, Doratopsylla, Hystrichopsylla, Chaetopsylla, Ischnopsyllus, Malareus, Monopsyllus, Nosopsyllus, Nycteridopsylla, Paraceras, Pulex* and *Rhadinopsylla* (total 21 named species) (Supplementary Table S1). Since males are usually smaller and more agile, which makes their capture by direct collection more difficult, about 2/3 of all captured fleas were females. Moreover, males are also more sensitive to sub-optimal living conditions often associated with rodent nests (Schwan 1993).

The dissection and subsequent microscopic examination revealed infection with trypanosomatids in 239 flea specimens (15%). This rate of infection is consistent with results of a recent study of fleas from birds in the Czech Republic and

Slovakia (I.D. and J.V., unpubl. data). The rate of infection varied between different flea species ranging from 0% for *Chaetopsylla matina*, *C. rotschildi*, *Hystrichopsylla talpae* and *Rhadinopsylla pentacantha* to 58.2% in *Monopsyllus sciurorum*. The absence of infection was not statistically significant in the majority of the cases due to the low number of specimens collected. Nevertheless, some species were rarely infected despite the relatively high number of dissected individuals. One such example is *Archeopsylla erinacei*, where only two fleas were infected out of over 100 examined. Generally, rodents served as hosts of most infected fleas with rates of infection reaching 22.4%, followed by carnivores (13.9%), insectivores (9.0%) and bats (7.3%) (Supplementary Table S2). These observations may reflect different environmental conditions in rodent nests and bat resting sites, yet the possibility of varying susceptibility of individual flea species to trypanosomatid infection cannot be discarded.

Careful dissection of the fleas allowed precise determination of the site of the trypanosomatid infection in most cases, with parasites being found in all examined parts of host intestine: midgut, hindgut, and Malpighian tubules. Parasites were detected predominantly in the hindgut (proctodeum), mainly in the rectal ampoules (Table 2). Occasionally, flagellates were also found in the midgut and very rarely in the Malpighian tubules, which might have happened because of either extremely heavy parasite burden and/or backflow of parasites during the dissection. Most observed flagellates of the newly erected genus *Blechomonas* (see below) were found as either free or rosettes-associated nectomonads and haptomonads attached to the cuticle lining of their insect host's hindgut (data not shown). In contrast, cultures of the same flagellates were dominated by aggregated short promastigotes forming typical rosettes attached to the wall of cultivation flask.

Establishment of Cultures and RAPD Analysis

In order to identify different isolates out of 90 (38% success rate) stable axenic cultures established from dissected fleas, RAPD analysis has been carried out. Attempts to separate the actively moving trypanosomatids from the contaminating fungi using a U-shaped tube (Podlipaev and Frolov 2000) were helpful in just a few cases, probably because some isolates multiply very slowly and others proved to be not susceptible for cultivation at all (Yurchenko et al. 2009). DNA isolated from axenic cultures was subjected to RAPD with four primers, and 15 isolates, each representing a group with a unique RAPD pattern, were chosen for more detailed morphological and molecular analyses (data not shown). Seven isolates originated from rodents, six from carnivores and two were from fleas hosted by bats and insectivores (Table 2 and Supplementary Table S3).

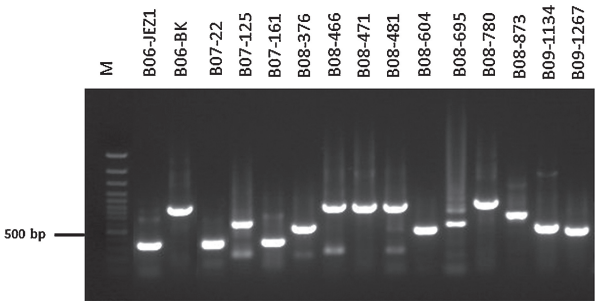


Figure 1. Amplification of SL RNA gene repeats from different isolates of flea trypanosomatids. Ethidium-bromide stained 1.0% agarose gel showing the SL products amplified from the axenic cultures of B06-JEZ1, B06-BK, B07-22, B07-125, B07-161, B08-376, B08-466, B08-471, B08-481, B08-604, B08-695, B08-780, B08-873, B09-1134, and B09-1267. Note that in some cases (B06-JEZ1, B07-161, and B08-873), an oligomer repeats are visible. Lane M is a "100-bp DNA ladder" (Life Technologies).

SL RNA-based Phylogenetic Analysis

PCR amplification using SL RNA-specific primers produced a spectrum of amplicons varying in size between ~300 and 1,000 bp (Fig. 1). Their sequencing revealed an unexpected diversity of detected flagellates and their association with different clades within the family Trypanosomatidae.

SL RNA sequences of isolates B08-466 (KF054152, 870 nt) and B08-481A (KF054151, 865 nt) were 97% identical and shared 86-87% homology with sequences from *Leptomonas tenua* of the subfamily Leishmaniinae. Based on the 10% threshold rule (Maslov et al. 2007), they represent a new typing unit (TU*146*). Sequences obtained from the isolate B08-471 (KF054155, 874 nt) clustered with several *Trypanosoma* species including *T. desterrensis* from South American bats (Grisard et al. 2003), *T. microti* from voles (Stevens et al. 1998, 1999) and *T. lewisi* from rats (53% identity overall). Based on a high 93% similarity of SL RNAs of the isolate B08-873 (KF054153, 656 nt) with *Herpetomonas nabiculae* Nfm (Kostygov et al. 2011), we considered both as members of the same species.

Table 1. GenBank accession numbers of the new sequences determined in the course of this work.

	Isolate	Primary host	Secondary host	SSU	gGAPDH	SL
non-*Blechomonas*	B08-471	*Monopsyllus sciurorum*	*Glis glis*	KF054111	KF054084	KF054155
	B08-466	*Monopsyllus sciurorum*	*Glis glis*	KF054114	KF054086	KF054152
	B08-481/B	*Monopsyllus sciurorum*	*Glis glis*	KF054112	KF054087	
	B08-481/A	*Monopsyllus sciurorum*	*Glis glis*		KF054089	KF054151
	B08-873	*Chaetopsylla globiceps*	*Vulpes vulpes*	KF054113	KF054088	KF054153
	B08-CTE2/B	*Ctenocephalides* sp.	*Felis silvestris* f. *catus*		KF054085	
Blechomonas	B08-CTE2/A	*Ctenocephalides* sp.	*Felis silvestris* f. *catus*	KF054134		KF054159
	B06-JEZ1	*Monopsyllus sciurorum*	*Erinaceus* sp.	KF054122	KF054098	KF054142
	B06-BK	*Ctenocephalides felis*	*Felis silvestris* f. *catus*	KF054133	KF054097	KF054141
	B07-22	*Nosopsyllus fasciatus*	Rodentia (nest)	KF054130	KF054102	KF054140
	B07-125	*Monopsyllus sciurorum*	*Sciurus vulgaris*	KF054118	KF054101	KF054143
	B07-161	*Ctenophthalmus* sp.	*Microtus arvalis*	KF054121	KF054099	KF054144
	B08-376	*Ctenophthalmus agyrtes*	Rodentia (nest)	KF054116	KF054093	KF054145
	B08-604	*Ctenocephalides canis*	*Vulpes vulpes*	KF054127	KF054100	KF054146
	B08-695	*Chaetopsylla globiceps*	*Vulpes vulpes*	KF054115	KF054094	KF054147
	B08-780	*Chaetopsylla globiceps*	*Vulpes vulpes*	KF054135	KF054095	KF054148
	B09-1134	*Pulex irritans*	*Vulpes vulpes*	KF054129	KF054091	KF054139
	B09-1267	*Nycteridopsylla eusarca*	*Nyctalus noctula*	KF054126	KF054092	KF054149
	ATCC 50186	*Pulex simulans*	*Canis lupus* f. *domesticus*	KF054128	KF054090	KF054138

Table 2. Host specificity and tissue localization of newly identified trypanosomatid species.

