Endangered monoxenous trypanosomatid parasites: a lesson from island biogeography

Jan Votýpka[1,2] · Petr Kment[3] · Vyacheslav Yurchenko[4,5] · Julius Lukeš[2,6]

Abstract

Most remote and oceanic islands are important, yet highly vulnerable biodiversity hotspots, which host a significant proportion of endemic species. Along with iconic endangered or extinct animals and plants, the disappearance of their co-inhabitants, including protist parasites, gets usually unnoticed from the conservation perspective. Here, we examined insects from Madagascar, Reunion, and Mauritius for the presence of trypanosomatid parasites (Kinetoplastea). Out of 660 specimens of the true bugs (Heteroptera) belonging to 87 species and 18 families, 95 individuals of 30 species were found to be infected (14% prevalence) by at least one trypanosomatid species, here referred to as typing units (TUs). Out of 141 flies (Diptera), 19 (13%) were infected. High diversity of the host species correlated with a high diversity of detected TUs belonging to 11 trypanosomatid genera, and representatives of 7 genera (*Angomonas*, *Blastocrithidia*, *Herpetomonas*, '*jaculum*', *Leptomonas*, *Wallacemonas*, and *Zelonia*) yielded axenic cultures. Of 39 detected TUs, more than half have not been encountered in other geographical regions and appear to be endemic. Altogether, 27 TUs, including 15 newly detected ones, were found exclusively in bugs, while flies hosted 11 TUs, out of which five were found exclusively on the studied islands. Only a single species, *Leptomonas moramango*, was found in both insect groups. Several new isolates have significantly extended the diversity of the plant-pathogenic *Phytomonas*. Geographically widespread as well as endemic TUs were detected in both widely distributed and (sub)endemic insects. The high proportion of endemic TUs suggests that the prominent role of islands in the global diversity of macroscopic organisms likely extends also to their protistan parasites and that the protection of macro-organisms in biodiversity hot spots can also protect the vast, yet mainly invisible, diversity of their parasitic companions.

Keywords Biodiversity loss · Conservation · Endemic · Host specificity · Coevolution · *Phytomonas* · Heteroptera

Introduction

The overwhelming majority of conservation efforts is focused on vertebrate animals and/or "higher" plants (see IUCN Red Data List, https://www.iucnredlist.org/), despite the already indisputable fact that from the extant eukaryotic groups, insects and protists are the most diverse ones (Novotný et al. 2006; Pawlowski et al. 2012). Eukaryotes with the parasitic lifestyle account for a large proportion of the overall diversity and play a major regulatory role in most ecosystems (Poulin 2014; Rocha et al. 2016). When a broader definition of this lifestyle is considered, parasites, somewhat counterintuitively, are a dominant group in terms of biodiversity, actually representing over half of all the species inhabiting our planet (Windsor 1998; Okamura et al. 2018). Still, they remain unjustifiably neglected by conservation biologists.

However, the ubiquity of parasites and their overwhelming diversity does not inform us about the vanishing species and those threatened with extinction. Unlike free-living organisms, most of them live in hiding, as the ecosystem for parasites is the body of their hosts. While the host specificity is, when trypanosomatids are concerned, not as high as previously believed (Grybchuk-Ieremenko et al. 2014; Kozminsky et al. 2015; Votýpka et al. 2015), extinction of a host species still poses a serious danger to its parasites. Although the term coextinction was introduced to describe this situation (Stork and Lyal 1993), very little data is available to assess its extent (Gómez and Nichols 2013). For example, it is estimated that a significant number of feather mites, lice, and ticks, which are highly host-specific parasites, have disappeared as a result of the extinction of their mammalian and bird hosts (Mihalca et al. 2011; Buckley et al. 2012; Rózsa and Vas 2015), and that along with extinct fishes and plants, their specific ectoparasites, primarily helminths and gall-forming or mining insects, have vanished (Dobson et al. 2008; Gómez and Nichols 2013). In a similar vein, one can safely assume that also many endoparasites, such as the highly host-specific monoxenous coccidia of the genera *Eimeria* and *Isospora*, were exterminated along with their hosts.

