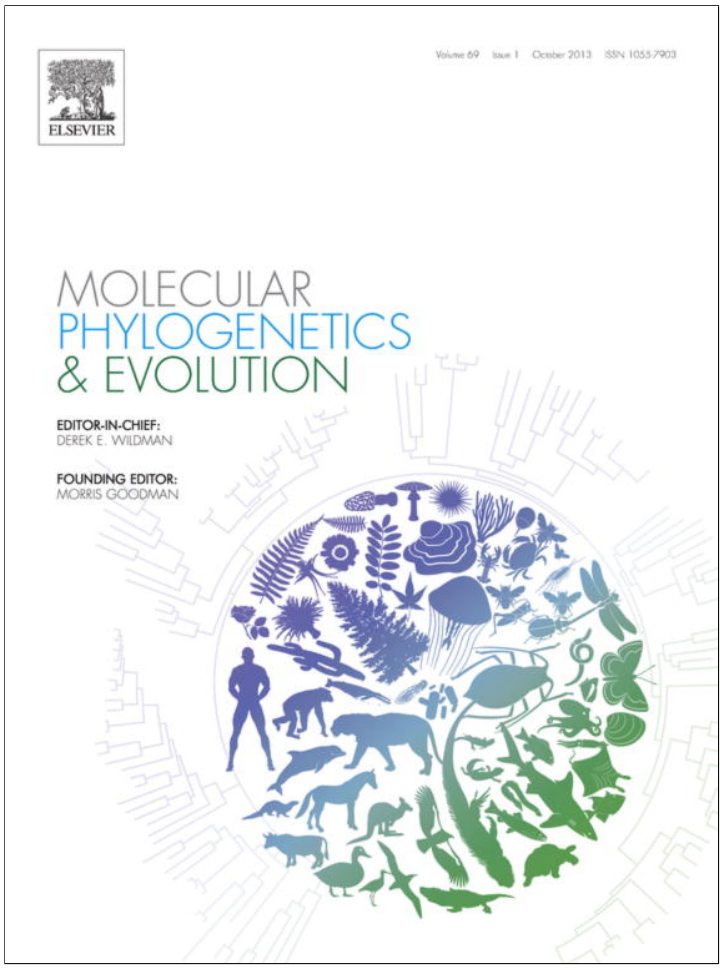

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Growing diversity of trypanosomatid parasites of flies (Diptera: Brachycera): Frequent cosmopolitism and moderate host specificity

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ABSTRACT

Widely distributed, highly prevalent and speciose, trypanosomatid flagellates represent a convenient model to address topics such as host specificity, diversity and distribution of parasitic protists. Recent studies dealing with insect parasites of the class Kinetoplastea have been focused mainly on trypanosomatids from true bugs (Heteroptera), even though flies (Diptera, Brachycera) are also known as their frequent hosts. Phylogenetic position, host specificity and geographic distribution of trypanosomatids parasitizing dipteran hosts collected in nine countries on four continents (Bulgaria, Czech Republic, Ecuador, Ghana, Kenya, Madagascar, Mongolia, Papua New Guinea and Turkey) are presented. Spliced leader (SL) RNA gene repeats and small subunit (SSU) rRNA genes were PCR amplified from trypanosomatids infecting the gut of a total of forty fly specimens belonging to nine families. While SL RNA was mainly used for barcoding, SSU rRNA was utilized in phylogenetic analyses. Thirty-six different typing units (TUs) were revealed, of which 24 are described for the first time and represent potential new species. Multiple infections with several TUs are more common among brachyceran hosts than in true bugs, reaching one third of cases. When compared to trypanosomatids from heteropteran bugs, brachyceran flagellates are more host specific on the genus level. From seven previously recognized branches of monoxenous trypanosomatids, the *Blastocrithidia* and "jaculum" clades accommodate almost solely parasites of Heteroptera; two other clades (*Herpetomonas* and *Angomonas*) are formed primarily by flagellates found in dipteran hosts, with the most species-rich Leishmaniinae and the small *Strigomonas* and "collosoma" clades remaining promiscuous. Furthermore, two new clades of trypanosomatids from brachyceran flies emerged in this study. While flagellates from brachyceran hosts have moderate to higher host specificity, geographic distribution of at least some of them seems to be cosmopolitan. Moreover, the genus *Angomonas*, so far known only from South America, is present on other continents as well.

1. Introduction

Trypanosomatids are a group of very successful and widespread parasites of vertebrates, invertebrates and plants. They belong to the class Kinetoplastea, which also encompasses other free-living, commensalic and parasitic flagellates (Moreira et al., 2004; Simpson et al., 2006). They are well known for several distinct oddities such as surface antigenic variation, huge mitochondrial (=kinetoplast) DNA and RNA editing of its transcripts, polycistronic transcription and *trans*-splicing in the nucleus, to name just a few prominent ones (Lukeš et al., 2005). Medically and/or economically important species, such as *Trypanosoma brucei* responsible for human African sleeping sickness and the livestock disease n'gana, *Trypanosoma cruzi* causing Chagas disease, and *Leishmania* spp., the causative agent of leishmaniases are studied most. Moreover, of significant economic interest are also members of the genus *Phytomonas* that infect plants (Camargo, 1999; Dollet, 1984; Hollar and Maslov, 1997). All parasites mentioned above have a dixenous life style, using various insects or invertebrates as vectors for their transmission.

The interest in members of the Trypanosomatidae confined to insect hosts in a monoxenous life cycle was initially motivated by their predicted extreme diversity, likely due to the ubiquitous presence and species richness of their hosts (Podlipaev, 2001). The few extensive studies of these flagellates, usually combining molecular phylogeny with morphology, unanimously exposed a conflict between the now classical morphology-based taxonomy (Hoare and Wallace, 1966; Wallace, 1966) and molecular data (Teixeira et al., 2011; Votýpka et al., 2010; Yurchenko et al.,

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2008). Moreover, it was shown recently that some trypanosomatid species have extremely diverse morphology that may even differ in the host and in the culture, rendering the few available morphological features virtually useless (Maslov et al., 2013; Podlipaev et al., 2004a; Votýpka et al., 2012b; Zídková et al., 2010).

Currently, there are two main views on the diversity, endemism and dispersal of free-living microorganisms. The ubiquity model postulates that "everything is everywhere", and the environment selects (Finlay, 2002), while the alternative view stresses moderate endemism (Foissner, 2006). However, the debate so far has mostly considered free-living protists, with the situation of parasitic microorganisms being much less addressed (Votýpka et al., 2012b). In this case the ultimate factor is the distribution and behavior of the host, unless the parasite has extensively relaxed its specificity.

As a matter of fact, trypanosomatids seem to vary widely in this character. High specificity was documented for *Leptomonas pulexsimulantis*, which was unable to experimentally infect other than the host flea species (Beard et al., 1989). Another example of narrow host specificity is the well-studied *T. brucei*, restricted to Africa due to *Glossina* spp. being its only known vectors (Balmer et al., 2011; Brun et al., 2010). Similarly, the range of *T. cruzi* correlates with the distribution of its vectors, the Triatominae bugs (Sturm and Campbell, 2010). On the other hand, the number of host switches and hosts infected with multiple species of flagellates is steadily growing (Podlipaev et al., 2004b; Votýpka et al., 2010, 2012a), inevitably leading to the abandonment of the "one host–one parasite" paradigm. The emerging scenario may be rather similar to the one described for the phytophagous insects (Novotný and Basset, 2005), with generalists being rare, and most species retaining certain level of specificity for a group of phylogenetically related hosts (Poulin and Keeney, 2008). Indeed, as shown recently, a heteropteran trypanosomatid *Leptomonas pyrrhocoris* was able to achieve cosmopolitan distribution by extending its host specificity to the level of a family, namely the Pyrrhocoridae (Votýpka et al., 2012b).

To shed further light on host-parasite specificity and especially diversity, several studies of trypanosomatids parasitizing insect hosts (mainly true bugs) sampled in Russia (Kostygov et al., 2004), Ecuador and Costa Rica (Maslov et al., 2007; Westenberger et al., 2004), Brazil (Teixeira et al., 2011), China (Votýpka et al., 2010) and Ghana and Kenya (Votýpka et al., 2012a) have been performed. While sequences of the SSU rRNA (Lukeš et al., 1997; Merzlyak et al., 2001) and/or glycosomal glyceraldehyde-3-phosphate dehydrogenase genes (gGAPDH) (Hamilton et al., 2004; Yurchenko et al., 2006) used in these surveys provide resolution for branches of the phylogenetic trees that represent more distantly related species, the kinetoplastid-specific spliced leader (SL) RNA gene and respective intergenic region are useful as group- or species-specific identification markers (Maslov et al., 2007; Westenberger et al., 2004).