Name	Type Isolate	SSU/ gGAPDH	Cult* RAPD	Envi* (SL)	Primary host	Secondary host	Localization
Herpetomonas nabiculae	B08-873			1	*Chaetopsylla globiceps*	*Vulpes vulpes*	HG
			1		*Paraceras melis*	*Vulpes vulpes*	HG
Herpetomonas sp. (TU*147*)**	B08-481/B		2		*Monopsyllus sciurorum*	*Glis glis*	HG, MG
Leptomonas cf. *tenua* (*TU146*)	B08-466	B08-481/A			*Monopsyllus sciurorum*	*Glis glis*	HG
Leptomonas sp. (TU*148*)**	B08-CTE2/A				*Ctenocephalides felis*	*Felis silvestris* f. *catus*	N.D.
Trypanosoma sp.	B08-471		1		*Monopsyllus sciurorum*	*Glis glis*	HG, (MG)
Blechomonas pulexsimulantis	B09-1134		1		*Pulex irritans*	*Vulpes vulpes*	HG, (MG), MT
			1		*Chaetopsylla globiceps*	*Vulpes vulpes*	HG, MT
Blechomonas ayalai	B08-376				*Ctenophthalmus agyrtes*	rodent nest	HG
Blechomonas juanalfonzi	B07-161		1		*Ctenophthalmus* (*assimilis*)	*Microtus arvalis*	HG
Blechomonas maslovi	B06-JEZ1				*Monopsyllus sciurorum*	*Erinaceus* sp.	HG
		B05-J13	3		*Paraceras melis*	*Meles meles*	HG
		B4, B6			*Ceratophyllus pullatus*	*Ficedula albicollis*	N.D.
Blechomonas campbelli	B06-BK	B-CTE2/A			*Ctenocephalides felis*	*Felis silvestris* f. *catus*	N.D.
Blechomonas keelingi	B07-22				*Nosopsyllus fasciatus*	*Rodentia, nest*	HG
		B86, B100			*Ceratophyllus pullatus*	*Parus major*	N.D.
Blechomonas englundi	B07-125				*Monopsyllus sciurorum*	*Sciurus vulgaris*	HG
		B55			*Ceratophyllus pullatus*	*Parus major*	N.D.
		B06-20			*Ceratophyllus pullatus*	*Ficedula albicollis*	N.D.
Blechomonas lauriereadi	B08-604				*Ctenocephalides canis*	*Vulpes vulpes*	HG
				1	*Ctenocephalides felis*	*Vulpes vulpes*	HG
				2	*Paraceras melis*	*Vulpes vulpes*	HG
Blechomonas wendygibsoni	B09-1267				*Nycteridopsylla eusarca*	*Nyctalus noctula*	HG
			6		*Nycteridopsylla eusarca*	*Nyctalus noctula*	HG
Blechomonas luni	B08-695	B08-658	43	28	*Chaetopsylla globiceps*	*Vulpes vulpes*	HG
				1	*Chaetopsylla trichosa*	*Vulpes vulpes*	HG
				1	*Archeopsylla erinacei*	*Vulpes vulpes*	HG
Blechomonas danrayi	B08-780	B08-864 B09-1372	5	10	*Chaetopsylla globiceps*	*Vulpes vulpes*	HG

*Belonging to the current species was based on RAPD analysis or by PCR product sizes of SL.
**Culture is not available.

The remaining set of unique sequences (B06-JEZ1, B06-BK, B07-22, B07-125, B07-161, B08-376, B08-604, B08-695, B08-780, B09-1134, B09-1267) and ATCC 50186 (described before as *Leptomonas pulexsimulantis* (Beard et al. 1989); to avoid confusion hereafter called ATCC 50186) ranged from 323 to 924 bp, with mutual identity ranging between 56 and 93% (Table 1). With a single exception, they may all represent new species based on the 90% similarity threshold rule (Jirků et al. 2012; Maslov et al. 2007, 2013). The only exception is the isolate B09-1134 which shares 93% identity with ATCC 51086, and hence represents just a new isolate of this previously described species. This isolate was excluded from further analysis. For all other sequences homology was limited to the 5′ region, and typically did not extend beyond 110 conserved nucleotides (26.1% consensus and 5.9% identity positions overall). SL RNA sequences of several additional isolates obtained in the course of this work also clustered together with this newly created group. However, they were not selected for further detailed morphological characterization because of their high similarity to the RAPD-selected representatives of novel TUs (B05-J13 (KF054161), B08-CTE2-A (KF054159), B07-66 (KF054158), and B09-1372 (KF054157)) (Table 2).

To reveal phylogenetic relationships among novel and known trypanosomatids, an extensive SL RNA-based dendrogram has been build (Fig. 2). It confirmed that B08-471 is closely affiliated with the *Trypanosoma microti / T. lewisi / T. desterrensis* group. Moreover, B08-466 and B08-481/A branch within the subfamily Leishmaniinae with *Leptomonas tenua* being their closest relative, whereas B08-873 is a new herpetomonad falling into the group represented by *H. nabiculae.* The degree of homology between *H. nabiculae* and B08-873 (93%) places them together as different isolates of the same species. All other SL sequences determined here, including the previously described ATCC 50186, form a novel large clade in the SL RNA dendrogram (Fig. 2). The level of homology among these sequences and their clear separation from other trypanosomatids suggest that these isolates may represent a new genus.

SSU rRNA and gGAPDH-based Phylogenetic Analyses

The fast-evolving SL RNA gene repeat preserves a limited phylogenetic signal and therefore is not suitable for the determination of relationships beyond the species level. Indeed, the deeper-branching order is not reliable and/or well supported in the SL RNA-based trees and thus needs to be independently confirmed by other phylogenetic markers. In order to do so, for each isolate investigated here, the SSU rRNA and gGAPDH genes were sequenced and analyzed. Concatenated sequences were then aligned for all known monoxenous and selected dixenous trypanosomatids, and analyzed using the Bayesian algorithms, maximum likelihood and maximum parsimony (Fig. 3).

In the initial SL RNA-based analysis, B08-466 represented a new TU clustering with *L. tenua* (Fig. 2) and this was fully corroborated by the concatenated SSU+gGAPDH dataset. Interestingly, a more detailed analysis of B08-481 revealed it to be a mix of isolates B08-481/A and B08-481/B, both derived from the flea *Monopsyllus sciurorum*. While thriving under cultivation conditions, isolate B08-481/A (KF054151, KF054087) was almost identical to B08-466 (and thus represented another isolate of this new TU, *TU146*), B08-481/B (KF054089, KF054112) proved refractory to cultivation and constituted a new TU (TU*147*) belonging to the genus *Herpetomonas* (Fig. 3). Similar mixed infection of two unrelated flagellates was noted in the case of the isolate B08-CTE2. Whereas B08-CTE2/B (KF054085) represented a new TU (*TU148*) within the clade '*Wallaceina*', B08-CTE2/A was a member of the newly erected genus (see below). It should be noted that the '*Wallaceina*' clade is questionable due to a possible contamination of the primary cultures of morphologically characterized *Wallaceina* sp. by an unknown *Leptomonas* sp. (Podlipaev 2003), which was later sequenced and mislabeled as '*Wallaceina*' (see discussion in (Yurchenko et al. 2008)).

A nice correlation between the SL RNA trees and an analysis based on the SSU+gGAPDH sequences is apparent from the fact that both datasets confirm the species identity of B08-873 and *Herpetomonas nabiculae*, as well as by the appurtenance of B08-471 to the genus *Trypanosoma*. However, we consider assigning B08-471 to a given trypanosome species based solely on its SSU rRNA, gGAPDH or SL RNA sequences as premature.