Due to specific features of the parasitic lifestyle, complex transmission modes and epidemiological "regularity", parasites may get extinct when the population density of their hosts is reduced below a certain threshold. Indeed, since parasitic species tend to decline at a faster rate than their hosts, even when a limited population of the latter survives, their parasites may already be gone (Dougherty et al. 2015; Strona 2015). Similarly, yet as an even more extreme example—if a host species disappears (albeit temporarily) from natural habitat and is maintained in rescue centers or zoos, its parasites may already be exterminated by drugs and/or breeding conditions (Gómez and Nichols 2013; Dougherty et al. 2015; Rózsa and Vas 2015).

Are parasites threatened or threatening? This is an unusual but completely legitimate issue that we should be addressing in more detail than previously. Although parasitic species may appear to be innumerable, many of them are (critically) endangered or extinct. Hence, the unique parasitofauna together with various commensals and endo/ectosymbionts (Windsor 1995; Yurchenko and Lukes 2018; Dheilly et al. 2019) should be protected along with their hosts. As such, parasites represent neglected wildlife and their diversity should be perceived as a conservation target (Gómez and Nichols 2013).

General appreciation of parasite diversity is inevitably biased by research on macroscopic parasites, such as helminths that infect terrestrial vertebrate hosts (Strona and Fattorini 2014; Carlson et al. 2020). Since protists parasitizing hosts with limited geographic occurrence remained largely neglected, in this study we have focused on the

occurrence of members of the family Trypanosomatidae infecting insects on the remote and oceanic islands. This was done to assess their geographic occurrence and host specificity and, consequently, their rarity and potential risk of extinction.

Since the formulation of the theory of island biogeography (MacArthur and Wilson 1967), the extreme biological diversity of remote islands has long stimulated research in ecology and evolution, with the islands being widely viewed as model systems for studying fundamental principles in ecology, evolutionary biology and biogeography (Emerson 2002; Warren et al. 2015; Graham et al. 2017). Remote islands host very vulnerable ecosystems that often lose a large part of their wildlife. Long isolation from mainland terrestrial ecosystems produced fauna and flora that display a high degree of endemism; however, island wildlife is generally badly affected by human activities including agriculture, destruction of ecosystems, and the introduction of alien invasive species. A rapid decline of many native species has led to numerous extinctions, including the most famous one—that of the flightless dodo of Mauritius (Turvey and Cheke 2008).

The majority of trypanosomatid flagellates is represented by monoxenous species restricted to the insect hosts (Maslov et al. 2013; Votýpka et al. 2015; Lukeš et al. 2018). Within the last decade, many new species have been described and several new genera and subfamilies of monoxenous trypanosomatids have been erected, overhauling the taxonomy of this speciose group, and substantially increasing our knowledge about their prevalence, diversity, pathogenicity, geographic distribution, as well as their intracellular symbionts (Kostygov et al. 2016; Barratt et al. 2017a, b; Grybchuk et al. 2018; Lukeš et al. 2018; Maslov et al. 2019). Examination of environmental samples from primarily heteropteran but also dipteran hosts collected worldwide seems to show initial signs of saturation (d'Avila-Levy et al. 2015). As a corollary, the available information allows us to characterize, for the first time, general rules regarding the distribution of trypanosomatids. Several studies focusing on their occurrence on islands have recently been published (Králová et al. 2019; Votýpka et al. 2019), allowing to address the island biography theory from the perspective of these protistan parasites. The current study analyzes their occurrence on the large remote island of Madagascar and two smaller oceanic islands in its distant neighborhood, Reunion and Mauritius.

Materials and methods

Localities, collection of insects and cultivation

The study was conducted on three remote islands of the southwestern Indian Ocean, namely Madagascar and the two largest of the Mascarene Islands, Reunion and Mauritius (Fig. S1). Madagascar, the fourth largest island in the world, is approximately 400 km off the southeast coast of Africa. Reunion is 700 km east of Madagascar and 175 km southwest of Mauritius. The habitats of these islands significantly differ in size, topography, rainfalls, geographic history, human settlements, and proximity to the nearest major landmass. Insects, predominantly true bugs (the suborder Heteroptera) and flies (Diptera: Brachycera), were captured in March 2010 in Madagascar and in March 2017 in Reunion and Mauritius. In Madagascar, seven different localities (Morondava village 20°18′2.82″S, 44°16′17.93″E; Ambatolampy 19°22′58.38″S, 47°25′57.86″E; Ambatofosty 19°16′46.79″S, 47°28′44.47″E; Moramango/Andasibe 18°55′33.89″S, 48°25′4.33″E; Mahambo 17°28′38.49″S, 49°27′50.84″E; Foulpointe (Mahavelona) 17°40′54.15″S, 49°30′9.80″E; Ivoloina Zoological Park (Toamasina) 18°3′32.95″S, 49°21′31.65″E), in