So far, all studies addressing diversity and host–parasite relationships of insect trypanosomatids have been performed on flagellates from Heteroptera, known to be frequently infected. However, Diptera, which represent the other globally distributed, speciose and heavily parasitized insect group (Podlipaev, 1990, 2001), was not subjected to similar studies. Dipteran insects are vectors of the most medically relevant species, such as African trypanosomes transmitted by tse–tse and horse flies, as well as widespread *Leishmania* species transmitted by phlebotomine sand flies. Interestingly, the blood-sucking behavior of some dipterans predisposes them as opportunistic transmitters of monoxenous flagellates to vertebrates, and such infections were already encountered, usually in immunocompromised humans and dogs (Maslov et al., 2013; Morio et al., 2008; Podlipaev et al., 2004b; Srivastava et al., 2010). It is worth noting that elegant, albeit unexpected evidence for the long term co-existence of Diptera with their trypanosomatid parasites comes from the findings of infected specimens trapped in amber (Poinar, 2008; Poinar and Poinar, 2005).

Even though the circulation of insect monoxenous trypanosomatids among their hosts is still predominantly undisclosed, they can be transmitted by contamination, coprophagy, necrophagy or predation (Wallace, 1966), with the fecal transmission being probably the predominant way (Tieszen and Molyneux, 1989). Although few species are also known to produce resistant cyst-like stages called straphangers (Peng and Wallace, 1982; Romeiro et al., 2000), most flagellates are unable to survive outside the host body. Since the life style of heteropterans is generally promiscuous and includes predation, coprophagy, necrophagy, cannibalism, as well as piercing other insects, it is rather difficult to predict which flagellates are specific and which have been randomly acquired via any of these behaviors. Hence, Heteroptera are predisposed to take flagellates from their vicinity as well as their prey, and such transmissions are reflected in previously published phylogenetic trees (Westenberger et al., 2004; Yurchenko et al., 2006; Votýpka et al., 2012a). In this context, it is interesting to note that insects with predatory life styles, such as Mantodea, Ensyfera and Hymenoptera are only very rarely infected with trypanosomatids (Podlipaev, 2001).

Few reports on monoxenous trypanosomatids found in dipterans are available, including morphological descriptions of members of the genera *Angomonas*, *Strigomonas* and *Herpetomonas* from Brazilian flies (Borghesan et al., 2013; Teixeira et al., 1997, 2011) and morphological and phylogenetic characterization of *Sergeia* from European biting midges (Svobodová et al., 2007). It is only an extensive study that can potentially address the following general questions: (i) Can conclusions drawn from studying flagellates from heteropteran hosts be also applied to those found in dipteran hosts? (ii) If so, is there the same level of diversification? (iii) Do dipterans and heteropterans carry parasites specific for each group? (iv) Are flagellates able to cross boundaries between the heteropteran and dipteran hosts?

To answer these questions we combined culture-dependent and -independent approaches, the latter allowing PCR-amplification of trypanosomatid SL and SSU rRNA genes directly from the intestinal tract of brachycerans collected in various countries in Europe, South America, Africa and Asia. To narrow the possibilities of parasite transmission, we focused our interest at so-called higher Diptera, namely at members of the suborder Brachycera. There are no predators in our collection, the studied fly species do not invade tissues of other insects and their general feeding strategy as adults is to lick the surfaces of diverse biological materials (Séguy, 1950), which can be a source of various trypanosomatids. However, since these parasites are likely to originate from other fly species rather than from different insect orders, as is commonly the case of heteropteran bugs, we expect the flagellates found in brachyceran flies to be specific to this group of insects. We want to note that in this study the term "host specificity" refers mostly to specificity at higher taxonomic levels, as more detailed data are not available. We identified several new typing units (TUs) and hence likely new species, and showed that while most isolates were confined to just three previously described clades, several new isolates were found elsewhere in the tree, with a handful forming two newly emerged clades. Throughout the SSU rRNA tree, there is a clear difference between the distribution of trypanosomatids from heteropterans and their relatives from dipterans. Moreover, brachyceran parasites are neither as broadly, nor as evenly distributed in phylogenetical trees as their heteropteran counterparts. Combined with various food sources and the predatory behavior of the heteropteran hosts, the observed distribution pattern supports our hypothesis that the bugs tend to accumulate trypanosomatids

from the environment, serving as a sink and possible dead end for various insect flagellates including the dipteran ones.

2. Materials and methods

2.1. Field work

Insect collections were conducted from 2008 to 2011 in five biogeographical areas: South America (Ecuador), Africa (Ghana, Kenya and Madagascar), Asia (Mongolia and Turkey), Europe (Bulgaria and Czech Republic), and Papua New Guinea. The following sites were sampled in Ecuador: Loja (3°56′37″S; 79°12′1″W) and Otongatchi (0°19′35″S; 78°55′19″W). In Ghana collections were performed in Kokrobite (5°29′42″N; 0°22′8″W), Abrafo (5°20′29″N; 1°22′58″W) and Cape Coast – Fort Victoria (5°6′24″S; 1°14′57″W), in Kenya sampling occurred in Todognang (4°27′22″N; 35°55′48″E) and Nairobi (1°28′88″N; 36°79′45″E), and in Madagascar flies from Ambatolampy (19°22′58″S; 47°25′58″E) and Moramango (18°55′34″S; 48°25′4″E) were examined. Samples from Mongolia originate from Ondorkhaan (47°19′24″N; 110°39′4″E and 47°21′43″N; 110°48′32″E) and Ulan Batar (47°51′28″N; 106°55′53″E). Bulgaria was surveyed and samples were acquired from Pirin Mountains (23°25′34″N; 41°46′5″E), while Czech Republic was examined around Předboř (49°46′5″N; 15°42′41″E) and Prague (50°03′65″N; 14°22′52″E). Finally, samples from Turkey were collected around the Sümella monastery (40°41′31″N; 39°39′28″E), and on Papua New Guinea research took place at Madang's Nagada – Binatang Research Center (5°9′23″S; 145°47′41″E) (also see Table 1). Collections of insect hosts were performed by sweep netting on vegetation. Within 24 h of captivity, insects were killed and rinsed in 70% ethanol. After washing in 0.85% saline physiological solution, they were carefully dissected by pulling out the intact intestine. The gut tissue was then squeezed by cover slip and carefully examined for the presence of flagellates by using 400 × total magnification of a portable microscope. When positive, the infected gut material was transferred from the slide to 1 ml of 2% SDS, 100 mM EDTA solution and samples were stored in ambient temperatures, and after reaching laboratory at −20 °C. In order to establish cultures, aliquot of the freshly obtained sample was also inoculated into 1 ml Brain Heart Infusion medium (BHI) enriched with RPMI and Schneider medium (Sigma) and containing 10 μg/ml hemin as described previously (Westenberger et al., 2004). To suppress the growth of bacteria, antibiotics were added at following concentrations: penicillin 500 units/ml, streptomycin 0.5 mg/ml and gentamycin 500 μg/ml. Obtained cultures are deposited in cryobanks of the Faculty of Science, Charles University, Prague and Institute of Parasitology, České Budějovice. Infected host fly specimens were stored after dissection in 70% ethanol for determination. Determination was based on morphology; species (or higher taxonomic level if the specimen was badly damaged by dissection procedure) are listed in Table 1.

2.2. DNA isolation, PCR amplification, cloning and sequencing

Total DNA was isolated from the preserved environmental samples or from axenic cultures by a High Pure PCR Template Preparation Kit (Roche) according to the manufacturer's manual, or by Chelex (Sigma–Aldrich). Isolated DNA was then used for amplification of SL RNA and SSU rRNA genes. For SL RNA two primer pairs were used: (i) M167 and M168 (Maslov et al., 2007) or (ii) SL-1S and SL-2A (Westenberger et al., 2004); For the SSU rRNA, primers S762 and S763 and R1 and SSU1B were utilized as describer elsewhere (Maslov et al., 1996; Westenberger et al., 2004). The SL RNA amplicons were gel purified by QIAquick ® gel extraction kit (Qiagen), subsequently cloned into the pCR-TOPO vector (Invitrogen) and sequenced, while the SSU rRNA amplicons were sequenced directly.