In the concatenated SSU+gGAPDH tree, a strongly supported clade comprising of TUs representing new species (B06-JEZ1, B06-BK, B07-22, B07-125, B07-161, B08-376, B08-604, B08-695, B08-780, B09-1134, and B09-1267) along with ATCC 50186 and several additional isolates (see below) emerges (Fig. 3). The degree of separation of this group from all other known clades of


T. microti
T. desterrensis
Trypanosoma sp. B08-471
T. lewisi
Trypanosoma
B08-466
B08-481/A
(TU146)
Leptomonas tenua
Cr. thermophila
Leishmaniinae
'jaculum'
Angomonas
Herpetomonas (part)
L. sp. NFM
B08-857
B08-873
Herpetomonas nabiculae
Blastocrithidia
Phytomonas
Herpetomonas (part)
B. keelingi (B07-22)
B. lauriereadi (B08-604)
B. pulexsimulantis (ATCC 50186)
B09-1134
B. wendygibsoni (B09-1267)
B09-1372
B. danrayi (B08-780)
B08-CTE2/A
B. campbelli (B06-BK)
B. luni (B08-695)
B. englundi (B07-125)
B. ayalai (B08-376)
B. juanalfonzi (B07-161)
B05-J13
B. maslovi (B06-JEZ1)
Blechomonas
0.1

trypanosomatids allowed us to erect a new taxonomic unit at the general level – *Blechomonas* gen. n. All these isolates, except for the one similar to the previously described ATCC 50186 and the conspecific B09-1134, constitute new species based on their SL RNA, SSU rRNA and gGAPDH sequences. In many instances these new species are represented by multiple isolates established in the course of this work and the list of isolates from fleas parasitizing mammalian hosts is further supplemented by several isolates from fleas captured in bird nests (B4, B6, B55, B86, B100 – see Fig. 3 and Table 2). For example, B08-658 (KF054103) and B08-CTE2/A (KF054134, KF054159) are indistinguishable from B08-695 and B06-BK, respectively. Both isolates B06-20 (KF054107, KF054119) and B55 (KF054108, KF054120) belong to the same species as B07-125, while B05-J13 (KF054104, KF054123, KF054160), B4 (KF054105, KF054124), and B6 (KF054106, KF054125) come together conspecific with B06-JEZ1. Similar cases are isolates B08-864 (KF054136) and B09-1372 (KF054137, KF054157), which are almost identical to B08-780, and B100 (KF054109, KF054131) and B86 (KF054110, KF054132) which are almost indistinguishable from B07-22 (Fig. 3 and Table 2).

The only previously known member of this clade is "*Leptomonas" pulexsimulantis* (ATCC 50186). It was named *Leptomonas* because its morphological appearance fitted the generic description (Beard et al. 1989). As shown here, based on molecular data ATCC 50186 is a typical member of the genus *Blechomonas.* Moreover, we have encountered the isolate B09-1134 (KF054091, KF054129 and KF054139) with sequences very similar to those derived from ATCC 50186 (KF054128, KF054090 and KF054138). Based on the high degree of sequence similarity we conclude that these isolates belong to the same species with a wide geographic distribution (USA and Czech Republic), yet with considerably narrow host specificity restricted to the genus *Pulex* (*P. simulans* and *P. irritans* for ATCC 50186 and B09-1134, respectively). Because of this, we herein rename *Leptomonas pulexsimulantis* to *Blechomonas pulexsimulantis* comb. n. Beard 1989 emend. Yurchenko, Votýpka et Suková 2013 (see the Taxonomic summary).

Whereas the above-described isolates belonging to the already known genera and subfamilies are only assigned to new TUs (TU*146-148*), all isolates belonging to the newly erected genus *Blechomonas* gen. n., are formally described as new species below.

Taxonomic summary

Class Kinetoplastea Honigberg, 1963 emend. Vickerman, 1976

Subclass Metakinetoplastina Vickerman, 2004

Order Trypanosomatida Kent, 1880 stat. nov. Hollande, 1952

Family Trypanosomatidae Doflein, 1901

Subfamily Blechomonadinae, subfam. n. Votýpka et Suková 2013

Diagnosis: This well-supported new subfamily is a clade defined by SSU rRNA and gGADPH gene sequences which is distinct from all other trypanosomatid genera. Morphologically, this subfamily comprises extremely polymorphic cells varying from very small to very large flagellates of the following morphotypes: promastigotes, choanomastigotes and amastigotes. The presence of other morphotypes cannot be excluded. The type genus is *Blechomonas* gen. n. from which the subfamily name is derived.

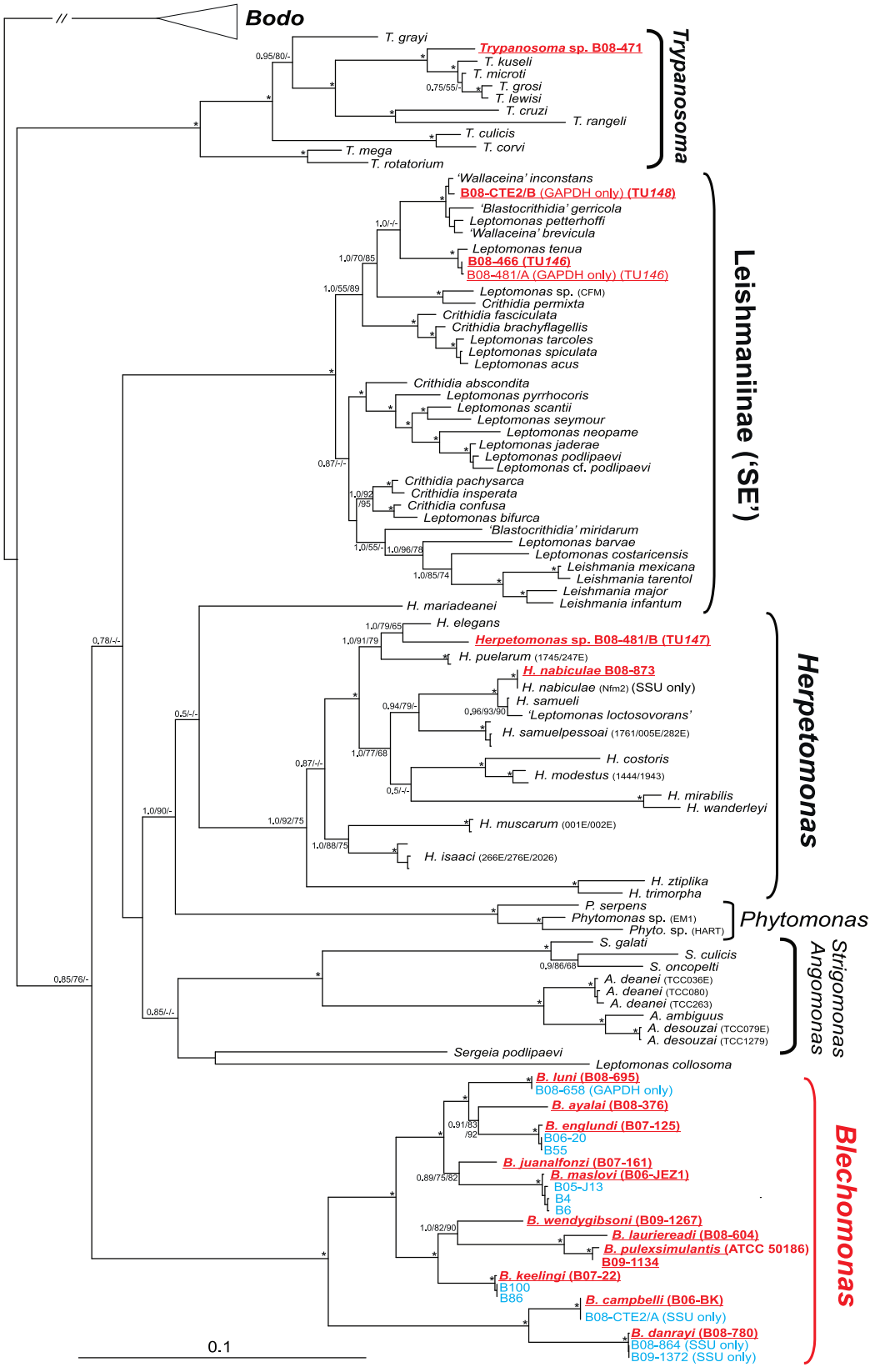
Etymology: See below.

Comment: The authorship of the new taxa (the subfamily, the genus and the species) proposed in this study should be attributed to Votýpka et Suková 2013.

Genus *Blechomonas*, gen. n. Votýpka et Suková 2013

Diagnosis: Same as for the thus far monogenic subfamily Blechomonadinae, no unique and/or unifying morphological features are known. While members of other clades of monoxenous trypanosomatid are generally faithful to a specific taxonomic group of their insect hosts with occasional switching to other insect groups, the genus *Blechomonas* is strictly bound to fleas (Siphonaptera). However, occurrence in other hosts cannot be excluded at present and thus the development of flagellates in a flea host cannot be used as a diagnostic character. The type species of the genus is *Blechomonas ayalai* sp. n. Morphological data are briefly described below and summarized in Suppl. Tables S4 and S5.