Reunion five localities (Cilaos 21°9′23.59″S, 55°28′15.07″E; Takamaka 21°4′40.98″S, 55°37′41.11″E; Salazie 21°0′44.97″S, 55°33′35.50″E; Saint-Denis 20°56′38.31″S, 55°28′49.52″E; Mafate Cirque—Dos d'Ane 20°59′21.13″S, 55°22′52.69″E), and one site in Mauritius (Vanille 20°30′15.10"S, 57°34′19.80″E) were inspected (Fig. S1).

Insects were collected predominantly by net sweeping from the vegetation, but also by manual picking or by light attraction to local light sources. Within 24 h after capture, the insects were processed as described in detail elsewhere (Lukeš and Votýpka 2020).

Host insect identification

To facilitate host identification, most specimens were dry-mounted and deposited to the collections of the Department of Entomology, National Museum, Prague, Czech Republic. When appropriate, the material was sent for identification to specialists on particular groups (see "Acknowledgements" section). The remaining specimens were compared with available taxonomic revisions and/or the collections of Muséum nationale d'Histoire naturelle, Paris, France, and The Natural History Museum, London, UK. Based on the available catalogs and revisions, the distribution of species and genera of the insect host was sorted into the following three categories: (i) endemic for the particular island; (ii) sub-endemic, distributed in Madagascar, and the Mascarene Islands, the Comoro Islands, and Seychelles (or at least some of the islands); (iii) widely distributed taxa, including other biogeographic realms, such as the studied island(s) plus continental Africa, and/or beyond.

DNA extraction, PCR amplification, and sequencing

Total genomic DNA was extracted from the insect gut tissue, or in the case of successful cultivations, from 1 ml of axenically grown cultures, as described previously (Kostygov et al. 2014; Votýpka et al. 2014), using either a DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) or a DNA tissue isolation kit (Roche Diagnostics, Indianapolis, USA) according to the manufacturers' protocols.

The extracted DNA was used for amplification of the 18S rRNA gene with primers S762 and S763, producing an almost full-length gene ($\sim$ 2.1 kb), as described previously (Maslov et al. 1996). When amplification with the first primer pair resulted in a weak or invisible PCR product, which occurs frequently for DNA samples extracted from the insect tissues, the second round of PCR was performed with nested primers TRnSSU-F2 and TRn-SSU-R2 (Seward et al. 2017). The obtained amplicons were directly sequenced, the sequences were aligned using Geneious software (version 10.0.6), and phylogenetic trees were inferred using related sequences available in GenBank. Alignments for phylogenetic analyses were generated by MAFFTv7, with ambiguously aligned positions in the trimmed alignment being removed manually in Geneious. The final dataset contained 398 taxa (Table S2) and 2484 characters. Analyses were performed in PhyMLv3.0.1 and MrBayes v3.2.2 with model optimization in ModelTest v3.06. A general time-reversible substitution model with a mixed model for among site rate variation (GTR $+ \Gamma + $ I) was chosen as the best fitting model of sequence evolution. Bootstrap analyses involved heuristic searches with 1000 replicates (Maximum likelihood). Bayesian inference analysis was run for five million generations with covarion and sampling every 100 generations. All other parameters were left in their default states.

Results and discussion

Field examination of insect hosts for trypanosomatid infection

Altogether, 660 specimens of heteropteran bugs belonging to 87 species and 18 families were captured and inspected for the presence of trypanosomatid flagellates in their gut (Table 1). Out of them, 95 (overall prevalence 14%) specimens belonging to 30 species (35%) and 8 (45%) families were infected by these parasites (Tables 1, 2 and S1; Fig. S1). Out of 57 trypanosomatid-free species, a significant fraction (52 species; 91%) was represented by only a very small number of dissected individuals, mostly less than five (Table 1). More than 10 specimens were inspected for the following trypanosomatid-free species: *Deraeocoris howanus* (14 individuals), *Pachygrontha angularis* (23), *Pachygrontha* sp. (18), *Coptosoma depulsa* (19), and *Diniella marginata* (28) (Table 1). The overall prevalence of heteropteran bug infection on the studied islands (14%) was similar to that in China (16%) and Papua New Guinea (15%), but significantly lower when compared to 26–38% prevalence documented in Africa and the Neotropics (Maslov et al. 2007; Votýpka et al. 2010, 2019; Jirků et al. 2012; Kozminsky et al. 2015; Lukeš et al. 2018; Králová et al. 2019). However, these differences may be explained not only by different geographic origin but also by varying number of dissected bugs within various heteropteran and dipteran families.

