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Phylogenetic relationships of trypanosomatids parasitising true bugs (Insecta: Heteroptera) in sub-Saharan Africa

Jan Votýpka [a,b], Helena Klepetková [b], Milan Jirků [a,c], Petr Kment [d], Julius Lukeš [a,c,*]

[a] Biology Centre, Institute of Parasitology, Czech Academy of Sciences, Branišovská 31, 370 05 České Budějovice (Budweis), Czech Republic
[b] Department of Parasitology, Faculty of Science, Charles University in Prague, Viničná 7, 128 44 Prague, Czech Republic
[c] Faculty of Sciences, University of South Bohemia, Branišovská 31, 370 05 České Budějovice (Budweis), Czech Republic
[d] Department of Entomology, National Museum, Golčova 1, 148 00 Prague 4 – Kunratice, Czech Republic

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ABSTRACT

Three hundred and eighty-six heteropteran specimens belonging to more than 90 species captured in Ghana, Kenya and Ethiopia were examined for the presence of trypanosomatid flagellates. Of those, 100 (26%) specimens were positive for trypanosomatids and the spliced leader RNA gene sequence was obtained from 81 (80%) of the infected bugs. Its sequence-based analysis placed all examined flagellates in 28 typing units. Among 19 newly described typing units, 16 are restricted to sub-Saharan Africa, three belong to previously described species and six to typing units found on other continents. This result was corroborated by the analysis of the ssrRNA gene, sequenced for at least one representative of each major spliced leader RNA-based clade. In all trees obtained, flagellates originating from sub-Saharan Africa were intermingled with those isolated from American, Asian and European hosts, revealing a lack of geographic correlation. They are dispersed throughout most of the known diversity of monoxenous trypanosomatids. However, a complex picture emerged when co-evolution with their heteropteran hosts was taken into account, since some clades are specific for a single host clade, family or even species, whereas other flagellates display a very low host specificity, with a capacity to parasitise heteropteran bugs belonging to different genera/families. The family Reduviidae contains the widest spectrum of trypanosomatids, most likely a consequence of their predatory feeding behaviour, leading to an accumulation of a variety of flagellates from their prey. The plant pathogenic genus *Phytomonas* is reported here from Africa, to our knowledge for the first time. Finding the same typing units in hosts belonging to different heteropteran families and coming from different continents strongly indicates that the global diversity of the insect trypanosomatids is most likely lower than was predicted on the basis of the "one host–one parasite" paradigm. The analysis presented significantly extends the known diversity of monoxenous insect trypanosomatids and will be instrumental in building a new taxonomy that reflects their true phylogenetic relationships.

1. Introduction

Trypanosomatid flagellates are extremely successful obligatory parasites occurring in hosts ranging from insects to all classes of vertebrates, as well as plants. Due to the nearly ubiquitous dispersal of their hosts, these protists can be found in virtually any aquatic or terrestrial environment (Simpson et al., 2006). While the medically and economically relevant members of the dixenous genera, *Trypanosoma* and *Leishmania*, have received substantial attention and thus belong to the best studied protists, all other trypanosomatids have remained largely overlooked. A common feature of nearly all monoxenous trypanosomatids is their confinement to "unimportant" insects in economic, medical or veterinary terms, with the majority of species recorded from heteropteran and dipteran hosts (Podlipaev, 1990, 2000). However, they have also been rarely, but repeatedly, encountered in warm-blooded vertebrates including humans (Morio et al., 2008; Srivastava et al., 2010).

Within the last decade, insect trypanosomatids have started to receive increasing attention, the main aim of which was to map their diversity, understand their transmission routes and lay foundations for a new taxonomy that would reflect true phylogenetic relationships (Podlipaev et al., 2004a,b; Yurchenko et al., 2006a,b; Maslov et al., 2007; Svobodová et al., 2007; Yurchenko et al., 2008, 2009; Votýpka et al., 2010; Teixeira et al., 2011). The current classification of trypanosomatids is largely based on the presence in the life cycle of one or more of the seven established

* Corresponding author at: Biology Centre, Institute of Parasitology, Czech Academy of Sciences, Branišovská 31, 370 05 České Budějovice (Budweis), Czech Republic. Tel.: +420 38 7775416; fax: +420 38 5310388.
E-mail address: jula@paru.cas.cz (J. Lukeš).

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morphologically distinct cell types (Hoare and Wallace, 1966; Wallace, 1966; Vickerman, 1976). This system was useful before the acquisition of biochemical and molecular data but it has outlived its virtue, as the data available to date show that there is almost no correlation between features observable by light and electron microscopy and molecular phylogeny (Yurchenko et al., 2009; Teixeira et al., 2011; Jirků et al., 2012). The latter method is based on sequences of the ssrRNA, spliced leader (SL) RNA, and to a lesser extent the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene. Regardless of the sequence used, the inclusion of newly isolated strains into phylogenetic trees has progressively rendered the monoxenous genera *Leptomonas*, *Crithidia*, *Blastocrithidia*, *Wallaceina* and *Herpetomonas* paraphyletic, whereas all three dixenous genera (*Trypanosoma*, *Leishmania* and *Phytomonas*) have retained their well-supported monophyly (Simpson et al., 2006). Moreover, detailed characterisation of cultured and host-dwelling stages has shown that at least some strains are morphologically highly variable (Zídková et al., 2010; Votýpka et al., in press), further underlining the unsuitability of morphological characters for the higher-level taxonomy of insect trypanosomatids.

Due to their extremely wide host range, insect parasites are particularly suitable to address potential correlations with hosts, environment and/or geography. In order to investigate these questions, we have assembled a large set of isolates from sub-Saharan Africa, a region from which not a single monoxenous insect trypanosomatid has been available to date. However, from this biogeographical region, information is not lacking for the dixenous genera *Trypanosoma* and *Leishmania*, of which hundreds of strains have been isolated from dipteran vectors since the beginning of the 20th century (Adams et al., 2010). Although some novel "African" clades have emerged, an extensive SL- and ssrRNA-based phylogenetic analysis convincingly showed that correlation with geography is absent for most flagellates studied. Interestingly, some well-supported clades parasitise heteropterans from a single family, while other species cross the family or even order boundaries of their hosts. Finally, although it was predicted that species richness of insect trypanosomatids may, due to their possibly high host specificity, reach millions (Stevens, 2001), making the attempt to map their diversity a challenging task, the fact that we have encountered some already known typing units (TUs) indicates much lower diversity (Votýpka et al., 2010).

2. Materials and methods

2.1. Collection of insects

In July and August 2009, two of the authors (Votýpka and Lukeš) intensely sampled the following localities in Ghana for heteropteran insects: villages Abutia-Kloe (6°28′54″N 0°25′19″E; 60 m above sea level (a.s.l.)), Dzolo (6°40′36″N 0°24′28″E; 60 m a.s.l.) and Maste (6°39′22″N 0°30′53″E; 60 m a.s.l.) near Ho (6°34′48″N 0°29′2″E; 60 m a.s.l.), Kokrobite Beach (5°29′42″N 0°22′8″W; 10 m a.s.l.) near Accra, Cape Coast (Fort Victoria) (5°6′24″N 1°14′57″W; 20 m a.s.l.), outskirts of the Kakum National Park close to Abrafo (5°20′29″N 1°22′58″W; 30 m a.s.l.), around the village of Beyin (5°0′0″N 2°39′6″W; 20 m a.s.l.) and in the vicinity of Elubo (5°14′40″N 2°43′44″W; 150 m a.s.l.). In May 2010, insects were captured at South Horr (2°5′52″N 36°55′16″E; 1020 m a.s.l.) in northern Kenya. Finally in March 2011, heteropterans were sampled at Shiraro (14°24′17″N 37°47′16″E; 1030 m a.s.l.) in north-western Ethiopia. For more information on the DNA isolates studied, their insect hosts and localisation and intensity of infection see Table 1.