Figure 2. The dendrogram of SL sequences. Neighbor-joining analyses of all available SL RNA gene repeats of monoxenous trypanosomatids and selected ones of the dixenous species only. The most conserved region (from "–100" position upstream of the exon to the 3′ end of the intron, excluding the amplification primer sequences) was selected and aligned with Clustal X. The sequences determined in this work and deposited to GenBank™ are listed in Table 1. The bar represents number of substitutions *per* site. For clarity, some major clades of Trypanosomatidae are substituted by triangles of the identical shape and size as in the original dendrogram.


Bodo
T. grayi
Trypanosoma sp. B08-471
T. kuseli
T. microti
T. grosi
T. lewisi
T. cruzi
T. rangeli
T. culicis
T. corvi
T. mega
T. rotatorium
Trypanosoma
'Wallaceina' inconstans
B08-CTE2/B (GAPDH only) (TU148)
'Blastocrithidia' gerricola
Leptomonas petterhoffi
'Wallaceina' brevicula
Leptomonas tenua
B08-466 (TU146)
B08-481/A (GAPDH only) (TU146)
Leptomonas sp. (CFM)
Crithidia permixta
Crithidia fasciculata
Crithidia brachyflagellis
Leptomonas tarcoles
Leptomonas spiculata
Leptomonas acus
Crithidia abscondita
Leptomonas pyrrhocoris
Leptomonas scantii
Leptomonas seymour
Leptomonas neopame
Leptomonas jaderae
Leptomonas podlipaevi
Leptomonas cf. podlipaevi
Crithidia pachysarca
Crithidia insperata
Crithidia confusa
Leptomonas bifurca
'Blastocrithidia' miridarum
Leptomonas barvae
Leptomonas costaricensis
Leishmania mexicana
Leishmania tarentol
Leishmania major
Leishmania infantum
Leishmaniinae ('SE')
H. mariadeanei
H. elegans
Herpetomonas sp. B08-481/B (TU147)
H. puelarum (1745/247E)
H. nabiculae B08-873
H. nabiculae (Nfm2) (SSU only)
H. samueli
'Leptomonas loctosovorans'
H. samuelpessoai (1761/005E/282E)
H. costoris
H. modestus (1444/1943)
H. mirabilis
H. wanderleyi
H. muscarum (001E/002E)
H. isaaci (266E/276E/2026)
H. ztiplika
H. trimorpha
Herpetomonas
P. serpens
Phytomonas sp. (EM1)
Phyto. sp. (HART)
Phytomonas
S. galati
S. culicis
S. oncopelti
A. deanei (TCC036E)
A. deanei (TCC080)
A. deanei (TCC263)
A. ambiguus
A. desouzai (TCC079E)
A. desouzai (TCC1279)
Strigomonas
Angomonas
Sergeia podlipaevi
Leptomonas collosoma
B. luni (B08-695)
B08-658 (GAPDH only)
B. ayalai (B08-376)
B. englundi (B07-125)
B06-20
B55
B. juanalfonzi (B07-161)
B. maslovi (B06-JEZ1)
B05-J13
B4
B6
B. wendygibsoni (B09-1267)
B. lauriereadi (B08-604)
B. pulexsimulantis (ATCC 50186)
B09-1134
B. keelingi (B07-22)
B100
B86
B. campbelli (B06-BK)
B08-CTE2/A (SSU only)
B. danrayi (B08-780)
B08-864 (SSU only)
B09-1372 (SSU only)
Blechomonas
0.1

Etymology: *Blecho-* – refers to the host (flea) name in many Slavic languages; *monas* (Greek) – monad; third declension (*monas*); feminine; the word *monas* is included in many generic names of flagellates.

***Blechomonas ayalai* sp. n. Votýpka et Suková 2013**

Diagnosis: The species is identified by the unique sequences with GenBank accession numbers KF054116 (SSU), KF054093 (gGAPDH), and KF054145 (SL).

Morphology: Light microscopy examination of the Giemsa-stained smears revealed morphological heterogeneity of oval-shaped cultured cells, most of which were choanomastigotes and some elongated promastigotes or other morphotypes. The first type (Fig. 4A) – length from 3.8 to 9.1 μm (6.5 ± 1.1 μm; n = 50), distance between the kinetoplast and the anterior end of the cell (K-a distance) from 1.0 to 3.2 μm (2.1 ± 0.5 μm; n = 50), distance between the nucleus and the anterior end of the cell (N-a) from 1.8 to 5.8 μm (4.0 ± 0.8 μm; n = 50), and highly variable long flagellum of 5.5 to 19.9 μm long (11.3 ± 3.4 μm; n = 50) – resembled cells observed in the insect host. The second morphotype – promastigotes of 7.2 to 15.6 μm (10.8 ± 1.9 μm; n = 50), the K-a distance from 1.8 to 4.8 μm (3.0 ± 0.7 μm; n = 50), the N-a distance from 0.7 to 8.5 μm (5.7 ± 1.3 μm; n = 50), and a flagellum of 5.5 to 20.3 μm (10.0 ± 3.0 μm; n = 50) – were detected only in culture (Fig. 4B). It is likely that the second morphotype, minor in the host, became abundant only under culture conditions. Interestingly, prolonged axenic cultivation of B08-376 cells *in vitro* revealed yet another morphotype – oval amastigote-like cells firmly attached to the plastic cultivation flask (Fig. 4C). These cells were 1.7 to 3.5 μm (2.5 ± 0.5 μm; n = 70) long, 0.8 to 2.1 μm (1.5 ± 0.3 μm; n = 70) wide with K-a within 0.5 to 1.3 μm (0.8 ± 0.2 μm; n = 20) and N-a within 1.0 to 2.0 μm (1.5 ± 0.3 μm; n = 20). TEM analysis revealed a standard set of trypanosomatid structures – a flagellum with a canonical 9+2 axoneme protruding from the flagellar pocket, subpellicular microtubules, a kinetoplast juxtapositioned to the basal body, extended mitochondrion, and acidocalcisomes (Fig. 4D). The width of the kinetoplast disk ranges from 63.9 to 93.9 nm (78.7 ± 7.1 nm; n = 50).

Type host and material: The hapantotype is the Giemsa-stained slide and the axenic culture of B08-376 isolated from the hindgut of *Ctenophthalmus agyrtes* (Ctenophthalmidae) captured in a rodent nest. The hapantotype and xenotype are deposited in the collection of the Department of Parasitology, Charles University, Prague.

Type locality: In the vicinity of Příbram (N49°41′23″, E14°00′36″), Czech Republic.

Etymology: The species name is after Francisco J. Ayala, for his major contribution to our understanding of the transmission and diversity of trypanosomatids.

***Blechomonas maslovi* sp. n. Votýpka et Suková 2013**

Diagnosis: The species is identified by unique sequences KF054122 (SSU), KF054098 (gGAPDH), and KF054142 (SL).

Morphology: The most abundant morphotypes observed in the host were slightly twisted promastigotes and choanomastigotes (Figs 5A and 5B), while long promastigotes predominated in the culture (Fig. 5C). Their cell length without flagellum ranged from 6.8 to 23.5 μm (15.3 ± 3.1 μm; n = 50), cell width from 1.1 to 2.0 μm (1.4 ± 0.2 μm; n = 50), and the length of the flagellum varied from 9.2 to 23.8 μm (15.3 ± 3.1 μm; n = 50). The elongated nucleus was located within 3.6 to 9.6 μm (6.4 ± 1.2 μm; n = 50), and the kinetoplasts within 2.2 to 4.6 μm (3.0 ± 0.5 μm; n = 50) from the anterior end of the cell. These cells were accompanied by small amastigote-like forms (Fig. 5C) varying from 2.3 to 4.3 μm (3.3 ± 0.4 μm; n = 50) in length, and from 1.6 to 2.8 μm (2.3 ± 0.2 μm; n = 50) in width. Their nuclei were placed within 1.6 to 3.5 μm (2.5 ± 0.4 μm; n = 50), and the kinetoplasts within 0.8 to 2.0 μm (1.5 ± 0.3 μm; n = 50) from the anterior end of the cell. Kinetoplasts were 71.6 – 111.6 nm (93.2 ± 8.8 mm; n = 50) wide. TEM of cultured promastigotes revealed all the typical features of a trypanosomatid cell (Fig. 5D).