2.3. Phylogenetic analysis

A SL RNA alignment was constructed after trimming of the sequences as described earlier (Votýpka et al., 2010, 2012a). Briefly, for species comparisons, only the most conserved section of the SL RNA repeats, starting at position 100 upstream of the exon and ending at position 30 of the intron, was used. All SL RNA sequences available from insect trypanosomatids were aligned with Clustal-X (ver. 2.0; gap opening penalty 12; gap extension penalty 5) and neighbor joining clustering with K2P distances was performed on the unmodified alignment using PAUP (4.0, beta version). The 90% cut-off level applied to the SL RNA sequence was used to delineate individual TUs (Maslov et al., 2007). The SSU rRNA alignment was generated using Kalign, with the ambiguous positions and poorly alignable sequences being manually removed using BioEdit. The final SSU rRNA alignment included 1926 characters. Analyses were performed using Bayesian, maximum likelihood and maximum parsimony approaches with programs and settings as described elsewhere (Votýpka et al., 2010, 2012a).

3. Results

3.1. SL RNA based analysis and barcoding

Forty new isolates of trypanosomatids from brachyceran hosts collected in nine countries in five biogeographical areas were examined in this study (Table 1). After their capture, the intestinal tract of fly hosts was examined by microscopy, and positive samples were preserved for future isolation of total DNA as described above. Moreover, introduction of flagellates into culture was attempted in all cases (see Section 3.4).

The first step in determination of discovered parasites was PCR amplification of the SL RNA gene repeat, which is a kinetoplastid-specific marker widely employed for barcoding of species within this group (Maslov et al., 2007; Pawlowski et al., 2012; Votýpka et al., 2010, 2012a; Westenberger et al., 2004). The most conserved part of the tandemly arranged SL RNA repeats is the region starting – 100 nucleotides upstream from the exon up to the start of the T-tract located downstream from the intron (Maslov et al., 2007), which was further used for multiple sequence alignment and analyzed by neighbor-joining (NJ) method. Due to their highly variable nature and short length, SL RNA sequences can only be used to identify groups – TUs of closely related trypanosomatids, which on the NJ dendrogram form terminal clusters and can be aligned reliably (data not shown). Individual TUs represent arbitrarily defined molecular species at 90% identity in the SL RNA gene sequence (Maslov et al., 2007; Westenberger et al., 2004).

Along with the new isolates, all TUs available up to date were included into the SL RNA-based analysis. Hence, the NJ dendrogram was composed of more than five hundred sequences (Suppl. Fig. 1). The newly obtained sequences were separated into 32 genotypes representing distinct TUs. Furthermore, the SSU rRNA-based analysis identified additional four TUs, for which the SL RNA sequences were not available, resulting in the total number of 36 TUs (Table 2). In the absence of the SL RNA sequence, final assignment to the currently named species was in three cases based on the 95% similarity rule, since the SSU rRNA gene is more conserved than the SL RNA gene. All 36 determined TUs can be divided into three categories, with the first one including 10 already known and named species: *Leishmania tarentolae* (SL RNA sequence not available [N/A]), *Herpetomonas muscarum*, *H. mariadeanei*, *H. samuelpessoai*, *H. modestus* (SL N/A), *H. isaaci* (=TU*107*), *H. puella-*

Table 1
Summary of the samples positive for trypanosomatid infections showing their geographic origin (country and locality), year of collection, hosts (family, genus, species and sex), indication of mixed infection, detected TUs determined in this study based on the SSU rRNA (in bold) or only SL RNA genes, and the GenBank™ accession numbers of the SSU rRNA sequences.

Country	Locality	Year	Code	Family	Genus	Species	Sex	Mixed infection	TUs (1)	SSU Acc. No
Ecuador	Loja	2008	ECU-03	Lauxaniidae					TU*126*	
Ecuador	Otongatchi	2008	ECU-05	Lauxaniidae					TU*114*	
Ecuador	Otongatchi	2008	ECU-06	Lauxaniidae					**TU*117***	KC206003
Ecuador	Otongatchi	2008	ECU-07	Sarcophagidae	*Ravinia*	sp.	F	YES	**TU*110*/TU*116*/*A. desou*.**	KC205996/KC206002/KC205980
Ecuador	Otongatchi	2008	ECU-08	Syrphidae			M	YES	**TU*104*/**TU*127*	KC205989
Bulgaria	Pirin	2010	MB-18	Syrphidae	*Eristalis*	*tenax*	M		TU*114*	
Bulgaria	Pirin	2010	MB-19	Calliphoridae	*Calliphora*	*vomitoria*	M	YES	*H. maria.*/**TU*113***	KC205999
Bulgaria	Pirin	2010	MB-22	Sarcophagidae	*Sarcophaginae*	sp.	F		**TU*17***	KC205987
Ghana	Abrafo	2009	GMO-01	Calliphoridae	*Chrysomya*	*megacephala*	F		***H. mariadeanei***	KC205981
Ghana	Cape Coast	2009	GMO-02	Sarcophagidae	*Sarcophaginae*	sp.	F		**TU*72***	
Ghana	Kokrobite	2009	GMO-04	Sarcophagidae	*Sarcophaginae*	sp.	M	YES	*A. deanei*/***H. sam.***/**TU*115***	KC205983/KC206001
Ghana	Kokrobite	2009	GMO-05	Muscidae	*Musca*	sp.	M	YES	***H. puellarum***/**TU*112***/TU*114*	KC205994/KC205998
Mongolia	Ondorkhaan	2009	M-08	Sarcophagidae			M	YES	***Leishmania tarent.***/TU*128*	KC205986
Mongolia	Ondorkhaan	2009	M-09	Muscidae				YES	***H. modestus***/TU*114*	KC709668
Mongolia	Ulan Batar	2009	M-19	Sarcophagidae	*Sarcophaginae*	sp.	M		TU*128*	
Kenya	Todognang	2009	Ke-19	Sarcophagidae	*Wohlfahrtia*	*nuba*	F		***A. deanei***	KC205976
Kenya	Nairobi	2009	Ke-22	Calliphoridae	*Chrysomya*	*marginalis*	F		**TU*114***	KC206000
Kenya	Nairobi	2009	Ke-23	Calliphoridae	*Chrysomya*	*putoria*	M		TU*114*	
Papua NG	Nagada	2011	PNG-M01	Calliphoridae	*Chrysomya*	*megacephala*	M	YES	***A. deanei***/***H. isaaci***/TU*124*	KC205974/KC709667
Papua NG	Nagada	2011	PNG-M02	Calliphoridae	*Chrysomya*	*megacephala*	F	YES	***A. ambiguus***/TU*125*	KC205973
Turkey	Sumella monastir	2011	TG-09	Syrphidae	*Eristalis*	*tenax*	M	YES	TU*115*/TU*127*	
Turkey	Sumella monastir	2011	TG-10	Syrphidae	*Eristalis*	*tenax*	M		TU*127*	
Turkey	Sumella monastir	2011	TG-11	Sarcophagidae	*Sarcophaginae*	sp.	F		***A. deanei***	KC205975
Madagascar	Ambatolampy	2010	MMO-01	Calliphoridae	*Chrysomya*	*putoria*	M		***H. muscarum***	KC205982
Madagascar	Ambatolampy	2010	MMO-02	Calliphoridae	*Chrysomya*	*putoria*	M	YES	***H. isaaci***/**TU*108***/TU*115*	KC205992/KC205993
Madagascar	Moramango	2010	MMO-09	Lauxaniidae	*Pachycerina*	cf. *vaga*	F		**TU*105***	KC205990
Madagascar	Moramango	2010	MMO-10	Muscidae	*Acritochaeta*	*orientalis*	F		***A. deanei***	KC205979
Czechia	Predbor	2010	MCZ-01	Calliphoridae	*Lucilia*	*caesar*	M		***A. deanei***	KC205977
Czechia	Predbor	2010	MCZ-02	Calliphoridae	*Lucilia*	*silvarum*	F	YES	TU*120*/TU*121*	
Czechia	Predbor	2010	MCZ-03	Anthomyiidae			F		***H. samuelpessoai***	KC205984
Czechia	Predbor	2010	MCZ-04	Muscidae	*Coenosia*	*albicornis*	F	YES	***H. sam.***/TU*103*	KC205985
Czechia	Predbor	2010	MCZ-06	Calliphoridae	*Calliphora*	*vicina*	F		TU*123*	
Czechia	Predbor	2010	MCZ-07	Calliphoridae	*Calliphora*	*vicina*	F		TU*122*	
Czechia	Prague	2011	MCZ-08	Muscidae	*Coenosia*	*tigrina*	F		***H. puellarum***	KC205995
Czechia	Prague	2011	MCZ-09	Lauxaniidae					**TU*111***	KC205997
Czechia	Prague	2011	MCZ-10	Drosophilidae					**TU*106***	KC205991
Czechia	Prague	2011	MCZ-11	Scathophagidae	*Cordilura* (*Cordilurina*)	*albipes*	F		**TU*103***	KC205988
Czechia	Prague	2011	MCZ-12	Calliphoridae	*Onesia*	*austriaca*	F		***A. deanei***	KC205978
Czechia	Prague	2011	MCZ-13	Muscidae	*Coenosia*	*tigrina*	F		**TU*119***	KC206005
Czechia	Prague	2011	MCZ-14	Opomyzidae	*Opomyza*	*germinationis*	F		**TU*118***	KC206004

(1) TUs were determined according to SL RNA or SSU rRNA genes (in bold).