Heteropterans were collected on vegetation by sweep-netting during the day, and less frequently using light traps during the night. Within 12 h after capture, insects were killed with 96% ethanol, washed and dissected in 0.9% sterile saline solution, and checked for parasite infection under a portable microscope as described elsewhere (Votýpka et al., 2010). Heteropteran specimens captured in Ghana were dissected by carefully pulling the intact intestinal tract from its body by removing the last abdominal segments. From most specimens, midgut and hindgut with Malpighian tubes were squeezed separately with a cover slip and examined for the presence of motile flagellates. During microscopic examination, the parasite stages were ranked by morphology into one of the three morphotypes described for trypanosomatid flagellates: (medium) promastigotes, (long slender) leptomonads and (short) choanomastigotes. The intensity (four categories) and location (midgut occasionally quoted as abdominal or thoracic midgut, hindgut and Malpighian tubes) of the infection in the intestinal tract were established.

Upon detection of trypanosomatids, part of the infected tissue was smeared upon a microscopic slide, fixed with methanol, air dried and stored until further use. Using sterile tools, the rest of the tissue sample was carefully transferred in 100 µl of 2% SDS and 100 mM of EDTA solution and kept at the ambient temperature until transfer to the laboratory (1–2 weeks), where it was kept at −20 °C until further use. Whereas all heteropteran bugs captured in Ghana were dissected as described above, the specimens obtained in Kenya and Ethiopia, after collection in the field, were stored in alcohol and individually inspected for trypanosomatid infection by PCR.

The dissection was performed so that the insect hosts were preserved for subsequent determination and they have been deposited in the collection of The National Museum, Prague, Czech Republic. The host species were identified according to available taxonomic revisions and by comparison with collections of particular specialists and The Natural History Museum, London, UK. The following specialists are responsible for identifications: F. Chérot (Miridae; Université Libre de Bruxelles, Belgium), D. Chłond (Reduviidae from Ethiopia and Kenya; University of Silesia, Katowice, Poland), É. Guilbert (Tingidae; Muséum National d'Histoire Naturelle, Paris, France), E. Kondorosy (Lygaeoidea from Kenya; Pannon University, Keszthely, Hungary), N. Nieser (Nepomorpha and Gerromorpha; Tiel, the Netherlands), D. Rédei (Reduviidae from Ghana; Hungarian Natural History Museum, Budapest, Hungary), J.L. Stehlík (Pyrrhocoroidea; Moravian Museum, Brno, Czech Republic), and P. Kment (remaining groups).

2.2. DNA isolation, PCR amplification and sequencing

Total DNA was isolated from the infected samples using a High Pure PCR Template Preparation Kit (Roche, Germany) and 10–50 ng were used for PCR amplification of the target genes. The SL RNA and ssrRNA genes were amplified using the kinetoplastid-specific primer pairs M167 and M186, and S762 and S763, respectively, as described elsewhere (Maslov et al., 1996; Westenberger et al., 2004). Upon gel-purification using a Gel Extraction Kit (Roche), both strands of the PCR-amplified ssrRNA genes were directly sequenced using the above primers, as well as internal primers 577F (5′-GCC AGC ACC CGC GGT-3′), 577R (5′-ACC GCG GGT GCT GGC-3′), 1510F (5′-CAG GTC TGT GAY GCT G-3′) and 1510R (5′-CAG CRT CAC AGA CCT G-3′). GenBank™ accession numbers of small subunit genes are listed in Table 1. Due to persistent difficulties with direct cloning of PCR products, the SL RNA gene amplicons were cloned into the pCR-TOPO vector (Invitrogen, USA).

2.3. Phylogenetic analysis

SL RNA alignment was constructed after trimming of the sequences as described earlier (Votýpka et al., 2010, in press). Briefly, for species comparisons, only the most conserved section of the SL

repeats, starting at position-100 upstream of the exon and ending at the 3′ end of the intron, was used. For TU*6/7*, TU*44*, and TU*90* the entire repeat sequences were 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 neighbour-joining clustering with K2P distances was performed with unmodified alignment using PAUP* (4.0, beta version). The 90% cut-off level applied to the entire sequence was used to delineate individual TUs (Maslov et al., 2007). Similarly, the ssrRNA alignment was generated using Kalign, and the ambiguous positions and poorly alignable sequences were manually removed using BioEdit. The final small subunit alignment included 2,423 characters and is available from the authors on request. The small subunit alignment was analysed using Bayesian, maximum likelihood and maximum parsimony analyses with programs and settings as described elsewhere (Votýpka et al., 2010; in press).

3. Results

3.1. Obtaining of isolates

In 100 bug specimens (corresponding to 26% of examined specimens), infection was confirmed either by dissection (samples from Ghana) or by PCR (samples from Kenya and Ethiopia). The majority of DNA isolates (81) has been obtained from 355 mostly adult heteropteran bugs that have been captured in southern Ghana, ranging from the borders with Cote d'Ivoire to Togo. Less extensive samplings and examinations of insects have been performed in northern Kenya and northern Ethiopia, the countries of origin of two and 12 isolates out of 13 and 18 bugs examined, respectively.

As shown in Table 1, at least 90 heteropteran species belonging to 20 families were inspected for trypanosomatid parasites. Determination of preserved insect specimens to a species or at least genus was successful in virtually all cases. In all, 33 (37%) host species were positive for flagellates. However, this is certainly a considerable underestimate, as for a given host species the likelihood of finding an infected individual increases with the number of inspected specimens. It is estimated that infection with a prevalence of 5% or higher can be detected with 95% confidence using a sample containing 57 specimens per species (Lederman and Lloud, 1984). In most cases, the sampling was not exhaustive for technical reasons, so usually only the relatively abundant heteropteran species were captured.

3.2. Localisation in the host

Midgut, hindgut and Malpighian tubes of all captured insects were subjected to microscopic examination. This not only allowed localisation of the parasites, but also whether two or more parasite species (or morpho-species) co-habit in a single host. Such on-site microscopy revealed that, in numerous cases, morphological differences were indicative of more than one trypanosomatid species infecting a single host (designated as a 'MIX' in the Table 1). However, infections judged by microscopy to be mixed do not correspond with sequence-based analyses; only one (G66) out of eight multiple infections based on morphological examination was confirmed by PCR and subsequent sequencing. Such a discrepancy can be explained by recently documented extensive morphological variability within the same species (Zídková et al., 2010; Votýpka et al., in press). Sequence-based analyses, which are much more reliable than morphology, revealed that a monospecific infection existed in 64 bugs, while at least 12 specimens hosted two trypanosomatid TUs, and in five bugs a triple infection was documented. Multiple infections were associated with specimens belonging to a wide range of families: Alydidae, Coreidae, Gerridae, Lygaeidae (2), Pentatomidae (3), Pyrrhocoridae, Reduviidae (5), and Scutelleridae (3). Multiple infection can be a consequence of the predatory behaviour of the host (e.g. Reduviidae, Gerridae), but this argument does not apply for the representatives of the remaining families, which are phytophagous.

Moreover, the intensity of infections (Table 1) ranged from a few flagellates to massive infections of parts or the entire alimentary tract. Approximately half of the infections were localised in the hindgut, whereas the frequency of the infection in the midgut (occasionally confined only to its thoracic (TMG) or abdominal (AMG) parts) was about the same. Generalised infection in both compartments was encountered only in six hosts (Table 1). Unexpectedly, not a single infection was observed in the Malpighian tubes.

3.3. SL RNA-based analysis

The multicopy SL RNA gene repeats, a marker particularly suitable for high resolution phylogenetic analysis of trypanosomatids (Westenberger et al., 2004; Maslov et al., 2007; Votýpka et al., 2010; in press) was successfully amplified and sequenced for 81 out of 82 DNA samples. A typical gel is shown in Fig. 1, also revealing the occasional co-infections with one or more trypanosomatid species. While we have attempted to sequence all major SL RNA bands, we cannot exclude the possibility that in mixed infections, only genes from the more abundant or preferentially amplifiable parasite were recovered. If this was the case, the number of multiple infections is underestimated.