Most individuals were caught and subsequently examined in the island of Madagascar. Out of 438 heteropterans, which were determined to belong to 68 species and 16 families, 52 specimens belonging to 21 species were infected. In Reunion, out of 173 dissected specimens (22 species; 9 families), 33 individuals (10 species) were infected, and in Mauritius out of 49 bugs (10 species; 7 families), 10 (6 species) were found to be parasitized (Tables 1 and S1; Fig. S1). On the higher taxonomic level, the prevalence of trypanosomatids in heteropteran families is similar to the situation observed on the species level—out of 18 inspected families, only 8 were trypanosomatid-positive: Alydidae (10 positive specimens out of 52 examined, 19%), Coreidae (25/76, 33%), Lygaeidae (18/95, 19%), Miridae (15/82, 18%), Pachygronthidae (1/57, 2%), Pentatomidae (18/133, 16%), Rhopalidae (7/66, 11%), and Rhyparochromidae (2/43, 5%).

This is in good correlation with studies from other geographic areas, where Alydidae, Coreidae, Lygaeidae, Pentatomidae, and Rhopalidae showed a prominently high prevalence of infection (Votýpka et al. 2010, 2012a, b, 2019; Králová et al. 2019). In the current study, however, two true bug families, the Reduviidae and Pyrrhocoridae, elsewhere found to be heavily infected by trypanosomatids (Votýpka et al. 2010, 2012a, b; Kozminsky et al. 2015; Králová et al. 2019), are missing from the list of parasite-positive taxa. Still, this may reflect the fact that reduviids and pyrrhocorids were underrepresented in the present study.

The behavior of many species of the families Pyrrhocoridae, Lygaeidae, and Rhyparochromidae are gregarious and due to their feeding on seeds on the ground, there is a higher probability of them being contaminated by infective stages present in insect feces, acquired during co-feeding. These families are also well-known for accidental coprophagy and/or necrophagy. In contrast to the phytophagous seed-feeding or sap-sucking species, predatory bugs such as Reduviidae, Gerridae, Hydrometridae, and Nabidae may acquire, in addition to their own parasites, less specific monoxenous trypanosomatids from their prey and thus may represent a kind of "collection container" for parasites occurring in their vicinity. Indeed, this is what was observed when the heteropteran-trypanosomatid relationship was inspected on several continents (Votýpka et al. 2010, 2012a, b; Kozminsky

Table 1 Summarized information about true bug (Heteroptera) hosts from Madagascar, Reunion, and Mauritius and their trypanosomatids, including the insect species distribution, the prevalence of parasites (number of dissected/infected specimens), and the list of detected trypanosomatids (typing units (TUs) and genera/species)