Table 2
Summary of the trypanosomatid species or typing units (TUs) and the list of strains detected in the brachyceran flies, their availability in culture and the availability of SSU and SL RNA gene sequences together with their geographical origin and hosts families in which they were found.

Typing unit	New TU	Culture	SSU (1)	SL (2)	Strains	Distribution (3)	Host family (detected worldwide until now) (4)
L. tarentolae		No	Yes		M-08 (ENVI)	Mongolia/EU, AS	Sarcophagidae/Phlebotominae
H. muscarum		Yes	Yes	Yes	MMO-01 (CULT)	Madagascar/USA, Brasil	Calliphoridae/Muscidae, Phoridae, Syrphidae
H. mariadeanei		Yes	Yes	Yes	GMO-01 (CULT), MB-19 (ENVI)	Ghana, Bulgaria/Brasil	Calliphoridae/Muscidae
H. samuelpessoai							Yes
Yes	Yes	GMO-04 (CULT), MCZ-03&04 (ENVI)			Ghana, Czechia/Brasil, Guinea Bissau	Sarcophagidae, Muscidae, Anthomyiidae/Faniidae, Calliphoridae//Heteroptera	
H. modestus			Yes		M-09 (ENVI)	Mongolia/Brasil	Muscidae/Calliphiridae
H. isaaci (TU107)		Yes	Yes	Yes	MMO-02 (CULT), PNG-M01 (CULT)	Madagascar, PNG/Brasil, Guinea Bissau	Calliphoridae/Muscidae, Syrphidae
H. puellarum (TU109)		Yes	Yes	Yes	MCZ-08 (ENVI), GMO-05 (CULT)	Ghana, Czechia/Brasil, Guinea Bissau	Muscidae/Calliphoridae
A. deanei		Yes	Yes	Yes	GMO-04 (ENVI), MCZ-01&12 (ENVI), Ke-19 (ENVI), MMO-10 (CULT), TG-11 (ENVI), PNG-M01 (ENVI)	Ghana, Czechia, Kenya, Madagascar, Turkey, PNG/NW	Calliphoridae, Sarcophagidae, Muscidae/Syrphidae// Heteroptera
A. ambiguus		Yes	Yes	Yes	PNG-M02 (CULT)	PNG/Brasil	Calliphoridae
A. desouzai			(Yes)		ECU-07 (ENVI)	Ecuador/Brasil	Sarcophagidae/Calliphoridae, Syrphidae
TU17			(Yes)	Yes	MB-22 (ENVI)	Bulgaria/Ecuador, Czechia, Ghana	Sarcophagidae//Heteroptera
TU72				Yes	GMO-02 (ENVI)	Ghana/Sicily, Ghana	Sarcophagidae//Heteroptera
TU103	Yes	Yes	Yes	Yes	MCZ-04 (ENVI), MCZ-11 (CULT)	Czechia	Muscidae, Scathophagidae
TU104	Yes	Yes	Yes	Yes	ECU-08 (CULT)	Ecuador	Syrphidae
TU105	Yes	Yes	Yes	Yes	MMO-09 (CULT)	Madagascar	Lauxaniidae
TU106	Yes		Yes	Yes	MCZ-10 (ENVI)	Czechia	Drosophilidae
TU108	Yes		Yes		MMO-02 (ENVI)	Madagascar	Calliphoridae
TU110	Yes	Yes	Yes	Yes	ECU-07 (CULT)	Ecuador	Sarcophagidae
TU111	Yes		Yes	Yes	MCZ-09 (ENVI)	Czechia	Lauxaniidae
TU112	Yes		Yes	(Yes)	GMO-05 (ENVI)	Ghana	Muscidae
TU113	Yes		(Yes)	Yes	MB-19 (ENVI)	Bulgaria	Calliphoridae
TU114	Yes		(Yes)	Yes	ECU-05 (ENVI), MB-18 (ENVI), M-09 (ENVI), Ke-22&23 (ENVI), GMO-05 (ENVI)	Ecuador, Bulgaria, Mongolia, Kenya, Ghana	Muscidae, Calliphoridae, Syrphidae, Lauxaniidae
TU115	Yes		(Yes)	Yes	TG-09 (ENVI), MMO-02 (ENVI), GMO-04 (ENVI)	Turkey, Madagascar, Ghana	Calliphoridae, Sarcophagidae, Syrphidae
TU116	Yes		(Yes)	Yes	ECU-07 (ENVI)	Ecuador	Sarcophagidae
TU117	Yes		Yes	(Yes)	ECU-06 (ENVI)	Ecuador	Lauxaniidae
TU118	Yes		Yes	Yes	MCZ-14 (ENVI)	Czechia	Opomyzidae
TU119	Yes		Yes	Yes	MCZ-13 (ENVI)	Czechia	Muscidae
TU120	Yes			Yes	MCZ-02 (ENVI)	Czechia	Calliphoridae
TU121	Yes			Yes	MCZ-02 (ENVI)	Czechia	Calliphoridae
TU122	Yes			Yes	MCZ-07 (ENVI)	Czechia	Calliphoridae
TU123	Yes			Yes	MCZ-06 (ENVI)	Czechia	Calliphoridae
TU124	Yes			Yes	PNG-M01 (ENVI)	PNG	Calliphoridae
TU125	Yes			Yes	PNG-M02 (ENVI)	PNG	Calliphoridae
TU126	Yes			Yes	ECU-03 (ENVI)	Ecuador	Lauxaniidae
TU127	Yes			Yes	TG-09&10 (ENVI), ECU-08 (ENVI)	Turkey, Ecuador	Syrphidae
TU128	Yes			Yes	M-08&19 (ENVI)	Mongolia	Sarcophagidae

(1) Full or partial (in brackets) sequence of SSU rRNA; (2) sequence of SL RNA; assignment of the SSU and SL RNA sequences to the relevant TU may in some cases be ambiguous (in brackets); (3) findings of trypanosomatids from the geographical area referred to after the slash were published elsewhere; (4) host families referred to after the slash were published elsewhere. (*) TU107 and TU109 were renamed based on new sequences and species descriptions that were published after their establishment.

rum (=TU*109*), *Angomonas deanei*, *A. desouzai* (SL N/A) and *A. ambiguus*. In the case of *A. deanei* all sequences ranked under this taxon were around 90% cut-off value identity of the SL RNA gene, and therefore rather represent a complex of several closely related species. Second category is composed of two TUs already encountered in previous studies. These are TU*17* found worldwide in true bugs (Maslov, personal commun.; Votýpka et al., 2012a,b) and TU*72* found in Ghanian (Votýpka et al., 2012a,b) and Sicilian true bugs (unpublished data). The last and by far biggest category is composed of 24 TUs that are novel ones (TU*103–6*, *108*, *110–28*). Most of these newly identified TU clusters fell within the *Herpetomonas*, Leishmaniinae (formerly "SE" clade) and *Angomonas* clades, with just a handful of them forming their own separate branches. Among these new TUs, we were unable to amplify the SL RNA sequence in one case (TU*108*); however, for this isolate (MMO-02 ENVI) the SSU rRNA gene sequence has been obtained and unambiguously differs from any published SSU rRNA.

Footnote: TU*107* and TU*109* were assigned to isolates MMO-02 CULT plus PNG-M01 CULT and MCZ-08 ENVI plus GMO-5 CULT, respectively, because the sequences of *H. isaaci* (TU*107*) and *H. puellarum* (TU*109*) were published only following this assignment.

3.2. SSU rRNA-based phylogenetic analysis

Since in general the SL RNA sequences are unsuitable for resolving phylogenetic relationships among more distantly related trypanosomatids, in order to determine newly isolated parasites, we resorted to a marker with higher resolution power, namely the SSU rRNA gene. We intended to obtain a full length sequence from each TU, yet in 10 out of 36 cases multiple attempts failed and in six other cases only part of the SSU rRNA gene has been amplified (Table 2). Combined, 11 and four new TUs are supported by complete and partial SSU rRNA sequence data, respectively, while other SSU rRNA sequences correspond to the previously described *L. tarentolae*, *H. muscarum*, *H. mariadeanei*, *H. samuelpessoai*, *H. isaaci*, *H. puellarum*, *H. modestus*, *A. deanei*, *A. ambiguus*, *A. desouzai* and TU*17*.