Sequences obtained revealed the existence in our dataset of 28 different TUs from Ghana, Kenya and Ethiopia. Given the 90% sequence identity level for the full-length SL RNA gene (Westenberger et al., 2004; Maslov et al., 2007; Votýpka et al., 2010; in press), these TUs are considered to delineate separate species. Three of these TUs correspond to previously described species (TU*1*: *Leptomonas pyrrhocoris*, TU*53*: *Leptomonas* cf. *lactosovorans*, and TU*56*: *Crithidia confusa*); six SL RNA sequences fell into already established TUs (TU*17* from the New World, TU*44* (formerly Ch1), TU*61* (formerly Ch4), TU*62* (formerly Ch6) and TU*64* (formerly Ch8) from China, and TU*73* from France); three TUs were simultaneously encountered in European bugs (TU*6/7E*, TU*72* and TU*82*; Votýpka and Klepetková, unpublished data). These findings strongly indicate that the above-mentioned 12 TUs belong to a species previously sampled outside Africa, thus with an area covering more than one continent (Fig. 2, Tables 1 and 2). On the other hand, 17 TUs represent new unnamed species restricted to sub-Saharan Africa only. However, only TU*70* has been described in detail elsewhere (Votýpka et al., in press).

All SL RNA sequences obtained in this study were included in the alignment, which contains all TUs available to date. Although based on the most conserved part of the gene, the alignment is short and contains very limited information (Votýpka et al., 2010; in press). The neighbour-joining analysis is useful for the differentiation of closely related species, but it is unsuitable for resolving deeper relationships (data not shown) (see Supplementary Fig. 1 in Votýpka et al., in press).

It is of particular interest that the Ghanaian isolates obtained from heteropterans belonging to TU*76* (from *Aeptus singularis*) and TU*77* (from *Aspilocoryphus fasciativentris*, *Cletus unifasciatus*, *Coranus* sp., *Durmia varicornis varicornis*, *Eysarcoris ventralis* and *Stenozygum alienatum*), and the Kenyan isolate TU*75* from *Platymeris rhadamantha* are clearly affiliated with the genus *Phytomonas*, known for high pathogenicity of its members to various economically important plants (e.g. oil and coconut palms, manioc and tomatoes). These three new TUs form a novel, relatively well-supported branch within the *Phytomonas* clade and represent not only a substantial extension of the known diversity of the genus, but

Table 1
Summary of the examined host families and species showing their geographic origin (Locality), the number of inspected versus infected specimens (Prop. – proportion), developmental stage (Stage: Ad. – adult; La. – larvae) and geographic distribution of host species (Distribution: Afr. – Afrotropical; Er. – Eremian; Gui. – Hologuinean; NGui. – North-Guinean; Sud. – Holosudanese; ESud. – East-Sudanese; WSud. – West-Sudanese; Sud.-Er. – Sudano-Eremian; Med. – Mediterranean; Ptr. – Palaeotropical; Pal. – Palaearctic; Cos. – Cosmopolitan). For infected host specimens the following information are provided: infected part of the intestine (Site: MG – midgut; AMG – abdominal MG; TMG – thoracic MG; HG – hindgut), intensity of the infection (Int.: four categories), cell types of the flagellates (Type: S – short choanomastigotes; M – medium promastigotes; L – long slender leptomonads), the GenBank™ accession numbers of the ssrRNA determined in this study and the typing units (TUs), to which the detected flagellates belong.

Host family (Prop.)/No. TUs (list of TUs)	Host species	Distribution	Prop.	Isolate	Locality	Stage	Site	Int.	Type	ssrRNA Acc. Nos.	Typing unit [a]
Alydidae (49/8)/3	*Mirperus jaculus*	Afr.	7/4	G29	Ho(Abutia-Kloe)	Ad.	HG	+	S		(TU*6*/*7D*)
(TU6/7D, TU*6*/*7E*, TU*77*)				G31	Ho(Abutia-Kloe)	Ad.	HG	+	MIX	JQ658821	TU*6*/*7D*
				G39	Abrafo	Ad.	HG	++++	M	JQ658829	TU*6*/*7D*
				G44	Cape Cost	Ad.	HG	++	M	JQ658832	TU*6*/*7D*
	Riptortus tenuicornis	Afr.	10/3	G01	Kokrobite	Ad.	MG	+++	MIX	JQ658807	TU*6*/*7E*
				G02	Kokrobite	Ad.	TMG	++	L		(TU*6*/*7E*)
				G20	Ho(Dzolo)	Ad.	MG	++	M	JQ658815	TU*6*/*7E*
	Stenocoris apicalis	Gui.	6/0		Abrafo	Ad.					
	Stenocoris southwoodi	Afr.	14/0		Abrafo/Cape Cost	Ad.					
	Stenocoris sp.	Afr.	3/0		Abrafo	Ad.					
	Alydinae gen. sp.		9/1	G61	Kokrobite	La.	MG	+	M	JQ658838	TU*6*/*7E*,(TU*77*)
Coreidae (27/3)/3	*Anoplocnemis curvipes*	Afr.	1/0		Elubo	Ad.					
(TU*17*, TU*72*, TU*77*)	*Anoplocnemis tristator*	Gui.	1/0		Abrafo	Ad.					
	Cletus ochraceus fuscescens	WSud.	2/1	G60	Kokrobite	Ad.	HG	++	L		(TU*72*)
	Cletus unifasciatus	Gui.	11/2	G23	Ho(Abutia-Kloe)	Ad.	MG	+++	M	JQ658818	TU*72*
				G64	Kokrobite	Ad.	HG	++	L	JQ658840	(TU*17*),TU*72*,(TU*77*)
	Cletus sp.	Afr.	1/0		Kokrobite	La.					
	Coreinae gen. sp.		1/0		Ho(Abutia-Kloe)	La.					
	Homoeocerus pallens	Gui.	1/0		Abrafo	Ad.					
	Hydara tenuicornis	Afr.	5/0		Ho(Matse)	Ad.					
	Myla gracilis	Gui.	4/0		Ho(Abutia-Kloe)	Ad.					
Cymidae (2/0)/0	*Cymodema robusta*	Sud.	2/0		Elubo	Ad.					
Geocoridae (2/1)/1 (TU*44*)	*Geocoris aethiops*	WSud.	2/1	G41	Abrafo	Ad.	MG + HG	+	M		(TU*44*)
Gerridae (22/3)/2	*Limnogonus hypoleucus*	Sud.	9/2	G21	Ho(Abutia-Kloe)	Ad.	MG	+++	L	JQ658816	TU*89*
(TU*82*, TU*89*)				G43	Cape Cost	Ad.	MG	+	M		(TU*89*)
	Limnogonus poissoni	Afr.	1/0		Ho(Abutia-Kloe)	Ad.					
	Limnogonus sp.	Afr.	11/0		Cape Cost	La.					
	Tenagogonus albovittatus	Afr.	1/1	G22	Ho(Abutia-Kloe)	Ad.	MG + HG	+++	S	JQ658817	(TU*82*),TU*89*
Hydrometridae (8/0)/0	*Hydrometra* cf. *aegyptia*	Sud.	8/0		Beyin/Cape Cost	Ad.					
Largidae (1/1)/1 (TU*80*)	*Physopelta festiva*	Gui.	1/1	G40	Abrafo	Ad.	HG	+	M	JQ658830	TU*80*
Lygaeidae (23/17)/5	*Aspilocoryphus fasciativentris*	Afr.+Er.	19/16	G09	Kokrobite	Ad.	MG + HG	+++	M	JQ658809	TU*88*
(TU*64*, TU*77*, TU*78* TU*87*, TU*88*)				G10	Kokrobite	Ad.	HG	++	M	JQ658810	TU*64*,(TU*78*)
				G11	Kokrobite	Ad.	HG	++	M		(TU*88*)
				G12	Kokrobite	Ad.	HG	++	M		(TU*88*)
				G25	Ho(Abutia-Kloe)	Ad.	HG	++	MIX		(TU*88*)
				G26	Ho(Abutia-Kloe)	Ad.	HG	+	M		(TU*88*)
				G27	Ho(Abutia-Kloe)	Ad.	HG	+++	M		(TU*88*)
				G28	Ho(Abutia-Kloe)	Ad.	HG	+++	MIX		(TU*88*)
				G45	Beyin	Ad.	HG	++	M	JQ658833	TU*88*
				G68	Kokrobite	Ad.	HG	+	M	JQ658844	(TU*77*),TU*88*
				6 ex.	Kokrobite	Ad.	HG	+	M		N/A
	Graptostethus servus	Ptr.+Med.	1/0		Kokrobite	Ad.					