Family	Species	Genus	Species	MG	REU	MU	TUs	Genus/species
Alydidae	*Nariscus* sp.	WIDE	?		1/1		*TU217	*'jaculum'* sp.
	Stenocoris annulicornis	WIDE	WIDE	**1/28**		**1/2**	*TU6/7AB2*	*'jaculum'* sp.
	Alydinae gen. sp.	WIDE	?	**3/12**			*TU218*	*'jaculum'* sp.
							TU216	*'jaculum'* sp.
							TU6/7D2	*'jaculum'* sp.
	Mirperus jaculus	WIDE	WIDE	**1/2**			TU88b	*'jaculum'* sp.
							TU6/7D	*'jaculum'* sp.
	Riptortus fabricii	WIDE	ENDE	**3/5**			TU6/7E	*'jaculum'* sp.
							TU6/7C	*'jaculum'* sp.
	Tenosius proletarius	WIDE	WIDE	0/2				
Belostomatidae	*Appasus quadrivitattus*	WIDE	ENDE	0/2				
Coreidae	*Clavigralla madagascariensis*	WIDE	ENDE	**4/6**			TU6/7C	*'jaculum'* sp.
							TU248	*Phytomonas* sp.
	Cletoscellus spinijugis	ENDE	ENDE	0/2				
	Cletus capensis	WIDE	WIDE		**5/14**	**1/4**	TU72	*'jaculum'* sp.
	Cletus clavatus	WIDE	SUBE	0/1				
	Cletus ochraceus	WIDE	WIDE	0/1	**7/18**	0/3	TU32	*L. spiculata*
							TU72	*'jculum'* sp.
							TU48A	*L. podlipaevi*
	Cletus poikilus	WIDE	ENDE	0/1				
	Cletus presignus	WIDE	ENDE	**2/2**			*TU218*	*'jaculum'* sp.
	Hydara tenuicornis	WIDE	WIDE	**6/11**	0/1		TU6/7C	*'jaculum'* sp.
	Mevanidea spiniceps	WIDE	ENDE	0/2				

Table 1 continued

Family	Species	Genus	Species	MG	REU	MU	TUs	Genus/species
Gerridae	*Limnogonus guttatus*	WIDE	ENDE	0/6				
	Limnogonus cereiventris	WIDE	WIDE	0/6				
	Tenagometra lanuginea	WIDE	ENDE	0/2				
Geocoridae	*Geocoris* sp. nov	WIDE	ENDE		0/1			
Hydrometridae	*Hydrometra bifurcata*	WIDE	SUBE	0/6				
Lygaeidae	*Aspilocoryphus unimaculatus*	WIDE	WIDE	2/2			TU88	*'jaculum'* sp.
							TU88*b*	*'jaculum'* sp.
	Caenocoris croceosignatus	WIDE	WIDE	0/1				
	Nysius albipennis/euphorbiae	WIDE	SUBE	0/15	6/41	1/21	*TU251	*Phytomonas* sp.
							TU32	*L. spiculata*
							TU72	*'jaculum'* sp.
	Spilostethus furculus	WIDE	WIDE	1/6			TU88*b*	*'jaculum'* sp.
	Spilostethus pandurus	WIDE	WIDE	7/7			TU88*b*	*'jaculum'* sp.
	Stalagmostethus furcatus	WIDE	WIDE	1/2			*TU214	*Zelonia* sp.
							TU88*b*	*'jaculum'* sp.

Table 1 continued

Family	Species	Genus	Species	MG	REU	MU	TUs	Genus/species
Miridae	Collaria bourbonica	WIDE	ENDE		0/1			
	Corizidolon notaticolle	WIDE	SUBE		0/5			
	Creontiades pallidus	WIDE	WIDE			0/1		
	Creontiades cf. subpellucidus	WIDE	WIDE	0/1				
	Creontiades cf. suturalis	WIDE	WIDE	0/1				
	Deraeocoris howanus	WIDE	SUBE		0/14			
	Deraeocoris ostentans	WIDE	WIDE		0/5			
	Dolichomiris linearis	WIDE	WIDE	0/2				
	Gutrida gabonia	WIDE	WIDE	0/3				
	Hyalopeplus madagascariensis	WIDE	ENDE	0/1				
	Charagochilus sp.	WIDE	?	0/1				
	Proboscidocoris cf. intermedius	WIDE	WIDE	**1/1**			*TU**211**	*Leptomonas* sp.
	Proboscidocoris fuliginosus	WIDE	WIDE	**1/2**	**1/1**		*TU**212**	*Leptomonas* sp.
							*TU**213**	*Leptomonas* sp.
	Taylorilygus apicalis	WIDE	WIDE	0/5	**7/28**	**5/8**	TU**32**	*L. spiculata*
							*TU**251**	*Phytomonas* sp.
	Taylorilygus simonyi	WIDE	WIDE	0/1	0/1			
Nabidae	Arbela elegantula elegantula	WIDE	WIDE		0/4			
	Nabis capsiformis	WIDE	WIDE			0/1		
Naucoridae	gen. sp.	WIDE	?	0/6				
	Heleocoris humeralis	WIDE	ENDE	0/2				
	Macrocoris distinctus	WIDE	ENDE	0/5				
	Naucoris madagascariensis	WIDE	ENDE	0/3				
Nepidae	Laccotrephes pseudoampliatus	WIDE	ENDE	0/1				