The SSU rRNA-based phylogeny is shown in Fig. 1. In general, this tree is in agreement with the one obtained for the SL RNA sequences (Suppl. Fig. 1). The finding of *H. mariadeanei* in flies of the family Calliphoridae in Ghana and Bulgaria is unexpected, since the species was so far known from just a single isolate from Brazil. This isolate is particularly interesting from the phylogenetic point of view, as it probably constitutes a basal lineage to all other *Herpetomonas* species (Yoshida et al., 1978; Borghesan et al., 2013). TUs *103–105* cluster within the Leishmaniinae ("SE") clade with TU*106* appearing at its base. Within the *Angomonas* clade, *A. deanei* was identified in at least seven separate cases in flies captured in six countries from four continents and thus seems to be a frequent and cosmopolitan species, while *A. ambiguus* and *A. desouzai* were encountered just once. TU*17* is affiliated with the rather rare *Blastocrithidia* clade, which was up to this finding known solely from the heteropteran hosts. Another interesting case is TU*110* in the *L. collosoma* branch, which became the first representative of this clade available in culture and hence amenable to further investigations.

The remaining 24 TUs introduced in this study are novel, qualifying them as candidates for new taxonomic units within Trypanosomatidae. One cluster consists of TU*116* and TU*117*, being so far unique to Ecuador, while another one, composed of TU*112* to TU*115*, is related to TU*84* previously isolated from a Ghanian heteropteran bug. TU*118* and TU*119* represent two different lineages lacking close relatives. The phylogenetic position of all four lineages is relatively unstable, and the same applies for TU*111* and TU*106*. Since none of these TUs is currently available in culture, more thorough characterization of these intriguing trypanosomatids remains impossible.

We failed to amplify corresponding SSU rRNA genes for nine TUs (TU*120–128*). Their current phylogenetic position among the other trypanosomatids is therefore based only on the poorly informative SL RNA sequences and hence remains uncertain. Not unexpectedly, in two other cases, the sequence data for the SL and SSU rRNA genes for a given isolate were incongruent. This can be a result of mixed infections and/or random amplification from different parasite species.

3.3. Host specificity, geographic distribution and multiple infections

We have found unexpectedly strong host specificity on the genus level (Fig. 1). Our data show that the genera *Herpetomonas* and *Angomonas* are predominantly parasites of Diptera with occasional host switch for the heteropteran insects, which probably occurs by predation. The same situation applies in the case of the two new clades emerging around TU*116* + TU*117* ("new clade 1" in Fig. 1) and TU*84* + TU*112–115* ("new clade 2" in Fig. 1), both of which seem to be primarily brachyceran parasites. Members of the speciose Leishmaniinae clade are much more promiscuous when their hosts are considered, as they are found in dipterans and heteropterans. The two remaining monoxenous clades are confined to bugs with two exceptions: TU*17* and TU*72* isolated from Bulgarian and Ghanian flies of the family Sarcophagidae are related to the *Blastocrithidia* and "jaculum" clades, respectively (TU*72* is not marked in Fig. 1, as it is based on the SL RNA sequence only; see Table 2 and Suppl. Fig. 1). Due to the life style and feeding behavior of host flies, it is easy to imagine contaminative acquisition of their flagellates. As a major surprise came the finding of the dixenous *L. tarentolae* in a fly of the family Sarcophagidae, as it is known to be transmitted by blood-sucking sand flies of the subfamily Phlebotominae.

While the available data nicely demonstrate that there are clades of trypanosomatids primarily parasitizing bugs or flies, we also found evidence for multiple host switches and only more detailed study may reveal their frequency. When geographic distribution is considered, the genus *Angomonas* is no more restricted to South America, since *A. ambiguus* was identified in a fly from Papua New Guinea and *A. deanei* is not only common but a true cosmopolitan species found in all five biogeographical areas investigated (Table 2). At this point, however, we cannot exclude that this is in fact a cluster of very closely related species, as indicated by the rather divergent SL RNA sequences. Cosmopolitan distribution was also established for several members of the genus *Herpetomonas*: *H. muscarum*, *H. puellarum* and *H. samuelpessoai* all found in frame of this study in Africa and Europe, in addition to their already known occurrence in South America. Moreover, we have found *H. modestus* and *H. isaaci* in flies from Asia and Africa, and Papua New Guinea, respectively, while both were so far known only from South America. Wide geographic distribution is apparently no exception in our dataset, as TU*115* was found in Madagascar, Ghana and Turkey, while TU*127* was isolated from the Turkish and Ecuadorian hosts. Yet the most remarkable example of cosmopolitan species is TU*114* encountered not only on all four continents, but in fact in most locations subjected to detailed sampling, such as Ecuador, Bulgaria, Mongolia, Kenya and Ghana. The remaining new TUs were found only once (or twice, as in the cases of TU*103* and TU*128*) and are therefore known solely from one or two localities, although due to the patchiness of this study, conclusions on their distribution and host specificity would be rather premature. It is of interest that species with broader distribution have lower host specificity (Table 2), providing credibility to the notion that such a distribution may be facilitated by lower specificity of the parasite.

Our data also show generally higher incidence of multiple infections in the brachyceran flies as compared to the heteropteran

bugs. So far occasional multiple infections have been described from bugs (Maslov et al., 2013; Podlipaev et al., 2004b; Votýpka et al., 2010, 2012a,b), yet 13 co-infections out of 40 infected brachyceran specimens represent a substantial fraction (33%) of all cases (Table 1). Such a high incidence of multiple infections must be a consequence of the ecological and behavioral differences between bugs and flies, and asks for particular caution when the SSU rRNA sequences are assembled from two separately amplified fragments. We tried to avoid generating such chimeric molecules by carefully comparing alignments before running the phylogenetic analysis; however, in one case (TU*117*) we were unable to exclude such a possibility.

3.4. Cultivation

PCR amplification revealed a vast and until now hidden biodiversity of trypanosomatids in brachyceran (and potentially other dipteran) hosts. Unfortunately, attempts to introduce these flagellates into cultivation media in most cases failed. This may be caused either by their inability to grow in the tested media or by large number of contaminating bacteria regularly found in the digestive tract of flies. Finally, flagellates carrying endosymbionts sensitive to antibiotics, which are invariably added to the media in order to prevent bacterial growth, cannot usually be established in culture. As a consequence, some newly emerging and basal lineages do not have a cultivable representative, preventing their in-depth study.

Establishing axenic cultures was successful in the following 11 cases: *A. deanei*, *A. ambiguus*, *H. mariadeanei*, *H. muscarum*, *H. samuelpessoai*, *H. isaaci*, *H. puellarum*, TU*103*, TU*104*, TU*105* and TU*110* (Table 2). The representatives can be subjected to future thorough analysis, include whole genome sequencing.

4. Discussion

In this work an extensive probe into the diversity of monoxenous (insect-only) trypanosomatids parasitizing flies is presented. We focused on dipterans from the suborder Brachycera, the ecology and life style of which are quite different from those of Heteroptera, so far the best studied hosts of trypanosomatids (Maslov et al., 2013, 2010; Votýpka et al., 2010, 2012b). By comparing isolates from localities as geographically distant as the Czech Republic, Ecuador, Madagascar and Papua New Guinea, to name just a few sites, we tried to assess the global diversity of these widespread, yet overlooked parasites.

When abundance, prevalence and distribution are considered, trypanosomatids belong to the most common parasites on Earth (Vickerman, 1994), rivaled among parasitic protists only by apicomplexans (Pawlowski et al., 2012). Monoxenous trypanosomatids have been encountered in a broad range of arthropods, yet their highest prevalence is associated with Heteroptera and Diptera (Podlipaev, 1990). One possible explanation is that these insects are the original hosts, while the other insect groups are still under colonization, accompanied by sub-optimal and less efficient transmission. An alternative hypothesis postulates a key role of host ecology. The requirement for moist environment (and/or food sources) for efficient transmission may be the key factor making heteropterans and dipterans perfect target groups for trypanosomatids, as they predominantly do not consume dry food (Séguy, 1950; Schaefer and Panizzi, 2000). Another favorable factor might be the broad list and combination of feeding strategies, as even phytophagous bugs can enrich their menu by grazing on the youngsters of other species. Such extra meal will occasionally be spiced with parasites of their prey. Hence, heteropterans may play a role of a sink for parasites as they are able to feed on different resources and "actively" collect their flagellates. Indeed, the trypanosomatids of Miridae, which are known to be highly promiscuous in their feeding behavior (Wheeler and Skaftason, 2010), are distributed throughout the phylogenetic tree (Votýpka et al., 2012a).