Table 1 (*continued*)

Host family (Prop.)/No. TUs (list of TUs)	Host species	Distribution	Prop.	Isolate	Locality	Stage	Site	Int.	Type	ssrRNA Acc. Nos.	Typing unit [a]
	Nysius sp.	Afr. (?)	1/0		Kokrobite	Ad.					
	Spilostethus pandurus	Ptr.+Med.	1/1	E04	Shiraro	Ad.	N/A	N/A	N/A	JQ658846	TU*87*
	Tropidothorax sternalis	WSud.	1/0		Abrafo	Ad.					
Miridae (15/2)/1	*Charagochilus guineensis*	NGui.	2/0		Elubo	Ad.					
(TU*17*)	*Deraeocorinae* gen. sp.		1/0		Kokrobite	Ad.					
	Gutrida neavei	Sud.	1/0		Abrafo	Ad.					
	Proboscidocoris fuliginosus	Afr.	5/2	G18	Ho(Dzolo)	Ad.	MG + HG	+++	MIX		(TU*17*)
				G19	Ho(Dzolo)	Ad.	MG + HG	+++	MIX	JQ658814	TU*17*
	Stenotus affinis	Afr.	2/0		Ho(Abutia-Kloe)/ Kokrobite	Ad.					
	Stenotus pylaon	Afr.	1/0		Kokrobite	Ad.					
	? *Taylorilygus* sp.		2/0		Kokrobite	Ad.					
	Mirini gen. sp.		1/0		Kokrobite	Ad.					
Nepidae (1/0)/0	*Ranatra* sp.	Afr.	1/0		Beyin	La.					
Notonectidae (3/0)/0	*Anisops debilis debilis*	Afr.+Med.	3/0		Ho(Abutia-Kloe)	Ad.					
Oxycarenidae (35/0)/0	*Oxycarenus congoensis*	Gui.	22/0		Ho(Dzolo)/ Kokrobite	Ad.					
	Oxycarenus hyalinipennis	Ptr.+Med.	3/0		Kokrobite	Ad.					
	Oxycarenus sp.	Afr.	10/0		Ho(Abutia-Kloe)	Ad.					
Pachygronthidae (32/0)/0	*Opistholeptus* cf. *elegans*	Afr.	1/0		Ho(Dzolo)	Ad.					
	Pachygrontha bipunctata bipunctata	Ptr.	30/0		Ho(Abutia-Kloe)/ Kokrobite/ Abrafo/Elubo	Ad./ La.					
	Pachyphlegyas modigliani ethiopicus	Sud.	1/0		Ho(Abutia-Kloe)	Ad.					
Pentatomidae (44/10)/5	*Acrosternum millierei*	Med.+Sud.	3/0		Kokrobite	Ad.					
(TU*44*, TU*72*, TU*76*, TU*77*, TU*81*)	*Aeliomorpha griseoflava*	Sud.	1/0		Kokrobite	Ad.					
	Aeptus singularis	Afr.	1/1	G24	Ho(Abutia-Kloe)	Ad.	?	+	S	JQ658819	TU*76*
	Aspavia armigera	Gui.	1/0		Kokrobite	Ad.					
	Aspavia brunnea	Gui.	3/0		Abrafo	Ad.					
	Aspavia hastator	Afr.	1/0		Abrafo	Ad.					
	Carbula melanacantha breviscutum	NGui.	10/0		Ho(Dzolo)/ Kokrobite	Ad.					
	Durmia haedula	Afr.	1/0		Kokrobite	Ad.					
	Durmia varicornis varicornis	WSud.	2/1	G65	Kokrobite	Ad.	MG	++++	L	JQ658841	(TU*72*),TU*77*
	Eysarcoris ventralis	Ptr.+Pal.	2/1	G63	Kokrobite	Ad.	MG	++	M	JQ658839	TU*44*,(TU*77*),(TU*81*)
	Kayesia setiger	NGui.	1/0		Ho(Abutia-Kloe)	Ad.					
	Menida maculiventris	Afr.	2/0		Ho(Abutia-Kloe)	Ad.					
	Menida transversa transversa	Afr.	1/0		Kokrobite	Ad.					
	Sepontia nitens	Sud.	9/5	G16	Ho(Dzolo)	Ad.	MG	++++	M		(TU*44*)
				G17	Ho(Dzolo)	Ad.	MG	++++	M		(TU*44*)
				G53	Elubo	Ad.	MG	++++	M		(TU*44*)
				G55	Elubo	Ad.	?	+	M	JQ658836	TU*44*
				G56	Elubo	Ad.	MG	++++	M		(TU*44*)
	Stenozygum alienatum	Afr.	3/2	G66	Kokrobite	Ad.	MG	++	MIX	JQ658842	(TU*72*),(TU*77*),TU*81*
				G67	Kokrobite	Ad.	MG	+	S	JQ658843	TU*81*
	Pentatominae gen. sp. 1		1/0		Abrafo	La.					
	Pentatominae gen. sp. 2		1/0		Kokrobite	La.					

(*continued on next page*)

Table 1 (*continued*)