Table 1 continued

Family	Species	Genus	Species	MG	REU	MU	TUs	Genus/species
Pachygronthidae	*Pachygrontha angularis*	WIDE	ENDE	0/23				
	Pachygrontha bipunctata	WIDE	WIDE	0/6				
	Pachygrontha quadripunctata	WIDE	ENDE	0/3				
	Pachygrontha sp.	WIDE	?	0/18				
	Teracrius namaquensis	WIDE	WIDE	**1/1**			TU**88b**	*'jaculum'* sp.
Pentatomidae	*Aeliomorpha viridis*	WIDE	ENDE	0/1				
	Afrius flavirostrum	WIDE	ENDE	0/2				
	Antestia variabilis	WIDE	ENDE	0/8				
	Antestiella mauritii	WIDE	SUBE	**1/38**	1/7		TU**99**	*B. raabei*
	Aspavia albidomaculata	WIDE	WIDE	**1/38**			TU**44**	*Blastocrithidia* sp.
	Aspavia longispina	WIDE	ENDE	**3/11**			TU**67D**	*'jaculum'* sp.
							TU**77/177-1**	*Phytomonas* sp.
Plataspidae	*Bathycoelia* cf. *conferenda*	WIDE	ENDE	0/1				
	Carbula frappai	WIDE	ENDE	0/1				
	Diploxys fallax	WIDE	WIDE	0/1				
	Dorycoris pavonius	WIDE	WIDE	0/6				
	Eysarcoris v-flavum	WIDE	ENDE	**3/6**			TU**44**	*Blastocrithidia* sp.
	Nezara viridula	WIDE	WIDE		0/1			
	Phricodus hystrix	WIDE	WIDE	**7/9**			*TU**252**	*Phytomonas* sp.
	Sciocoris wolffi	WIDE	ENDE	**1/24**			TU**77/177-2**	*Phytomonas* sp.
	Scotinophara tibialis	WIDE	SUBE		0/1			
	Veterna nigromarginata	WIDE	ENDE	**2/16**			TU**62**	*Blastocrithidia* sp.
	Coptosoma depalsa	WIDE	ENDE	0/19				
Reduviidae	*Oncocephalus angulatus*	WIDE	SUBE	0/1				
	Sphedanolestes sp.	WIDE	?	0/2				

Table 1 continued

Family	Species	Genus	Species	MG	REU	MU	TUs	Genus/species
Rhopalidae	*Agraphopus brevicollis*	WIDE	ENDE	0/5				
	Leptocoris hexophthalma lateralis	WIDE	SUBE		0/1			
	Leptocoris mutilata	WIDE	WIDE		**4/18**		TU**48A** / *TU**249**	*L. podlipaevi* / *Phytomonas* sp.
	Liorhyssus hyalinus	WIDE	WIDE		0/6	**1/1**	TU**73**	*'jaculum'* sp.
	Stictopleurus scutellaris coquerelii	WIDE	SUBE	**1/32**	0/3		TU**105** / TU**73**	*L. moramango* / *'jaculum'* sp.
Rhyparochromidae	*Dieuches placidus*	WIDE	SUBE	0/2				
	Diniella marginata	WIDE	ENDE	0/28				
	Gyndes capito	WIDE	ENDE	0/2				
	Horridipamera sp.	WIDE	?		**1/1**		*TU**250**	*Phytomonas* sp.
	Paromius gracilis	WIDE	WIDE			0/7		
	Perimeda dimidiata	WIDE	ENDE	0/1				
	Pseudopachybrachius reductus	WIDE	WIDE	0/1				
	Stigmatonotum geniculatum	WIDE	WIDE			**1/1**	*TU**215**	*Blastocrithidia* sp.
Stenocephalidae	*Dicranocephalus punctipes*	WIDE	SUBE	0/1				

Distribution of species and genus of the insect host was sorted into the following categories: ENDE = taxon endemic for the particular island; SUBE = taxon sub-endemic, distributed in Madagascar, the Mascarene Islands, the Comoro Islands, and Seychelles; and WIDE = widely distributed taxa, including other biogeographic realms, such as the studied island(s) plus continental Africa, and/or beyond; ? = undeterminable distribution. MG, Madagascar; REU, Reunion; MU, Mauritius