Type host and material: The hapantotype is the Giemsa-stained slide and the axenic culture of B06-JEZ1 isolated from the hindgut of *Monopsyllus sciurorum* (Siphonaptera: Ceratophyllidae) captured on hedgehog (*Erinaceus* sp.). The hapantotype and xenotype are deposited in the collection of the Department of Parasitology, Charles University, Prague.

Type locality: In the vicinity of Nymburk (N50°07′59″, E15°07′01″), Czech Republic.

Etymology. The species name is after Dmitri A. Maslov, who made a significant contribution to mapping the diversity of insect trypanosomatids.

Comments: Based on SL (KF054160, KF054161), SSU (KF054123, KF054124, KF054125) and gGAPDH (KF054104, KF054105, KF054106) sequences the isolate B05-J13 from the intestine (hindgut) of *Paraceras melis* captured in the nest site of European badger (*Meles meles*) in the cave "Blešárna" in the vicinity of Beroun (N49°53′27″, E14°01′23″), and the isolates B4 and B6 from the intestine of *Ceratophyllus pullatus* captured in the nesting boxes of collared flycatcher

Figure 3. The Bayesian phylogenetic tree of Trypanosomatidae. This tree is based on concatenated small subunit (SSU) rRNA and glycosomal glyceraldehyde-3-phosphate dehydrogenase (gGAPDH) gene sequences and includes all available sequences of *Blechomonas* n. gen. Type isolates representing new *Blechomonas* species are underlined; the availability of only SSU rRNA or gGAPDH sequences is indicated in brackets. Bootstrap values from Bayesian posterior probabilities (5 million generations), maximum likelihood and maximum parsimony (1,000 replicates) are shown at the nodes. Asterisks (*) denote Bayesian posterior probabilities and bootstraps of 95% or higher. Dashes (-) indicate bootstrap support below 50% or posterior probability below 0.5 or different topology. The tree was rooted with five bodonid sequences. The scale bar denotes the number of substitutions *per* site. The sequences determined in this work and deposited to GenBank™ are listed in Table 1.

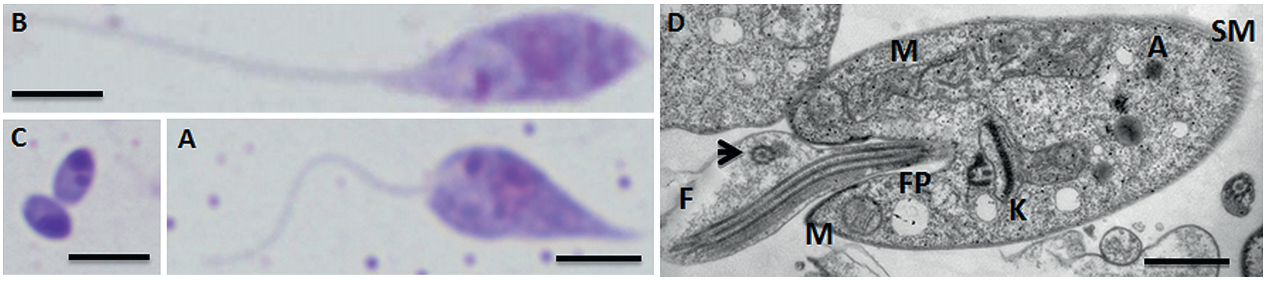


Figure 4. *Blechomonas ayalai* sp. n. (isolate B08-376). Giemsa-stained choanomastigotes (**A**), a morphotype prevalent in the environmental samples obtained from the insect intestine, proved refractory to cultivation. The axenic cultures were dominated by promastigotes (**B**, Giemsa-stained; **D**, TEM) and, under certain experimental conditions, such as prolonged cultivation, by amastigote-like flagellum-lacking cells (**C**, Giemsa-stained). All the typical features of trypanosomatids including a flagellum ("F"), a canonical 9+2 axoneme (labeled by an arrow in Fig. 4D), a flagellar pocket ("FP"), subpellicular microtubules ("SM"), a kinetoplast ("K") juxtapositioned in a close proximity to the basal body, an extended mitochondrion ("M"), and acidocalcisomes ("A"), can be detected. Scale bars are 3 μm (A-C) and 1 μm (D).

(*Ficedula albicollis*) in the vicinity of Lanžhot (N48°41′46″, E16°58′29″) represent three additional isolates of the same species.

***Blechomonas campbelli* sp. n. Votýpka et Suková 2013**

Diagnosis: The species is identified by unique sequences KF054133 (SSU), KF054097 (gGAPDH), and KF054141 (SL).

Morphology: The predominant morphotype in culture were bended promastigotes from 7.0 to 12.1 μm (9.5 ± 1.2 μm; n = 50) long, and 1.4 to 2.8 μm (1.9 ± 0.3 μm; n = 50) wide. They were further subdivided into two distinct groups with very short and medium flagella ranging from 2.0 to 11.3 μm (5.3 ± 2.1 μm; n = 50). Other minor morphotypes (e.g. amastigote-like) were also detected. The kinetoplast of cultured promastigotes ranged between 56.6 and 7.3 nm (65.6 ± 5.0 nm; n=50).

Type host and material: The hapantotype is the Giemsa-stained slide and the axenic culture of strain B06-BK isolated from the intestine of *Ctenocephalides felis* (Pulicidae). The hapantotype and xenotype are deposited in the collection of the Department of Parasitology, Charles University, Prague.

Type locality: In the vicinity of Prague (N50°05′00″, E14°28′00″), Czech Republic.

Etymology: The species name is after David A. Campbell, who contributed substantially to our understanding of diversity of American trypanosomatids.

Comments: Based on SL (KF054159) and SSU (KF054134) sequences the isolate B-CTE2-A from the intestine of *C. felis* captured on a domestic cat in the vicinity of Košice, Slovakia (N48°42′23″, E21°16′04″) is another isolate of the same species.

***Blechomonas keelingi* sp. n. Votýpka et Suková 2013**

Diagnosis: The species is identified by unique sequences KF054130 (SSU), KF054102 (gGAPDH), and KF054140 (SL).

Morphology: All B07-22 cells in the insect host were typical epimastigotes. In contrast, B07-22 cells recovered in culture were twisted promastigotes of sizes from 8.3 to 21.3 μm (14.3 ± 2.8 μm; n = 50), and the flagellum of 5.3 to 15.3 μm (9.2 ± 2.2 μm; n = 50) with occasional appearance of cells resembling choanomastigotes. The kinetoplast disk is of typical shape and size, with the width varying from 65.0 to 85.0 nm (75.2 ± 5.5 nm; n = 50).

Type host and material: The hapantotype is the Giemsa-stained slide and the axenic culture of strain B07-22 isolated from the hingut of *Nosopsyllus fasciatus* (Ceratophyllidae)

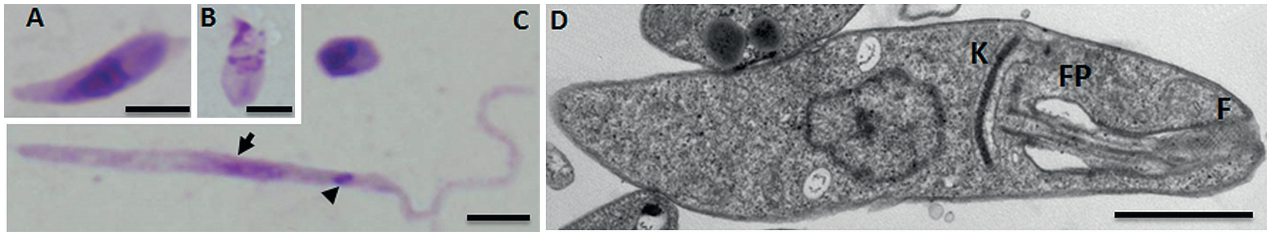


Figure 5. *Blechomonas maslovi* sp. n. (isolate B06-JEZ1). Giemsa-stained promastigotes (**A**) and choanomastigotes (**B**) from the insect guts were substituted in culture by elongated promastigotes (**C**) with well-visible kinetoplast (arrowhead) and nucleus (arrow). A typical kinetoplast ("K") at the base of a relatively deep flagellar pocket ("FP") and a flagellum ("F") can be detected by TEM (**D**). Scale bars are 3 μm (A-C) and 1 μm (D).

captured in a rodent nest. The hapantotype and xenotype are deposited in the collection of the Department of Parasitology, Charles University, Prague.