Host family (Prop.)/No. TUs (list of TUs)	Host species	Distribution	Prop.	Isolate	Locality	Stage	Site	Int.	Type	ssrRNA Acc. Nos.	Typing unit [a]
	Phyllocephalinae gen. sp.		1/0		Ho(Abutia-Kloe)	La.					
Plataspidae (3/0)/0	*Coptosoma stali*	Afr.	2/0		Kokrobite	Ad.					
	Coptosoma cf. *stali*	Afr.	1/0		Kokrobite	Ad.					
Pyrrhocoridae (24/20)/3	*Dysdercus fasciatus*	Afr.	21/20	G03	Kokrobite	Ad.	MG	+++	L		(TU*1*)
(TU*1*, TU*70*, TU*72*)				G04	Kokrobite	Ad.	MG	+++	L		(TU*1*)
				G05	Kokrobite	Ad.	MG	+++	L		(TU*1*)
				G06	Kokrobite	Ad.	MG	+++	L		(TU*1*)
				G07	Kokrobite	Ad.	MG	+++	L	JQ658808	TU*1*,(TU*70*)
				G08	Kokrobite	Ad.	MG	+++	L		(TU*1*)
				G14	Ho(Matse)	Ad.	MG	+++	M	JQ658812	TU*72*
				G58	Kokrobite	Ad.	AMG	+++	L	JQ658837	TU*1*
				G59	Kokrobite	Ad.	AMG	+++	L		(TU*1*)
				11 ex.	Kokrobite	Ad.	AMG	+	L		N/A
	Dysdercus voelkeri	Gui.	1/0		Ho(Abutia-Kloe)	Ad.					
	Scantius forsteri	Afr.+Er.	2/0		South Horr	Ad.					
Reduviidae (30/12)/13	*Acanthaspis bilineolata*	Afr.	1/0		Ho(Abutia-Kloe)	Ad.					
(TU*6/7E*, TU*44*, TU*53*, TU*56*, TU*61*, TU*62*, TU*72*, TU*75*, TU*77*, TU*83*, TU*84*, TU*90*)	*Coranus dulichoides*	NGui.	1/0		Ho(Abutia-Kloe)	Ad.					
	Coranus sp.	Afr.	2/2	G30	Ho(Abutia-Kloe)	Ad.	TMG	+++	L	JQ658820	TU*53*,(TU*72*),(TU*77*)
				G38	Abrafo	Ad.	TMG	++	M	JQ658828	TU*53*,(TU*72*),(TU*77*)
	Cosmolestes aethiopicus	Afr.	1/0		Abrafo	Ad.					
	Ectomocoris fenestratus	Sud.-Er.	1/1	E03	Shiraro	Ad.	N/A	N/A	N/A	JQ658845	TU*61*
	Fusius dilutus	Gui.	1/0		Abrafo	Ad.					
	Lisadra soudanica	ESud	1/0		Shiraro	Ad.					
	Reduvius minutus	Afr.+Med.	1/0		Shiraro	Ad.					
	Reduvius montadoni	Sud.-Er.	1/1		Shiraro	Ad.					
	Reduvius tabidus	Sud.-Er.	2/0		Shiraro	Ad.					
	Nagusta cf. *punctaticollis*	Afr.	4/1	G42	Cape Cost	Ad.	?	+	L	JQ658831	TU*84*,(TU*90*)
	Peprius sp.	Afr.	1/0		Ho(Abutia-Kloe)	Ad.					
	Platymeris rhadamantha	ESud.	10/2	Kn01	South Horr	Ad.	N/A	N/A	N/A	JQ658849	TU*75*
				Kn07	South Horr	Ad.	N/A	N/A	N/A	JQ658850	TU*62*
	Rhynocoris albopilosus	Sud.	4/2	G34	Abrafo	Ad.	TMG	++	L	JQ658824	TU*53*,(TU*72*)
				G52	Elubo	Ad.	MG	+	M	JQ658834	TU*44*
	Rhynocoris bicolor	Afr.	1/1	G37	Abrafo	Ad.	MG + HG	++	MIX	JQ658827	TU*56*
	Rhynocoris rapax	Afr.	3/1	G15	Ho(Matse)	Ad.	HG	+++	S	JQ658813	(TU*6/7E*),TU*83*
	Sphedanolestes picturellus	Afr.	3/1	G32	Ho(Abutia-Kloe)	Ad.	HG	+	S	JQ658822	TU*56*
Rhopalidae (8/2)/1	*Leptocorisa* sp.	Afr.	2/0		Ho(Abutia-Kloe)	Ad.					
(TU*73*)	*Liorhyssus hyalinus*	Cos.	6/2	G33	Ho(Abutia-Kloe)	Ad.	HG	+++	M	JQ658823	TU*73*
				G62	Kokrobite	Ad.	MG	+	L		N/A
Rhyparochromidae (17/12)/3	*Dieuches albostriatus*	Afr.	9/6	G35	Abrafo	Ad.	HG	++	M	JQ658825	TU*86*
(TU*79*, TU*85*, TU*86*)				E07	Shiraro	Ad.	N/A	N/A	N/A	JQ658847	TU*79*
				E11	Shiraro	Ad.	N/A	N/A	N/A		(TU*85*)
				E13	Shiraro	Ad.	N/A	N/A	N/A		(TU*79*)
				E15	Shiraro	Ad.	N/A	N/A	N/A		(TU*85*)
				E16	Shiraro	Ad.	N/A	N/A	N/A		(TU*85*)
	Dieuches armatipes	Afr.+Med.	1/1	G13	Ho(city)	Ad.	HG	+	M	JQ658811	TU*85*
	Dieuches mucronatus	Sud.Er.	1/0		South Horr	Ad.					
	Elasmolomus squalidus	Ptr.	5/5	E06	Shiraro	Ad.	N/A	N/A	N/A		(TU*85*)
				E09	Shiraro	Ad.	N/A	N/A	N/A		(TU*85*)
				E12	Shiraro	Ad.	N/A	N/A	N/A	JQ658848	TU*85*
				E17	Shiraro	Ad.	N/A	N/A	N/A		(TU*85*)
				E21	Shiraro	Ad.	N/A	N/A	N/A		(TU*85*)

Table 1 (*continued*)

Host family (Prop.)/No. TUs (list of TUs)	Host species	Distribution	Prop.	Isolate	Locality	Stage	Site	Int.	Type	ssrRNA Acc. Nos.	Typing unit [a]
	Naphius zavattarii	ESud.	1/0		Shiraro	Ad.					
Scutelleridae (29/10)/3	*Deroplax* cf. *nigropunctata*	Afr.	1/0		Ho(Abutia-Kloe)	Ad.					
(TU*44*, TU*88*, TU*90*)	*Hotea subfasciata*	Afr.	15/0		Ho(Abutia-Kloe & Dzolo)/Cape Cost	Ad./La.					
	Sphaerocoris annulus	Afr.	4/2	G50	Elubo	Ad.	HG	++++	M		(TU*90*)
				G51	Elubo	Ad.	HG	++++	M		(TU*90*)
	Sphaerocoris cf. *testudogrisea*	Afr.	2/2	G54	Elubo	La.	MG	++++	M	JQ658835	TU*44*
				G57	Elubo	La.	?	+	S		(TU*90*)
	Sphaerocoris testudogrisea	Afr.	7/6	G36	Abrafo	Ad.	HG	++	S	JQ658826	TU*44*,(TU*90*)
				G46	Elubo	Ad.	HG	++++	M		(TU*88*)
				G47	Elubo	Ad.	HG	++++	M		(TU*44*),(TU*90*)
				G48	Elubo	Ad.	HG	++++	M		(TU*44*),(TU*90*)
				G49	Elubo	Ad.	HG	++++	M		(TU*90*)
				1 ex.	Elubo	Ad.	HG	++++	M		N/A
Total: 20 families	>90 species		386/100								28 TUs

[a] (TU in brackets) – determined based on the spliced leader (SL) RNA gene sequence only.

also the first report on these economically important parasites from Africa. Unfortunately, we were unable to introduce these isolates into culture, which would allow further investigation.

3.4. ssrRNA-based phylogeny

Isolates based on the SL RNA-derived phylogeny which clearly extend the known diversity of trypanosomatids were selected for PCR amplification of their ssrRNA gene. Due to its substantially larger size and higher conservation, this gene is much more informative for phylogenetic analysis. Thus, the ssrRNA sequences were obtained for the following 25 TUs: TU*1* (*L. pyrrhocoris*), TU*6*/*7D*, TU*6*/*7E*, TU*17*, TU*44*, TU*53* (*L.* cf. *lactosovorans*), TU*56* (*C. confusa*), TU*61-62*, TU*64*, TU*72-73*, TU*75-77*, TU*79-81*, and TU*83-89*. We were unable to amplify this gene from the remaining four new SL RNA-based TUs (TU*70*, TU*78*, TU*82* and TU*90*). All small subunit sequences obtained were added to the alignment containing all homologues available from *Phytomonas* spp. and the monoxenous insect trypanosomatids (Fig. 2), and supplemented with sequences of selected members of the dixenous genera *Leishmania* and *Trypanosoma*. Several more distantly related members of the genera *Bodo* and *Neobodo* were used as outgroups (Moreira et al., 2004).

Phylogenetic analysis of this dataset by Mr Bayes confirmed and extended conclusions derived from the SL RNA-based alignment. Namely, the newly included ssrRNA sequences from African isolates fell within the same clades as in the SL RNA-based tree (data not shown), confirming the suitability of both genes.

In the ssrRNA based tree, all insect trypanosomatids fell into at least nine major clades (Fig. 2). The earliest branching double-clade is composed of species recently ranked into the well-defined genera *Angomonas* and *Strigomonas* (Teixeira et al., 2011). All remaining monoxenous flagellates isolated from heteropterans worldwide are then split into seven major clades. However their phylogenetic relationships are not stable. One clade is formed by a handful of species belonging to TU*69*, TU*84* and TU*89* (from bugs captured in Ghana and China) and represents a candidate for a novel taxonomic unit. Another clade is formed by the genera *Herpetomonas* and *Phytomonas*, and yet another includes *Leptomonas collosoma*, *Herpetomonas mariadeanei* and *Sergeia podlipaevi* (Fig. 2).