Families and specimens positive for trypanosomatids are in bold; * TU newly detected in this study

Table 2 Summarized information about the detected trypanosomatids (species and/or typing unit; TUs), insect host species (including the number of infected specimens and species distribution), localization of the infection in the host intestine, the GenBank™ accession numbers of the 18S rRNA sequences determined in this study, isolate designation, and availability in culture

Trypanosomatid species	TUs	Madagascar	Reunion	Mauritius	Family
Leptomonas moramango	TU105	Pachycerina cf. vaga			DIPTERA
		Stictopleurus scutellaris coquerelii			Rhopalidae
Leptomonas podlipaevi	TU48A		Leptocoris mutilata (2)		Rhopalidae
			Cletus ochraceus		Coreidae
Leptomonas spiculata	TU32		Taylorilygus apicalis (7)	Taylorilygus apicalis (5)	Miridae
			Nysius albipennis/euphorbiae	Nysius albipennis/euphorbiae	Lygaeidae
			Cletus ochraceus		Coreidae
Leptomonas sp. 1	*TU211	Proboscidocoris cf. intermedius			Miridae
Leptomonas sp. 2	*TU212	Proboscidocoris fuliginosus			Miridae
Leptomonas sp. 3	*TU213	Proboscidocoris fuliginosus			Miridae
Zelonia sp. 1	*TU214	Stalagmostethus furcatus			Lygaeidae
Blastocrithidia raabei	TU99		Antestiella mauritii		Pentatomidae
Blastocrithidia sp.	TU44	Eysarcoris v-flavum (3)			Pentatomidae
		Aspavia albidomaculata			Pentatomidae
Blastocrithidia sp.	TU62	Veterna nigromarginata (2)			Pentatomidae
Blastocrithidia sp. A	*TU215			Stigmatonotum geniculatum	Rhyparochromidae
'jaculum' sp.	*TU6/7AB2			Stenocoris annulicornis	Alydidae
'jaculum' sp.	TU6/7C	Hydara tenuicornis (6)			Coreidae
		Clavigralla madagascariensis			Coreidae
'jaculum' sp.	TU6/7D	Mirperus jaculus			Alydidae
		Riptortus fabricii			Alydidae
		Aspavia longispina			Pentatomidae
'jaculum' sp.	*TU6/7D2	Alydinae gen. sp.			Alydidae
'jaculum' sp.	TU6/7E	Riptortus fabricii			Alydidae

Table 2 continued

Trypanosomatid species	TUs	Madagascar	Reunion	Mauritius	Family
'jaculum' sp.	TU73	Stictopleurus scutellaris coquerelii			Rhopalidae
				Liorhyssus hyalinus	Rhopalidae
'jaculum' sp.	TU72		Cletus ochraceus (10)	Cletus ochraceus	Coreidae
			Nysius albipennis/euphorbiae		Lygaeidae
'jaculum' sp.	TU88	Aspilocoryphus unimaculatus			Lygaeidae
'jaculum' sp.	TU88b	Spilostethus pandurus (7)			Lygaeidae
		Spilostethus furculus			Lygaeidae
		Stalagmostethus furcatus			Lygaeidae
		Aspilocoryphus unimaculatus			Lygaeidae
		Teracrius namaquensis			Pachygronthidae
'jaculum' sp. A	*TU216	Alydinae gen. sp. (2)			Alydidae
'jaculum' sp. B	*TU217		Nariscus sp.		Alydidae
'jaculum' sp. C	*TU218	Cletus presignus (2)			Coreidae
		Alydinae gen. sp. (2)			Alydidae
Phytomonas sp.	TU77/177-1	Aspavia longispina (2)			Pentatomidae
Phytomonas sp.	TU77/177-2	Sciocoris wolffi			Pentatomidae
Phytomonas sp. 1	*TU248	Clavigralla madagascariensis (4)			Coreidae
Phytomonas sp. 2	*TU249		Leptocoris mutilata		Rhopalidae
Phytomonas sp. 3	*TU250		Horridipamera sp.		Rhyparochromidae
Phytomonas sp. 4	*TU251		Nysius albipennis/euphorbiae (6)		Lygaeidae
			Taylorilygus apicalis		Miridae
Phytomonas sp. 5	*TU252	Phricodus hystrix (7)			Pentatomidae
Herpetomonas muscarum	N/A	Chrysomya putoria			DIPTERA
Herpetomonas isaaci	TU107	Chrysomya putoria			DIPTERA
Herpetomonas puellarum	N/A		fly (3)		DIPTERA
Herpetomonas sp. A	TU108	Chrysomya putoria + Musca sp.			DIPTERA