Type locality: In the vicinity of Pardubice (N50°02′26″, E15°46′35″), Czech Republic.

Etymology: The species name is after Patrick J. Keeling, an outstanding protistologist.

Comments: Based on SSU (KF054132, KF054131) and gGAPDH (KF054110, KF054109) sequences, the isolates B86 and B100 from the intestine of *Ceratophyllus pullatus* captured in the nesting boxes of great tit (*Parus major*) in the vicinity of Lanžhot (N48°41′46″, E16°58′29″) are other two isolates of the same species.

***Blechomonas englundi* sp. n. Votýpka et Suková 2013**

Diagnosis: The species is identified by unique sequences KF054118 (SSU), KF054101 (gGAPDH), and KF054143 (SL).

Morphology. Small choanomastigotes identified in the host were substituted by long needle-like promastigotes in culture. They ranged between 9.2 and 35 3 μm (18.4 ± 5.5 μm; n = 50) in length, 1.0 to 1.9 μm (1.4 ± 0.2 μm; n = 50) in width, K-a distance from 2.0 to 5.6 μm (3.3 ± 0.8 μm; n = 50), N-a distance from 3.7 to 15.1 μm (8.2 ± 2.8 μm; n = 50) and a relatively long flagellum from 10.1 to 29.0 μm (19.6 ± 4.3 μm; n = 50). The kinetoplast disk was of typical shape and size, with the width ranging from 66.6 to 100.0 nm (82.0 ± 7.9 nm; n = 50). Small numbers of wider promastigotes and round choanomastigotes similar to the dominant morphotype in the host were also detected.

Type host and material: The hapantotype is the Giemsa-stained slide and the axenic culture of strain B07-125 isolated from the hindgut of *Monopsyllus sciurorum* (Ceratophyllidae) captured on a Eurasian red squirrel (*Sciurus vulgaris*). The hapantotype and xenotype are deposited in the collection of the Department of Parasitology, Charles University, Prague.

Type locality: In the vicinity of Brno (N49°11′27″, E16°35′33″), Czech Republic.

Etymology: The species name is after Paul T. Englund, whose research was instrumental for our understanding of the kDNA.

Comments: Based on SSU (KF054120, KF054119) and gGAPDH (KF054108, KF054107) sequences, the isolates B55 and B06-20 from the intestine of *Ceratophyllus pullatus* captured in the nesting box of great tit (*Parus major*) and collared flycatcher (*Ficedula albicollis*) in the vicinity of Lanžhot (N48°41′46″, E16°58′29″) are other two isolates of the same species.

***Blechomonas juanalfonzi* sp. n. Votýpka et Suková 2013**

Diagnosis: The species is identified by unique sequences KF054121 (SSU), KF054099 (gGAPDH), and KF054144 (SL).

Morphology: There were two types of B07-161 cells observed in the host: relatively wide promastigotes and smaller cells with the posterior-localized nucleus. Axenically cultured B07-161 cells contained a range of promastigotes 9.5 to 21.1 μm (14.9 ± 2.9 μm; n = 50) long, and 0.8 to 1.6 μm (1.1 ± 0.2 μm; n = 50) wide, with K-a distance from 1.4 to 4.3 μm (2.8 ± 0.6 μm; n = 50), N-a distance from 3.6 to 10.4 μm (6.7 ± 1.4 μm; n = 50), flagellum of 5.8 to 14.4 μm (10.3 ± 1.9 μm; n = 50), and the kinetoplast disk varying in width between 66.6 to 105.0 nm (89.0 ± 7.8 nm; n = 50). In addition, medium-sized choanomastigotes were occasionally detected.

Type host and material: The hapantotype is the Giemsa-stained slide and the axenic culture of strain B07-161 isolated from the hindgut of *Ctenophthalmus* sp. (Ctenophthalmidae) captured on a common vole (*Microtus arvalis*). The hapantotype and xenotype are deposited in the collection of the Department of Parasitology, Charles University, Prague.

Type locality: In the vicinity of Nové Městečko (N49°58′00″, E13°10′00″), Czech Republic.

Etymology: The species name is after Juan D. Alfonzo, who made key contributions to our understanding of mitochondrial tRNAs in trypanosomatids.

***Blechomonas lauriereadi* sp. n. Votýpka et Suková 2013**

Diagnosis: The species is identified by unique KF054127 (SSU), KF054100 (gGAPDH), and KF054146 (SL).

Morphology: Medium-sized drop-like choanomastigotes in the culture were 7.8 to 17.0 μm (11.9 ± 2.0 μm; n = 50) long, 3.5 to 7.5 μm (5.2 ± 1.0 μm; n = 50) wide with their kinetoplasts located within 1.6 to 4.7 μm (3.4 ± 0.7 μm; n = 50), and nuclei within 3.2 to 6.7 μm (4.8 ± 1.0 μm; n = 50) from the anterior end of the cells. Their long flagella measured 9.9 to 37.5 μm (19.3 ± 5.7 μm; n = 50).

Type host and material: The hapantotype is the Giemsa-stained slide and the axenic culture of strain B08-604 isolated from the hindgut of *Ctenocephalides canis* (Pulicidae) captured on a red fox (*Vulpes vulpes*). The hapantotype and xenotype are deposited in the collection of the Department of Parasitology, Charles University.

Type locality: In the vicinity of Prague (N50°05′00″, E14°28′00″), Czech Republic.

Etymology: The species name is after Laurie K. Read, who made important contributions to our understanding of RNA editing in trypanosomatids.

***Blechomonas wendygibsoni* sp. n. Votýpka et Suková 2013**

Diagnosis: The species is identified by unique sequences KF054126 (SSU), KF054092 (gGAPDH), and KF054149 (SL).

Morphology: The range of morphotypes in the host included small choanomastigotes (predominant form, Fig. 6A), epimastigotes, cyst-like forms, and slim long promastigotes (Fig. 6B). The most abundant choanomastigotes were 2.8 to 4.8 μm (3.5 ± 0.5 μm; n = 45) long, 1.3 to 3.0 μm (2.0 ± 0.3 μm; n = 45) wide, their N-a and K-a distances 2.1 to 3.9 μm (2.7 ± 0.4 μm; n = 45) and 1.0 to 2.4 μm (1.7 ± 0.3 μm; n = 45), respectively. The very long and thin promastigote morphotype that predominated in the culture (Fig. 6C) ranged between 13.5 and 37.0 μm (24.8 ± 4.9 μm; n = 50) in length, and 0.7 to 2.0 μm (1.2 ± 0.3 μm; n = 50) in width, with N-a and K-a distances 3.6 to 11.4 μm (7.5 ± 1.5 μm; n = 50) and 2.4 to 8.9 μm (3.8 ± 1.0 μm; n = 50), respectively; flagellum measured between 11.1 and 35.5 μm (19.1 ± 5.1 μm; n = 50). The kinetoplast (Fig. 6D) measured between 66.6 to 100.0 nm (78.7 ± 7.7 nm; n = 50).

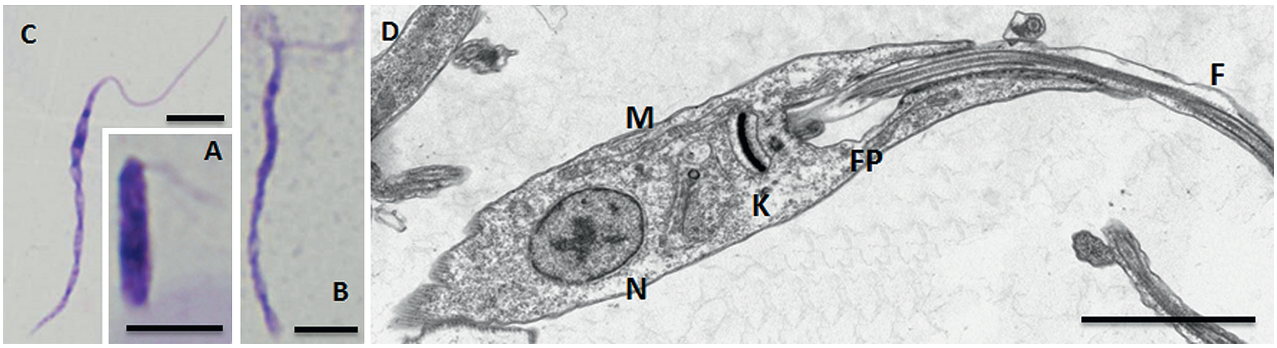


Figure 6. *Blechomonas wendygibsoni* sp. n. (isolate B09-1267). Giemsa-stained choanomastigotes (**A**) and very slim and long promastigotes (**B**) obtained from the insect host and axenic culture (**C**) have all the typical features, such as kinetoplast ("K"), flagellum ("F") protruding from marked flagellar pocket ("FP"), nucleus ("N"), and an extended mitochondrion ("M") detectable by TEM (**D**). Scale bars are 5 μm (A-C) and 2 μm (D).