From the growing diversity of insect trypanosomatids, approximately two-fifths of known monoxenous trypanosomatids are embraced by the SE (slowly evolving) clade. This fastest expanding clade established by Hollar et al. (1998) and recently raised to the rank of subfamily Leishmaniinae (Jirků et al., 2012), embraces species with particularly short branches in the ssrRNA-based tree. While the dixenous genus *Leishmania* covers only a fraction of the diversity of the globally distributed members of this clade, its sister groups contain a diverse assortment of flagellates currently ranked into the four monoxenous genera *Crithidia*, *Leptomonas*, *Blastocrithidia* and *Wallaceina*, as well as into the unnamed TU*79-81*, TU*83* and TU*91*. No common morphological trait or life cycle feature is currently known to unify this assemblage, and we doubt that any exists.

Numerous new TUs (including ssrRNA sequences of African trypanosomatids belonging to TU*17*, TU*44* and TU*61-62*) fell into another clade that is, based on its inclusion of *Blastocrithidia tria-*

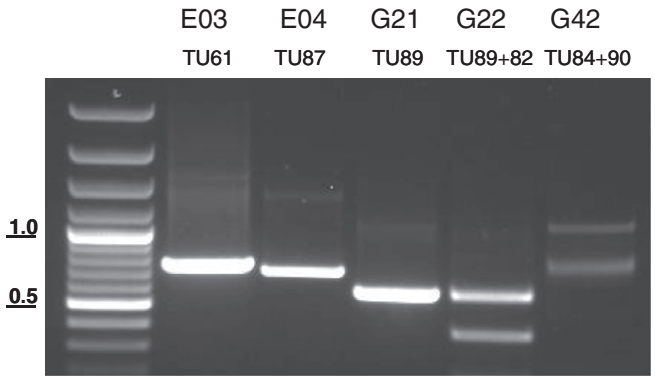

Fig. 1. PCR amplification of spliced leader RNA gene repeats from a panel of selected intestinal DNA samples shows variability of gene repeats and occasional co-infections of various trypanosomatid species. In the first three lanes are single infections and each of those represents different typing unit (TU). Individual TU may vary in size (isolate E04 and G21), although a similar size of amplification product of different TU is possible (isolates E03 and E04). Isolates G22 and G42 represent co-infections of two different trypanosomatid species. The amplification products were resolved in 1% agarose gels and the sizes (in kb) of the marker bands (GeneRuler™ 100 bp DNA Ladder) are shown.

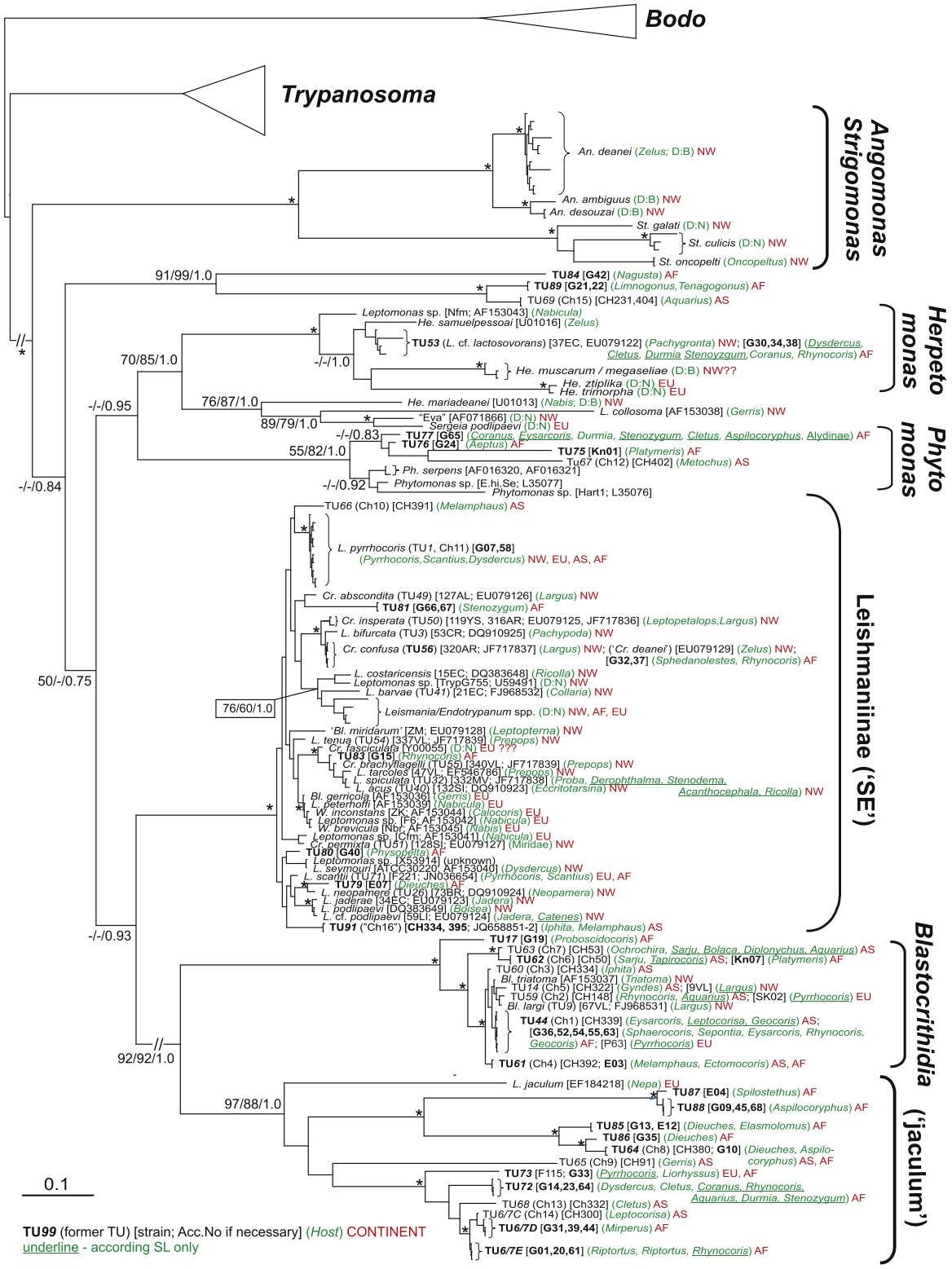

Fig. 2. Phylogenetic tree of trypanosomatids based on ssrRNA gene sequences and reconstructed by the Bayesian method. The ssr sequences of the new trypanosomatid species (typing units) described in this study (in bold) were amplified directly from the respective gut DNA samples. Information about typing units is given as follows: current typing unit occasionally followed by former typing units (in round brackets), strain and accession No. [in square brackets], Host species (in green and in round brackets: for trypanosomatids originating from heteropteran bugs the genus name is given; for other hosts D:B – Diptera/Brachycera and D:N – Diptera/Nematocera; underlined heteropteran hosts – typing unit was assigned according to spliced leader only) and Continent of origin (in uppercase red letters: NW – New World; EU – Europe; AS – Asia; AF – Africa). Bootstrap values from maximum-parsimony and maximum-likelihood (1,000 replicates) and Bayesian posterior probabilities (5 million generations) are shown at the nodes. Asterisks (*) denote Bayesian posterior probabilities and bootstrap percentages 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 ssrRNA sequences determined in this work were deposited under the GenBank™ Accession Nos. JQ658807–JQ658852. The accession numbers of the reference ssrRNA gene sequences were retrieved from GenBank™. The scale bar denotes the number of substitutions per site. Some of the long branches were arbitrarily shortened. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Summary of the typing units detected in heteropteran bugs from sub-Saharan Africa, showing the availability of small subunit and spliced leader RNA sequences, distribution and the list of infected insect families.