Reduviid bugs are predators and consequently their parasites are highly diverse, with multiple infections being common. Moreover, many of their TUs are shared with other heteropteran families, which are likely their original hosts (Westenberger et al., 2004; Votýpka et al., 2012a). The available data shows that flagellates isolated from reduviids are related to those found also in mosquitoes, demonstrating switching even among evolutionary distant hosts. Other predatory or omnivorous families such as Lygaeidae and Pentatomidae were also shown to host extended number of TUs. Interestingly, there is an exception for aquatic predatory water striders from the family Gerridae that seem to have their own separate clade of parasites (Votýpka et al., 2012a). The proposed explanation holds that aquatic life is somehow separated from its terrestrial kin, together with the fact that their own specific trypanosomatids are probably able to better survive in the aquatic environment.

The life style of Brachycera predetermines them as rather specific hosts. With reference to their mouth part and absent proboscis, they are generally not predators and do not feed on other living organisms. Their feeding behavior includes mainly licking the surfaces containing sugar (such as flowers, honeydew etc.) and other nutrients (various biological materials including feces, carrions, etc.). Within the frame of this study, 40 Brachyceran specimens from nine families were found to be infected with trypanosomatids (Table 1). By using SL RNA sequence for barcoding and supplemented by sequencing of the SSU rRNA gene, these isolates were separated into 36 distinct TUs with 24 being novel.

An important result of this study is that the brachyceran parasites from geographically distant locations are more related to each other than to the trypanosomatids of heteropterans from the same locality. The SSU rRNA-based analysis shows that trypanosomatid clades isolated from Diptera are spread throughout the phylogenetic tree, yet they are practically missing from certain clades, such as *Blastocrithidia* and "jaculum", while the heteropteran parasites are present in all well sampled groups of trypanosomatids.

Moreover, two new Diptera-specific clades emerged in the present study. First one is composed of two Ecuadorian isolates, while the second group is in fact an expansion of an already described branch of TU*84* from a Ghanian bug. Only the most numerous Leishmaniinae clade is relatively equally shared by both groups of hosts. Findings of *Herpetomonas*, *Angomonas* and other trypanosomatids, so far associated with dipteran insects, also in heteropteran bugs can be explained by predation and feeding behavior of the latter hosts. It has to be mentioned that opposite cases have also been encountered, such as the finding of TU*17* and TU*72* in a Sarcophagidae fly, however, such occurrences cannot be explained on the basis of predation. The finding of *L. tarentolae* in another fly from this group can be best explained by a rather speculative scenario involving licking of a wound of an injured gecko infected by this dixenous parasite, cycling between reptiles and phlebotomine sand flies. Consequently, it can be concluded that Sarcophagidae may be exceptional among brachycerans, when specificity of their parasites is considered, as a consequence of their scavenging on contaminated sources. These findings demonstrate that even though flies are generally more restricted to their own trypanosomatids, some exceptions exist and the brachyceran flies could accommodate even parasites which are typical for other insect groups. All in all, dipteran trypanosomatids are clearly different from those infecting Heteroptera (Maslov et al., 2013; Votýpka et al., 2010, 2012a).

Another examined aspect was the geographic distribution of these parasites throughout the world. Until recently one group that

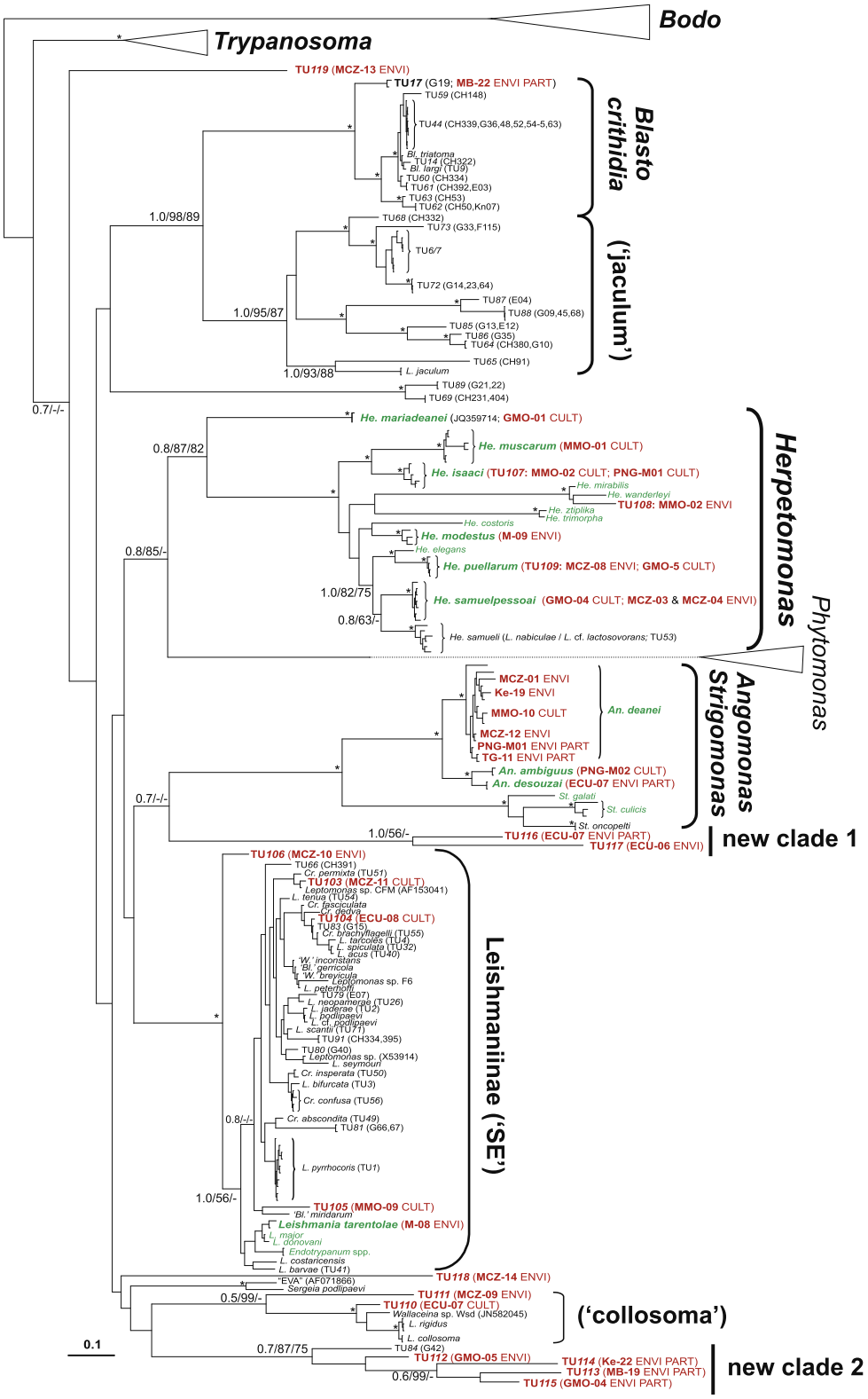

Fig. 1. SSU rRNA-based Bayesian phylogenetic tree of trypanosomatids. Name of species or number of TU (strain; Acc. No. if necessary) are indicated. Full or partial (PART) SSU rRNA genes of the new typing units (TUs) from the brachyceran hosts described in this study are shown in red. Sequences were amplified either directly from the gut samples (ENVI) or from cultures (CULT) derived from the gut samples. Species described from the dipteran host in previous studies are highlighted in green. Bootstrap values from Bayesian posterior probabilities (5 million generations), maximum parsimony and maximum likelihood (1000 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. SSU rRNA sequences determined in this work were deposited under the GenBank™ Accession Numbers KC205973–KC206005 and KC709667–KC709668 (see Table 1).

appeared to be confined to a single continent was the *Angomonas–Strigomonas* clade of South America (Teixeira et al., 2011). However, *Angomonas* spp. were encountered in almost all localities across four continents examined in frame of this study. *A. ambiguus* was found in flies from Papua New Guinea, and *A. deanei* is a true cosmopolitan, which may in the future turn into a congregation of very closely related species, since the divergence of the SL RNA gene between the individual isolates was around 90%. A member of the genus *Strigomonas* was recently also identified outside South America, namely in Europe (Wilfert et al., 2011).