Type host and material: The hapantotype is the Giemsa-stained slide and the axenic culture of B09-1267 isolated from the hindgut of *Nycteridopsylla eusarca* (Ischnopsyllidae) captured on common noctule (*Nyctalus noctula*). The hapantotype and xenotype are deposited in the collection of the Department of Parasitology, Charles University, Prague.

Type locality: In the vicinity of Prague (N50°05′00″, E14°28′00″), Czech Republic.

Etymology: The species name is after Wendy Gibson, for her key studies of taxonomy and life cycles of trypanosomatids.

***Blechomonas luni* sp. n. Votýpka et Suková 2013**

Diagnosis: The species is identified by unique sequences KF054115 (SSU), KF054094 (gGAPDH), and KF054147 (SL).

Morphology: The predominant morphotype in the insect host was a choanomastogote, while all cells recovered in the culture were slightly twisted promastigotes 7.5 to 15.0 μm (11.2 ± 1.6 μm; n = 50) long, 0.9 to 1.8 μm (1.3 ± 0.2 μm; n = 50) wide with their K-a distance 1.7 to 3.6 μm (2.6 ± 0.5 μm; n = 50), and N-a distance 3.6 to 6.8 μm (4.9 ± 0.7 μm; n = 50); flagellum was between 7.7 to 19.1 μm (11.4 ± 2.5 μm; n = 50) long.

Type host and material: The hapantotype is the Giemsa-stained slide and the axenic culture of strain B08-695 isolated from the intestine of *Chaetopsylla globiceps* (Vermipsyllidae) captured on a red fox (*Vulpes vulpes*). The hapantotype and xenotype are deposited in the collection of the Department of Parasitology, Charles University, Prague.

Type locality: In the vicinity of Most (N50°30′11″, E13°38′12″), Czech Republic.

Etymology: The species name is after Zhao-Rong Lun, who made important discoveries in the life cycle and transmission of trypanosomes.

Comments: Based on gGAPDH (KF054103) sequences, B08-658 from the hindgut of *Chaetopsylla globiceps* captured on a red fox (*Vulpes vulpes*) in the vicinity of Benešov (N49°43′24″, E14°38′23″) is another isolate of the same species.

***Blechomonas danrayi* sp. n. Votýpka et Suková 2013**

Diagnosis: The species is identified by unique sequences KF054135 (SSU), KF054095 (gGAPDH), and KF054148 (SL).

Morphology: The predominant morphotypes in both the insect host and the culture were small choanomastigotes between 2.7 to 4.7 μm (3.7 ± 0.5 μm; n = 50) long, and 1.0 to 1.8 μm (1.3 ± 0.2 μm; n = 50) wide; kinetoplasts was located within 0.8 to 1.9 μm (1.3 ± 0.2 μm; n = 50), and nuclei within 1.5 to 3.0 μm (2.2 ± 0.3 μm; n = 50) from the anterior end of the cells; flagellum varied between 1.3 to 4.5 μm (2.7 ± 0.8 μm; n = 50).

Type host and material: The hapantotype is the Giemsa-stained slide and the axenic culture of B08-780 isolated from the hindgut of *Chaetopsylla globiceps* (Vermipsyllidae) captured on a red fox (*Vulpes vulpes*). The hapantotype and xenotype are deposited in the collection of the Department of Parasitology, Charles University, Prague.

Type locality: In the vicinity of Trutnov (N50°33′38″, E15°54′47″), Czech Republic.

Etymology: The species name is after Dan S. Ray, whose research was instrumental for our understanding of the kDNA.

Comments: Based on SL (KF054157) and SSU (KF054136, KF054137) sequences, B08-864 and B09-1372 from the hindgut of *Chaetopsylla globiceps* captured on two red foxes (*V. vulpes*) in the vicinity of Česká Lípa (N50°40′56″, E14°32′13″) belong to the same species.

***Blechomonas pulexsimulantis* comb. n. Beard 1989 emend. Yurchenko, Votýpka et Suková 2013**

This species corresponds to former *Leptomonas pulexsimulantis* isolated from the intestine of *Pulex simulans* (type host) in Alachua County, Florida, USA (Beard et al. 1989, 1990).

Diagnosis: The species is identified by unique sequences KF054128 (SSU), KF054138 (SL), and KF054090 (gGAPDH).

Type material: The duplicate of the xenotype (culture ATCC 50186) is deposited in the collections of the Department of Parasitology, Charles University, Prague and Life Science Research Centre, University of Ostrava.

Comments: Based on molecular data (Figs 2 and 3) this species belongs to the genus *Blechomonas.* Isolate B09-1134 from the hindgut, midgut and Malpighian tubules of *Pulex irritans* captured on a red fox (*V. vulpes*) in the vicinity of Benešov (N49°46′58″, E14°41′19″) and identified by unique sequences KF054139 (SL), KF054129(SSU) and KF054091(gGAPDH), is clearly conspecific.

Discussion

Due to an increased interest in monoxenous insect trypanosomatids within the last decade, a major discrepancy between the widely used taxonomy and phylogeny of the family Trypanosomatidae has been uncovered. It resulted in the proposal of a molecular data-based system that would better reflect true relationships and the ever increasing diversity of this group. Numerous studies also demonstrated that all medically and veterinary important dixenous flagellates are derived from the monoxenous lineages (Borghesan et al. 2013; Flegontov et al. 2013; Jirků et al. 2012; Maslov et al. 2013; Teixeira et al. 2011; Týč et al. 2013). Therefore, insect-only parasites are critical for our understanding of the emergence of one of the most successful parasitic lifestyles on Earth.

The recent rapid expansion of known diversity is a consequence of extensive sampling in true bugs (Heteroptera) and flies (Diptera) throughout Europe, South and Central America, South-East Asia and central Africa (Borghesan et al. 2013; Jirků et al. 2012; Maslov et al. 2007; Teixeira et al. 2011; Týč et al. 2013; Votýpka et al. 2010, 2012a; Westenberger et al. 2004). Several new clades have emerged as a result of these studies. Interestingly, some of them have already exceeded the diversity of the genus *Trypanosoma*, which has been sampled extensively and studied for more than a century. While it is likely that the number of new species will still be growing at a fast rate, we argue that the number of known major clades will not increase as rapidly, if at all. Virtually all trypanosomatids sampled recently in one of the biological hot spots in Papua New Guinea fell into already known clades (J.V. and J.L., unpubl. data). Hence, the case of a novel species-rich clade reported herein is rather exceptional. The finding that 15% of fleas collected on birds, mammals and bats in different localities of the Czech Republic are infected suggests that these blood-sucking insects are likely to be massively parasitized by the flagellates worldwide. The well-supported monophyly of the *Blechomonas* clade indicates that this is a natural taxon and here we suggest recognizing it as a new subfamily Blechomonadinae subf. n. In molecular systematics, the apomorphy-based definition of the clade is routinely used when the evolutionary event at the origin of the clade is known (de Queiroz and Gauthier 1994). One such example concerns genera *Strigomonas* and *Angomonas*, for which the establishment of endosymbiosis with a β-proteobacterium by their common ancestor represents a unique and unifying event (Du et al. 1994; Motta et al. 2013; Teixeira et al. 2011). In most cases, however, such evolutionary events are not known, and hence it is not clear what are the distinct features, if any, defining such taxa (Jirků et al. 2012; Maslov et al. 2010; Yurchenko et al. 2008). Yet, as long as there is distinguishing molecular data, preferably represented by a combination of RNA- and protein-coding genes, morphological information is not indispensable (and can be added in the future if it becomes available) (Maslov et al. 2013). Following these criteria we have recently proposed to recognize a new subfamily Leishmaniinae (Jirků et al. 2012).