Typing unit	New typing unit	Small subunit	Spliced leader	Distribution [a]	Host family (detected worldwide until now)
TU***1*** [b]	No	Yes	Yes	NW, AS, EU, AF	Pyrrhocoridae
TU***6/7D***	Yes	Yes	Yes	AF	Alydidae
TU***6/7E***	Yes	Yes	Yes	(EU), AF	Alydidae, Reduviidae
TU***17***	No	Yes	Yes	NW, (EU), AF	Miridae, Coreidae
TU***44***	No	Yes	Yes	NW, AS, EU, AF	Pentatomidae, Coreidae, Alydidae, Geocoridae, Scutelleridae, Reduviidae, Oxycarenidae, Pyrrhocoridae
TU***53*** [c]	No	Yes	No	NW, AF	Reduviidae, Lygaeidae
TU***56*** [d]	No	Yes	Yes	NW, AF	Reduviidae, Lygaeidae, Calliphoridae (Brachycera)
TU***61***	No	Yes	Yes	AS, AF	Pyrrhocoridae, Reduviidae
TU***62***	No	Yes	Yes	AS, AF	Pentatomidae, Reduviidae
TU***64***	No	Yes	Yes	AS, AF	Rhyparochromidae, Lygaeidae
TU***70***	No	No	Yes	AF	Pyrrhocoridae
TU***72***	No	Yes	Yes	(EU), AF	Pyrrhocoridae, Reduviidae, Coreidae, Pentatomidae
TU***73***	No	Yes	Yes	EU, AF	Pyrrhocoridae, Rhopalidae
TU***75***	Yes	Yes	Yes	AF	Reduviidae
TU***76***	Yes	Yes	Yes	AF	Pentatomidae
TU***77***	Yes	Yes	Yes	AF	Pentatomidae, Reduviidae, Alydidae, Coreidae, Lygaeidae
TU***78***	Yes	No	Yes	AF	Lygaeidae
TU***79***	Yes	Yes	Yes	AF	Rhyparochromidae
TU***80***	Yes	Yes	Yes	AF	Largidae
TU***81***	Yes	Yes	Yes	AF	Pentatomidae
TU***82***	Yes	No	Yes	(EU), AF	Gerridae
TU***83***	Yes	Yes	Yes	AF	Reduviidae
TU***84***	Yes	Yes	Yes	AF	Reduviidae
TU***85***	Yes	Yes	Yes	AF	Rhyparochromidae
TU***86***	Yes	Yes	Yes	AF	Rhyparochromidae
TU***87***	Yes	Yes	Yes	AF	Lygaeidae
TU***88***	Yes	Yes	Yes	AF	Lygaeidae
TU***89***	Yes	Yes	Yes	AF	Gerridae
TU***90***	Yes	No	Yes	AF	Scutelleridae, Reduviidae

[a] EU – Europe; AS – Asia; AF – Africa; NW – New World; (EU in brackets) – unpublished data from European bugs (J. Votýpka and H. Klepetková).
[b] *Leptomonas pyrrhocoris.*
[c] *Leptomonas* cf. *lactosovorans.*
[d] *Crithidia confusa.*

toma, labelled as the genus *Blastocrithidia* (Fig. 2), with virtually all of its members being refractory to cultivation. Its substantial expansion by isolates obtained in this study is therefore caused only by environmental samples. No culture has been established.

Finally, the "jaculum" crown clade contains at its base the only named species, *Leptomonas jaculum* (Fig. 2). It was substantially expanded by the addition of the ssrRNA sequences of new African isolates forming TU*6/7*, TU*64*, TU*72-73* and TU*85-88*. This clade is clearly another candidate for the establishment of a novel taxonomic unit, at least at the level of genus. We propose that the TUs that have no known relatives represent not only new species, but in several cases new higher-level taxonomic units of the rank of genus or even higher.

3.5. Host-parasite co-evolution and correlation with geography

Globally distributed trypanosomatids that parasitise a significant fraction of their omnipresent insects are particularly suitable to address questions concerning co-evolution with their hosts and/or correlation between their phylogeny and geographic origin. However, prior to this study, the number of isolates obtained from insects from geographically distant localities was rather low. The addition of the African isolates (this study) to the collections of isolates from south-western China (Votýpka et al., 2010) and Ecuador (Maslov et al., 2007), now provides a dataset that allows us to address such a question. In Fig. 2 and Table 2, the geographic origin of individual isolates from heteropteran and dipteran insects is indicated (NW – New World; EU – Europe; AS – Asia; AF – Africa).

As is apparent from the ssRNA-based tree, isolates from hosts originating from geographically distant locations in Africa, China, South and central America and Europe are frequently genetically very closely related (Fig. 2, Table 2). Moreover, numerous isolates belonging to a single TU originate from different continents (TU*1*, TU*6/7*, TU*14*, TU*17*, TU*44*, TU*53*, TU*56*, TU*59*, TU*61*, TU*62*, TU*64*, TU*71*, TU*72*, TU*73* and TU*82*). Indeed, the overall outcome of the phylogenetic tree is that, in most clades, trypanosomatids originating from different continents are intermingled. The *Angomonas*/*Strigomonas* clade is a notable exception as it is confined to South America (Fig. 2). Although the "jaculum" crown clade is thus far composed of small subunit sequences solely from Old World trypanosomatids, the SL RNA analysis indicates that the complex of TU*6/7*, TU*8* and TU*43* is also present in the New World (data not shown) (Votýpka et al., 2010; in press).

A similarly ambiguous situation is encountered when host-parasite co-evolution is considered. Most well-supported and densely-sampled clades bring together trypanosomatids from phylogenetically unrelated heteropteran hosts (e.g. the *Blastocrithidia*, *Phytomonas*, *Herpetomonas* and SE clades) (Fig. 2). On the other hand, some clades demonstrate high host specificity. The most conspicuous case is the un-named early-branching clade containing TU*69* and TU*89*, thus far encountered only in heteropteran hosts of the family Gerridae caught in Africa and Asia. The strictly aquatic life style of these predatory hosts may preclude the transmission of their parasites to other insects, underpinning their co-evolution. A similar situation is encountered in the crown clade composed of TU*64* and TU*85-88*, which is confined to members of the superfamily Lygaeoidea (Fig. 2). It should be noted, however, that both clades

are currently relatively poorly sampled, so the inclusion of more isolates may expand their host range and weaken their present specificity. Nevertheless, the observed correlation supports, at least in these cases, limited co-evolution of these parasites with their hosts.

The transmission cycle between predator and prey may be reflected in the situation observed in the reduviids. Among sub-Saharan isolates, 13 TUs occur in this family (Tables 1 and 2). In addition, these predatory hosts are parasitised by flagellates which form a sister group to isolates from unrelated flies and mosquitoes (Fig. 2). It is conceivable that the heteropteran predators became infected from their dipteran prey. Higher numbers of TUs (five just for African representatives – see Tables 1 and 2) were found also for the mostly phytophagous families Lygaeidae and Pentatomidae.

4. Discussion

In this work we have presented the first characterisation of monoxenous insect trypanosomatids from Africa. This novel set of isolates from a previously unsampled continent has allowed us to address the prevalence, biodiversity and host specificity of monoxenous trypanosomatids. In Ghana, Kenya and Ethiopia, we examined a representative collection of nearly 400, mostly adult, heteropterans, of which 26% was found to be infected with at least one trypanosomatid species. From 27 out of 28 TUs, the SL RNA has been sequenced, allowing their placement in a phylogenetic tree (data not shown). The only non-amplifiable TU was TU*53* (*L.* cf. *lactosovorans*), for which the problematic nature of cloning SL RNA is known (Maslov, personal communication). Moreover, in order to confirm or disprove SL RNA-based branching, the more informative ssrRNA gene was sequenced for most of the new TUs. This approach failed in the case of four TUs (TU*70*, TU*78*, TU*82* and TU*90*), as they were present only as minor components of mixed infections, allowing (serendipitous) amplification of their SL RNA, while ssrRNA was repeatedly amplified only from the most abundant species.

It has recently been shown that mixed infections of trypanosomatid species in a single host are more frequent than previously appreciated (Yurchenko et al., 2009). Indeed, examination of the Chinese (Votýpka et al., 2010) and the sub-Saharan datasets (this study) was quite similar in this respect. Similarly, it has been shown (see Table 2) that some TUs have low host specificity and their broad host spectra cover insects from different heteropteran families (e.g. TU*44*, TU*72* and TU*77*), and in some cases even from different orders such as TU56 (*C. confusa*) parasitising heteropterans and dipterans. These findings dispel the "one host–one parasite" paradigm and show that although the global diversity of monoxenous parasites is high, it is not as enormous as earlier suggested (Podlipaev, 1990, 2000, 2001; Stevens, 2001).