Broad or even cosmopolitan presence can be achieved by low specificity, with the parasite not being restricted to the area of distribution of only one host. Such strategy may be exemplified by TU*17* and TU*72*, which have been found on at least two continents and in two different orders of insects, or the cosmopolitan TU*44* known to parasitize several families of the order Heteroptera (Votýpka et al., 2012a). Additional TUs with apparently cosmopolitan distribution are TU*114*, TU*115*, TU*127* and all *Herpetomonas* species detected in flies in the current study. It would be interesting to test whether the generalists TU*17* and TU*72* have the capacity to infect not only other insects but also vertebrates, as was documented for *Herpetomonas* (Podlipaev et al., 2004b) and a handful of other monoxenous species (Barreto-de-Souza et al., 2008; Morio et al., 2008; Srivastava et al., 2010). Alternative way how to accomplish a global distribution is not to extend specificity, but to find a cosmopolitan host. Here the most convincing example is *L. pyrrhocoris* that parasitizes solely the family Pyrrhocoridae, which is distributed throughout the world (Votýpka et al., 2012b).

Although infections with a single TU dominate, some dipterans host dual or even multiple infections with such cases being significantly more frequent then in Heteroptera (Votýpka et al., 2010, 2012a). Moreover, the same phenomenon was described for *Drosophila* spp. in Europe (Wilfert et al., 2011). It is easy to imagine that the brachyceran hosts, usually very active and found in large quantities licking and vacuuming surfaces of their food sources, frequently encounter parasites of their competitors, predominantly other fly species, and hence acquire multiple infections.

Out of 36 TUs, only 11 were successfully transferred into the culture. Unfortunately, none of them represents a newly emerged clade or basal lineage discovered in our survey. Various reasons responsible for that situation can be invoked, such as the requirement of special conditions or nutrients unavailable in our simple media designed for the broadest range of parasites, or sensitivity to antibiotics ordinarily added to suppress bacterial growth. This concern is highly relevant for isolates carrying bacterial endosymbionts, such as *Angomonas* and *Strigomonas* spp. (Teixeira et al., 2011).

Of particular interest is isolate GMO-01, identical with *H. mariadeanei*, which probably constitutes a basal lineage to all other *Herpetomonas* species (Teixeira et al., 2011) and was so far known from just a single isolate (Yoshida et al., 1978). Moreover, ECU-07 (=TU*110*) in the *L. collosoma* branch is the first representative of this clade available in culture and a candidate for whole genome sequencing. Such a project may shed light on the emergence of the dixenous life style from the monoxenous and presumably ancestral state. Another feature of species parasitizing Brachycera is their low specificity on the genus level and broad geographical distribution. Not only is the "one host–one parasite" paradigm untenable for monoxenous trypanosomatids, but their specificity is more relaxed than that of their dixenous siblings. It appears that the two very different environments they encounter in the course of their dixenous lifestyle confine these flagellates to a rather narrow group of insect vectors.

The general picture emerging for trypanosomatids collected from brachycerans favors the scenario postulating that there are not many parasite generalists, but parasites in general tend to be specific for a group of phylogenetically related hosts (Poulin and Keeney, 2008). There is a clear specificity of most trypanosomatids for either heteropteran or dipteran hosts. Moreover, under certain rare circumstances, flagellates confined to heteropterans are able to cross host barriers, infecting dipterans or *vice versa*.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2013.05.024.

References

Balmer, O., Beadell, J.S., Gibson, W., Caccone, A., 2011. Phylogeography and taxonomy of *Trypanosoma brucei*. PLoS Negl. Trop. Dis. 5, e961.
Barreto-de-Souza, V., Xavier Medeiros, T., Machado Motta, M.C., Chequer Bou-Habib, D., Saraiva, E.M., 2008. HIV-1 infection and HIV-1 Tat protein permit the survival and replication of a non-pathogenic trypanosomatid in macrophages through TGF-β1 production. Microbes Infect. 10, 642–649.
Beard, C.B., Butler, J.F., Greiner, E.C., 1989. In vitro growth characterization and host-parasite relationship of *Leptomonas pulexsimulantis* n. sp., a trypanosomatid flagellate of the flea *Pulex simulans*. J. Parasitol. 75, 658–668.
Borghesan, T.C., Ferreira, R.C., Takata, C.S.A., Campaner, M., Borda, C.C., Paiva, F., Milder, R.V., Teixeira, M.M.G., Camargo, E.P., 2013. Molecular phylogenetic redefinition of *Herpetomonas* (Kinetoplastea, Trypanosomatidae), a genus of insect parasites associated with flies. Protist 164, 129–152.
Brun, R., Blum, J., Chappuis, F., Burri, C., 2010. Human African trypanosomiasis. Lancet 375, 148–159.
Camargo, E.P., 1999. Phytomonas and other trypanosomatid parasites of plants and fruit. Adv. Parasitol. 42, 29–112.
Dollet, M., 1984. Plant-diseases caused by flagellate protozoa (*Phytomonas*). Annu. Rev. Phytopathol. 22, 115–132.
Finlay, B.J., 2002. Global dispersal of free-living microbial eukaryote species. Science 296, 1061–1063.
Foissner, W., 2006. Biogeography and dispersal of micro-organisms: a review emphasizing protists. Acta Protozool. 45, 111–136.
Hamilton, P.B., Stevens, J.R., Gaunt, M.W., Gidley, J., Gibson, W.C., 2004. Trypanosomes are monophyletic: evidence from genes for glyceraldehyde phosphate dehydrogenase and small subunit ribosomal RNA. Int. J. Parasitol. 34, 1393–1404.
Hoare, C.A., Wallace, F.G., 1966. Developmental stages of trypanosomatid flagellates – a new terminology. Nature 212, 1385–1386.
Hollar, L., Maslov, D.A., 1997. A phylogenetic view on the genus *Phytomonas*. Mol. Biochem. Parasit. 89, 295–299.
Kostygov, A.Y., Slisarenko, E.P., Merkulov, P.A., Podlipaev, S.A., 2004. Genetic diversity of insect trypanosomatids from subarctic and north west Russia revealed by UP PCR typing. Protistology 3, 257–264.
Lukeš, J., Hashimi, H., Zíková, A., 2005. Unexplained complexity of the mitochondrial genome and transcriptome in kinetoplastid flagellates. Curr. Genet. 48, 277–299.
Lukeš, J., Jirků, M., Doležel, D., Kral'ová, I., Hollar, L., Maslov, D.A., 1997. Analysis of ribosomal RNA genes suggests that trypanosomes are monophyletic. J. Mol. Evol. 44, 521–527.
Maslov, D.A., Lukeš, J., Jirků, M., Simpson, L., 1996. Phylogeny of trypanosomes as inferred from the small and large subunit rRNAs: implications for the evolution of parasitism in the trypanosomatid protozoa. Mol. Biochem. Parasit. 75, 197–205.
Maslov, D.A., Votýpka, J., Yurchenko, V.Y., Lukeš, J., 2013. Diversity and evolution of insect trypanosomatids: all that is hidden shall be revealed. Trends Parasitol. 29, 43–52.
Maslov, D.A., Westenberger, S.J., Xu, X., Campbell, D.A., Sturm, N.R., 2007. Discovery and barcoding by analysis of spliced leader RNA gene sequences of new isolates