In the case of *Blechomonas* the evolutionary event that led to its separation can be traced back to the exploration of fleas as potential hosts. Because the peculiarities of the fleas' life cycle may put extraordinary evolutionary pressure on their parasites, such exploration must have been a rare event. Therefore, the *Blechomonas* clade can be defined by the descent from a given common ancestor. It needs to be stressed that the parasitism of the order Siphonaptera does not define this taxon per se, since even in this study we have demonstrated that not all flagellates of fleas are members of this clade. Trypanosomatids unrelated to *Blechomonas* spp. seem to be exploring the same host niche independently, as can be exemplified by the rare cases of *Trypanosoma* sp., *Herpetomonas* spp. (*H. nabiculae* and a new *Herpetomonas* species) and *Leptomonas* spp. (isolates closely related to *L. tenua* and '*W. inconstans*') identified here.

One of the attributes of the genus *Blechomonas* might have evolved as a consequence of the unique life cycle of Siphonaptera (Jones 1984). Fleas are holometabolous insects meaning that they are undergoing radical metamorphosis, with the larval and adult stages differing considerably in their structure and behavior (Mito et al. 2010). Since adult fleas take blood meal exclusively from their hosts and *Blechomonas* spp. seem to be restricted to the Siphonaptera, the infections must be established at the larval stage and the parasites must accompany the host throughout its metamorphosis.

An interesting possibility is that these presumably monoxenous trypanosomatids may be occasionally transmitted into the warm-blooded vertebrates.

Adult fleas are vectors for many pathogens including etiological agents of serious human diseases. Whether they can transmit flagellates remains to be investigated. Interestingly, one species of Trypanosomatidae similar to *B. pulexsimulantis* was documented causing an opportunistic infection in HIV-positive patients (Pacheco et al. 1998).

The predominant presence of parasites in hindguts (mainly in rectal ampoules) correlates with previous studies (Mackinnon 1909; Molyneux et al. 1981). Occurrence of trypanosomatids in the rear part of the flea' intestine relates to the supposedly contaminative way of their transmission. Because of their high nutritional content the feces of adult fleas are often ingested by the larvae thus facilitating the spread of the infection (Beard et al. 1989).

Most new species described in the frame of this study are highly polymorphic and display an extensive variability in cell size, length of the flagellum and relative positions of the nucleus and kinetoplast. Moreover, usually two to three morphotypes, each variable, can be detected in axenic cultures derived from the infected fleas. In agreement with the results of the extensive studies of heteropteran and dipteran trypanosomatids, *Blechomonas* spp. display no unique morphological features that would distinguish them from other flagellates. This further confirms the now widely accepted notion that morphology is generally useless for taxonomy of monoxenous trypanosomatids (Maslov et al. 2013; Votýpka et al. 2012a).

Methods

Field work and DNA isolation: Fleas were manually collected from their hosts or from hosts' nests throughout the Czech Republic. The handling of vertebrate hosts was approved by the Ethical Committee of the Faculty of Science, Charles University (CZU 945/05). Fleas were killed by quick immersion into 96% ethanol within 12 hours after the capture, washed and dissected in 0.9% sterile saline solution. Their intestines were analyzed for the presence of trypanosomatids by light microscopy as described previously (Votýpka et al. 2010). When flagellates were detected, one third of the infected gut was smeared on microscopic slides and stained, another third was used for cultivation (see below) and the remaining part of the gut material was preserved in 0.5 ml of 2% SDS, 100 mM EDTA solution for DNA isolation. DNA was purified using High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany) per manufacturer's protocol as described (Maslov et al. 2007; Westenberger et al. 2004).

Cultures: Primary cultures were established in composite Schneider's SDM/RPMI-1640 medium supplemented with 10% FBS, amikacin (200 μg/ml), penicillin (100 U/ml), and fluorocytosine (1500 μg/ml) at 24 °C. If found contaminated with fungi, they were further purified using U-shaped tubes (Podlipaev and Frolov 2000). Axenic cultures were kept in horizontal flasks filled with media, and passaged as needed, usually once *per* two weeks, since the growth was relatively slow. In addition, the previously characterized *Leptomonas pulexsimulantis* (Beard et al. 1989) was obtained from the American Type Culture Collection, Manassas, USA (ATCC #50186) and included in our analysis. It was cultivated in Schneider's SDM supplemented with 10% FBS and antibiotics per supplier's protocol. This is the only species available from the survey of fleas' flagellates collected in Alachua County, Florida, USA (Beard et al. 1989, 1990).

Microscopy: Light and transmission electron microscopy were done as described elsewhere (Yurchenko et al. 2006a, b, 2008) and the kDNA disks were measured as reported before (Lukeš and Votýpka 2000; Svobodová et al. 2007). Every isolate analyzed in culture was observed as a spectrum of several morphotypes judged by the differences in cell morphology, relative position of the nucleus and kinetoplast, and length of the flagellum. At least 30 cells of the most abundant morphotype were analyzed for each isolate and compared to the environmental samples when available.

RAPD analyses, PCR amplification and sequencing: The random amplified polymorphic DNA (RAPD) analyses were performed as described elsewhere using the following oligonucleotides: OPA-3 (5′-AGTCAGCCAC), OPD-8 (5′-GTGTGCCCCA), OPA-10 (5′-GTGATCGCAG), and OPD-13 (5′-GGGGTGACGA) (Dvorak et al. 2006). The products were resolved in 1% agarose gel and visualized by SybrSafe (Life Technologies, Prague, Czech Republic). The RAPD analysis was reproducible with minor variations in the intensity of individual bands as previously reported (Svobodová et al. 2007).

PCR amplification of the SL RNA gene repeats was performed using primers M167 (5′-GGGAAGCTTCTGATTGGTTACTWTA) and M168 (5′-GGGAATTCAATAAAGTACAGAAACTG) (Maslov et al. 2007; Westenberger et al. 2004), or ME1 (5′-TGTACTTTCTTATTGGTA) and ME2 (5′-CAATAAAGTGAAACAACTG) (Podlipaev et al. 2004). Amplicons were cloned into the pGEM-T Easy (Promega, Madison, USA) or pCR2.1 (Life Technologies) vector systems, sequenced and analyzed as described elsewhere (Yurchenko et al. 2006b).

SSU rRNA and gGAPDH genes were amplified and sequenced as described previously (Maslov et al. 1996, 2010; Yurchenko et al. 2006b). The GenBank accession numbers for the sequences determined in the course of this work are summarized in Table 1.

Phylogenetic analyses: The SSU rDNA and gGAPDH sequences were aligned using Kalign (http://www.ebi.ac.uk/Tools/msa/kalign/) and the resulting alignments were edited manually using BioEdit software to remove the fast-evolving regions (mainly for the SSU rRNA sequences) preventing unambiguous alignment. One obvious drawback of this approach is the apparent loss of resolution in the consensus tree, which can be however compensated by high bootstrap support of the clades recoverable using this method (Jirků et al. 2012; Maslov et al. 2010; Yurchenko et al. 2008). The final concatenated SSU rDNA+gGAPDH alignment included 3099 characters and is available upon request.

Phylogenetic analyses of the SSU rDNA and gGAPDH datasets were performed with MrBayes 3.2.1 program (Bayesian criteria: rates for six different types of substitution; proportion of invariant sites and shape parameter of the γ-correction for the rate heterogeneity with four discrete categories were allowed to vary; the covarion model was used to allow the rate heterogeneity along the tree; the Markov chain Monte Carlo was run for five million generations), PhyML for maximum likelihood (ML: the

best-fitting model [GTR + *I* + Γ] of the sequence evolution was searched using Modeltest 3.7. and bootstrapped with 1000 replicates) and PAUP* for maximum parsimony (MP: using 10 replicates of heuristic search and bootstrapped with 1000 replicates).

The SL RNA alignment was done as described earlier (Votýpka et al. 2010, 2012b; Votýpka et al. 2010). For species comparisons, only conserved region of the SL RNA repeats, starting at position "-100" upstream of the exon and ending at the 3′ end of the intron, was used. SL RNA sequences were aligned with Clustal-X (gap opening penalty 12; gap extension penalty 5) and neighbor-joining clustering with K2P distances was performed with unmodified alignment using PAUP*. The 90% cut-off level applied to the entire sequence was used to delineate individual TUs (Maslov et al. 2007; Votýpka et al. 2010, 2012a).

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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.2013.08.002.

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