The distribution of microorganisms is addressed by two general and opposing models. The "ubiquitous" model postulates the occurrence of microorganisms everywhere (Finlay, 2002), whereas the second model favours the existence of moderate endemism (Foissner, 2006). The distribution of monoxenous trypanosomatids, as judged from the available data, seems to be compatible with both views. *Leptomonas pyrrhocoris* (TU*1*) has been found on several continents, where it retains a high specificity for a single heteropteran family, the Pyrrhocoridae (Votýpka et al., in press). A similar cosmopolitan distribution has been achieved by TU*44*, found in the family Coreidae in the New World (Maslov et al., 2007), in the families Alydidae, Geocoridae and Pentatomidae in Asia (Votýpka et al., 2010), in the family Pyrrhocoridae in Europe (Votýpka et al., in press), and in the families Geocoridae, Pentatomidae, Reduviidae and Scutelleridae in Africa (this work). Conversely, *L. pyrrhocoris* (TU*1*), TU*44* has developed a very wide host specificity. Based on the sub-Saharan dataset, TU*72* and TU*77* similarly have wide host specificity and we predict that extensive sampling will lead to their finding outside Africa.

We were particularly interested to compare the diversity of monoxenous versus dixenous trypanosomatids. For this comparison, the ssrRNA gene is particularly useful, as it is available for a number of monoxenous and dixenous species (Hughes and Piontkiwska, 2003; Hamilton et al., 2004, 2005; Simpson et al., 2006). As shown in Fig. 2, the diversity of most branches of insect trypanosomatids exceeds that of the genus *Leishmania*, which consistently appears among the monoxenous trypanosomatids. Diversity of the genus *Trypanosoma* is comparable with that of the *Herpetomonas*, *Angomonas*/*Strigomonas* and *Blastocrithidia* clades (Fig. 2). It should be mentioned, however, that any conclusions of diversity of individual clades needs to be supported by analyses of other genes, such as GAPDH. However, due to considerable difficulties with the amplification of GAPDH from environmental samples, the available set of GAPDH sequences remains very limited.

Most isolates found during this African survey clearly belong to monoxenous trypanosomatids. While monoxenous trypanosomatids appear to be much more diverse, dixenous trypanosomatids may be unexpectedly species-rich. We postulate that this pattern reflects the different life styles that are characteristic of each group. Due to the host life style, monoxenous trypanosomatids can enter new host species with relative ease, a step much less likely to occur with *Trypanosoma* spp. which use for their transmission in most cases a relationship between the final and intermediate hosts.

Moreover, we have attempted to introduce all isolates into culture by transferring small amounts of intestinal content into sterile cultivation media. This approach was successful in the case of six strains (G07, G15, G30, G37, G58 and G59), which thus extended the collection of flagellates available for future studies. Although their morphology has not yet been investigated, based on previous studies one can predict that it will be uninformative in determination of their phylogenetic position. There are multiple examples in which morphology, such as the presence of promastigotes in culture, has hinted at an association with the genus *Leptomonas*, while the ssrRNA sequences placed the same isolate within the genus *Crithidia* (Yurchenko et al., 2008; Jirků et al., 2012). It was not the intention of this study to revise the presently used, yet grossly outdated taxonomic system of trypanosomatids, as all isolates are evenly ranked at the TU level. However, this dataset will substantially contribute to the efforts of generating a system that would better reflect the true relationships of these morphologically uniform but extremely diverse protists.

The unexpected lack of correlation of monoxenous as well as dixenous trypanosomatids with geography (Podlipaev, 1990) can be explained either by radiation of these flagellates predating the separation of Pangaea into the present continents, or by numerous inter-continental transfers via their hosts, including massive exchanges of fauna when isolated continents came into contact. While we cannot at present distinguish between these scenarios, which are ultimately not mutually exclusive, it is worth noting that one of the infected bugs in Ghana was *Liorhyssus hyalinus*, a cosmopolitan species with the capability for widespread dispersal by wind (Hradil et al., 2007). Such hosts may travel with their parasites and allow distribution over huge distances. Hence we predict that the handful of Africa-only TUs and clades (Fig. 2 and Table 2), representing the newly discovered segments of diversity, will eventually be extended by the inclusion of isolates from distant locations. The distribution and bionomics of Afrotropical true bugs (Heteroptera) is unfortunately very poorly known. In fact some of the hosts examined are recorded for the first time from Ghana, and *Cymodema robusta*

and *Oxycarenus congolensis* were until our study known only from the original descriptions. The composition of the African bug fauna depends on the main biomes (forests, savannas and deserts), their changes in the Quaternary and recent human influence such as agriculture and deforestation. Each host insect can be ranked, based on its distribution, into one of the four faunal elements as defined by Linnavuori (1980, 1982): (i) Afrotropical element with wide distribution in most of tropical Africa, inhabiting both savannah and forest areas; (ii) Guinean element – forest species of equatorial Africa; (iii) Sudanese element – savannah species often widely distributed from the Atlantic to the Indian Ocean, and (iv) Eremian element, which includes species of the desert and semi-desert belts extending from the Sahara towards the Arabian Peninsula or even further east. The majority of the hosts examined (80%) occurs only in Africa (44% being Afrotropical, 17% Guinean, and 19% Sudanese), 5% belong to the Eremian element and 15% of species have a wide range, extending into the Mediterranean, Near East or even to the tropics of the Oriental and Australian regions (Table 1).

In accordance with studies from other continents (Maslov et al., 2007; Votýpka et al., 2010), monoxenous trypanosomatids were found almost exclusively in the intestinal tract of their insect hosts, most likely allowing their transmission via predation, contamination, coprophagy and/or necrophagy (Schaub et al., 1989; Tieszen and Molyneux, 1989). Although very little experimental evidence is available regarding the transmission routes of monoxenous trypanosomatids, faecal transmission is probably a dominant route of their perpetuation (Tieszen and Molyneux, 1989). If present, free or flagellar pseudocysts, also called "straphangers", may mediate efficient distribution in some species (Peng and Wallace, 1982; Romeiro et al., 2000). Even trypanosomatids without pseudocysts are able to survive for a limited period of time outside the intestines of their hosts, including water environments, where they may infect other hosts (Clark et al., 1964; Tieszen and Molyneux, 1989). Such an explanation can well explain the emerging water bug-only clade.

The available data present a rather complex picture, which may confound the relationships between the protists and their insect hosts. One way to explain the observed complex patterns is to imply numerous switches among hosts, a likely event when a predator–prey interaction is invoked. The SL RNA gene was to some extent instrumental in revealing that some host taxa are infected more frequently and/or with a higher diversity of parasites. As hosts of at least 13 TUs in sub-Saharan Africa, the predatory Reduviidae stand out, as they may frequently become infected from their prey. However, it is known that numerous phytophagous bugs occasionally graze on young larvae of other heteropteran species or even other insects, a behaviour frequently encountered in species of Miridae (Wheeler and Skaftason, 2010), which serve as hosts to flagellates scattered throughout trypanosomatid phylogenetic trees. The acquisition of the plant parasite *Phytomonas* by the reduviids of the genera *Coranus* (TU77) and *Platymeris* (TU75) is rather unexpected, although predatory heteropteran bugs may have obtained the parasite from insects on which they feed, such as vectors of *Phytomonas* spp. among host plants, as was described for the predatory bug *Zelus leucogrammus* (Carvalho and Deane, 1974). However, even the predatory heteropteran bugs sometimes feed on plant sap (Bérenger and Pluot-Sigwalt, 1998), and the direct acquisition of the flagellates from plants cannot be excluded.

It should be noted that even other insect groups which somehow come into contact with heteropteran bugs could be infected and thus further confound the complexity of transmission routes of trypanosomatids in nature. The very low host specificity of some trypanosomatid species supports such a scenario. However, it has to be stressed that our knowledge of monoxenous trypanosomatids in insects other than Heteroptera remains limited.

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