of Trypanosomatidae from Heteroptera in Costa Rica and Ecuador. J. Eukaryot. Microbiol. 54, 57–65.
Maslov, D.A., Yurchenko, V.Y., Jirků, M., Lukeš, J., 2010. Two new species of trypanosomatid parasites isolated from heteroptera in Costa Rica. J. Eukaryot. Microbiol. 57, 177–188.
Merzlyak, E., Yurchenko, V., Kolesnikov, A.A., Alexandrov, K., Podlipaev, S.A., Maslov, D.A., 2001. Diversity and phylogeny of insect trypanosomatids based on small subunit rRNA genes: polyphyly of *Leptomonas* and *Blastocrithidia*. J. Eukaryot. Microbiol. 48, 161–169.
Moreira, D., Lopez-Garcia, P., Vickerman, K., 2004. An updated view of kinetoplastid phylogeny using environmental sequences and a closer outgroup: proposal for a new classification of the class Kinetoplastea. Int. J. Syst. Evol. Microbiol. 54, 1861–1875.
Morio, F., Reynes, J., Dollet, M., Pratlong, F., Dedet, J.-P., Ravel, C., 2008. Isolation of a protozoan parasite genetically related to the insect trypanosomatid *Herpetomonas samuelpessoai* from a human immunodeficiency virus-positive patient. J. Clin. Microbiol. 46, 3845–3847.
Novotný, V., Basset, Y., 2005. Host specificity of insect herbivores in tropical forests. Proc. Roy. Soc. B: Biol. Sci. 272, 1083–1090.
Pawlowski, J., Audic, S., Adl, S., Bass, D., Belbahri, L., Berney, C., Bowser, S.S., Čepička, I., Decelle, J., Dunthorn, M., Fiore-Donno, A.M., Gile, G.H., Holzmann, M., Jahn, R., Jirků, M., Keeling, P.J., Kostka, M., Kudryavtsev, A., Lara, E., Lukeš, J., Mann, D.G., Mitchell, E.A.D., Nitsche, F., Romeralo, M., Saunders, G.W., Simpson, A.G.B., Smirnov, A.V., Spouge, J.L., Stern, R.F., Stoeck, T., Zimmermann, J., Schindel, D., de Vargas, C., 2012. CBOL protist working group: barcoding eukaryotic richness beyond the animal, plant, and fungal kingdoms. PLoS Biol. 10, e1001419.
Peng, P.L.-M., Wallace, F.G., 1982. The cysts of *Blastocrithidia triatomae* Cerisola et al., 1971. J. Eukaryot. Microbiol. 29, 464–467.
Podlipaev, S., 1990. Catalogue of world fauna of Trypanosomatidae (Protozoa). Proc. Zool. Inst. Leningrad 144, 1–177.
Podlipaev, S., 2001. The more insect trypanosomatids under study – the more diverse Trypanosomatidae appears. Int. J. Parasitol. 31, 648–652.
Podlipaev, S., Votýpka, J., Jirků, M., Svobodová, M., Lukeš, J., 2004a. *Herpetomonas ztiplika* n. sp (Kinetoplastida: Trypanosomatidae): a parasite of the blood-sucking biting midge *Culicoides kibunensis* Tokunaga, 1937 (Diptera: Ceratopogonidae). J. Parasitol. 90, 342–347.
Podlipaev, S.A., Sturm, N.R., Fiala, I., Fernandes, O., Westenberger, S.J., Dollet, M., Campbell, D.A., Lukeš, J., 2004b. Diversity of insect trypanosomatids assessed from the spliced leader RNA and 5S rRNA genes and intergenic regions. J. Eukaryot. Microbiol. 51, 283–290.
Poinar, G., 2008. *Leptoconops nosopheris* sp. n. (Diptera: Ceratopogonidae) and *Paleotrypanosoma burmanicus* gen. n., sp. n. (Kinetoplastida: Trypanosomatidae), a biting midge: trypanosome vector association from the Early Cretaceous. Mem. Inst. Oswaldo Cruz 103, 468–471.
Poinar Jr., G., Poinar, R., 2005. Fossil evidence of insect pathogens. J. Invertebr. Pathol. 89, 243–250.
Poulin, R., Keeney, D.B., 2008. Host specificity under molecular and experimental scrutiny. Trends Parasitol. 24, 24–28.
Romeiro, A., Solé-Cava, A., Sousa, M.A., de Souza, W., Attias, M., 2000. Ultrastructural and biochemical characterization of promastigote and cystic forms of *Leptomonas wallacei* n. sp. isolated from the intestine of its natural host *Oncopeltus fasciatus* (Hemiptera: Lygaeidae). J. Eukaryot. Microbiol. 47, 208–220.
Schaefer, C.W., Panizzi, A.R., 2000. Heteroptera of Economic Importance. CRC Press, pp. 828.
Séguy, E., 1950. La Biologie des Dipteres. Encycl. Entomol. 26, 609.
Simpson, A.G., Stevens, J.R., Lukeš, J., 2006. The evolution and diversity of kinetoplastid flagellates. Trends Parasitol. 22, 168–174.
Srivastava, P., Prajapati, V.K., Vanaerschot, M., Van der Auwera, G., Dujardin, J.C., Sundar, S., 2010. Detection of *Leptomonas* sp. parasites in clinical isolates of Kala-azar patients from India. Infect. Genet. Evol. 10, 1145–1150.
Sturm, N.R., Campbell, D.A., 2010. Alternative lifestyles: the population structure of *Trypanosoma cruzi*. Acta Trop. 115, 35–43.
Svobodová, M., Zídková, L., Čepička, I., Oborník, M., Lukeš, J., Votýpka, J., 2007. *Sergeia podlipaevi* gen. nov., sp. nov. (Trypanosomatidae, Kinetoplastida), a parasite of biting midges (Ceratopogonidae, Diptera). Int. J. Syst. Evol. Microbiol. 57, 423–432.
Teixeira, M.M.G., Borghesan, T.C., Ferreira, R.C., Santos, M.A., Takata, C.S.A., Campaner, M., Nunes, V.L.B., Milder, R.V., de Souza, W., Camargo, E.P., 2011. Phylogenetic validation of the genera *Angomonas* and *Strigomonas* of trypanosomatids harboring bacterial endosymbionts with the description of new species of trypanosomatids and of proteobacterial symbionts. Protist 162, 503–524.
Teixeira, M.M.G., Takata, C.S.A., Conchon, I., Campaner, M., Camargo, E.P., 1997. Ribosomal and kDNA markers distinguish two subgroups of herpetomonas among old species and new trypanosomatids isolated from flies. J. Parasitol. 83, 58–65.
Tieszen, K.L., Molyneux, D.H., 1989. Transmission and ecology of trypanosomatid flagellates of water striders (Hemiptera: Gerridae). J. Eukaryot. Microbiol. 36, 519–523.
Vickerman, K., 1994. The evolutionary expansion of the trypanosomatid flagellates. Int. J. Parasitol. 24, 1317–1331.
Votýpka, J., Klepetková, H., Jirků, M., Kment, P., Lukeš, J., 2012a. Phylogenetic relationships of trypanosomatids parasitising true bugs (Insecta: Heteroptera) in sub-Saharan Africa. Int. J. Parasitol. 42, 489–500.
Votýpka, J., Klepetková, H., Yurchenko, V.Y., Horák, A., Lukeš, J., Maslov, D.A., 2012b. Cosmopolitan distribution of a trypanosomatid *Leptomonas pyrrhocoris*. Protist 163, 616–631.
Votýpka, J., Maslov, D.A., Yurchenko, V., Jirků, M., Kment, P., Lun, Z.-R., Lukeš, J., 2010. Probing into the diversity of trypanosomatid flagellates parasitizing insect hosts in south-west China reveals both endemism and global dispersal. Mol. Phylogenet. Evol. 54, 243–253.
Wallace, F.G., 1966. The trypanosomatid parasites of insects and arachnids. Exp. Parasitol. 18, 124–193.
Westenberger, S.J., Sturm, N.R., Yanega, D., Podlipaev, S.A., Zeledon, R., Campbell, D.A., Maslov, D.A., 2004. Trypanosomatid biodiversity in Costa Rica: genotyping of parasites from Heteroptera using the spliced leader RNA gene. Parasitology 129, 537–547.
Wheeler, A.G., Skaftason, J.F., 2010. Notes on the feeding behavior of *Teratocoris saundersi* (Hemiptera: Miridae) in Iceland: phytophagy, zoophagy, and adventitious biting. Acta Entomol. Musei Nat. Pragae 50, 45–52.
Wilfert, L., Longdon, B., Ferreira, A.G.A., Bayer, F., Jiggins, F.M., 2011. Trypanosomatids are common and diverse parasites of *Drosophila*. Parasitology 138, 858–865.
Yoshida, N., Freymüller, E., Wallace, F.G., 1978. *Herpetomonas mariadeanei* sp. n. (Protozoa, Trypanosomatidae) from *Muscina stabulans* (Falléen, 1816) (Diptera, Muscidae). J. Eukaryot. Microbiol. 25, 421–425.
Yurchenko, V.Y., Lukeš, J., Jirků, M., Zeledon, R., Maslov, D.A., 2006. *Leptomonas costaricensis* sp n. (Kinetoplastea: Trypanosomatidae), a member of the novel phylogenetic group of insect trypanosomatids closely related to the genus *Leishmania*. Parasitology 133, 537–546.
Yurchenko, V.Y., Lukeš, J., Tesařová, M., Jirků, M., Maslov, D.A., 2008. Morphological discordance of the new trypanosomatid species phylogenetically associated with the genus *Crithidia*. Protist 159, 99–114.
Zídková, L., Čepička, I., Votýpka, J., Svobodová, M., 2010. *Herpetomonas trimorpha* sp. nov. (Trypanosomatidae, Kinetoplastida), a parasite of the biting midge *Culicoides truncorum* (Ceratopogonidae, Diptera). Int. J. Syst. Evol. Microbiol. 60, 2236–2246.