Table 2 continued

Trypanosomatid species	TUs	Madagascar	Reunion	Mauritius	GB Acc.No.	Family	Cult.
Herpetomonas sp. B	*TU221	*Musca* sp.				DIPTERA	Y
Lafontella marideanei	N/A	*Chrysomya putoria*				DIPTERA	Y
Wallacemonas raviniae	TU110	fly				DIPTERA	Y
Wallacemonas sp. 1	*TU220		fly (3)			DIPTERA	
'new clade II' sp. A	*TU253	*Chrysomya putoria* (2)	fly			DIPTERA	Y
Angomonas deanei	N/A	fly (2)				DIPTERA	
Kentomonas sp. A	*TU219		fly (3)			DIPTERA	

Trypanosomatid species	Genus	Species	Localization	Isolate for SSU	GB Acc.No.	Cult.
Leptomonas moramango			RA	MMO-09_cult	KC205990	Y
	WIDE	ENDE	MG, HG, MT	M05_cult	MT174471	Y
Leptomonas podlipaevi	WIDE	WIDE	MG	Re23_cult	MT174503	Y
	WIDE	WIDE	MG			
Leptomonas spiculata	WIDE	SUBE	MG	Re31_cult	MT174505	Y
	WIDE	WIDE	MG			
Leptomonas sp. 1	WIDE	WIDE	MT	M31_envi	MT174482	
Leptomonas sp. 2	WIDE	WIDE	MG	M32_envi	MT174483	Y
Leptomonas sp. 3	WIDE	WIDE	MG	Re13_envi	MT174502	
Zelonia sp. 1	WIDE	WIDE	MG	M08_cult	MT174474	Y
Blastocrithidia raabei	WIDE	SUBE	MG	Re12_envi	MT174501	
Blastocrithidia sp.	WIDE	ENDE	MG	M37_envi	MT174484	
Blastocrithidia sp.	WIDE	WIDE	MG			
Blastocrithidia sp. A	WIDE	ENDE	TMG	M07_cult	MT174473	Y
'jaculum' sp.	WIDE	WIDE	MG (?)	Re36_envi	MT174507	
	WIDE	WIDE	MG/HG	Re43A_envi	MT174508	

Table 2 continued

Trypanosomatid species	Genus	Species	Localization	Isolate for SSU	GB Acc.No.	Cult.
'jaculum' sp.	WIDE	WIDE	MG, (HG, MT)	M47_envi	MT174487	
	WIDE	ENDE	MG	M22_cult		Y
'jaculum' sp.	WIDE	WIDE	HG	M04_cult/envi	MT174470	Y
	WIDE	ENDE	MG			
	WIDE	ENDE	MG (?)			
'jaculum' sp.	WIDE	?	AMG	M21_cult	MT174478	Y
'jaculum' sp.	WIDE	ENDE	MG	M26_envi	MT174480	
'jaculum' sp.	WIDE	ENDE	MG, HG, MT	M05_envi	MT174472	
	WIDE	WIDE	MG	Re35_envi	MT174506	
'jaculum' sp.	WIDE	WIDE	MG, (HG)	Re02_envi	MT174498	
	WIDE	SUBE	MG			
'jaculum' sp.	WIDE	WIDE	MG	M09_cult	MT174475	Y
'jaculum' sp.	WIDE	WIDE	AMG	M12_cult	MT174476	Y
	WIDE	WIDE	MG			
	WIDE	WIDE	MG			
	WIDE	WIDE	AMG			
	WIDE	WIDE	AMG			
'jaculum' sp. A	WIDE	?	AMG	M20_cult	MT174477	Y
'jaculum' sp. B	WIDE	?	MG (?)	Re01_envi	MT174497	
'jaculum' sp. C	WIDE	ENDE	HG	M30_envi	MT174481	
	WIDE	?	AMG	M19/M20_cult		Y
Phytomonas sp.	WIDE	ENDE	AMG	M03_envi	MT174469	
Phytomonas sp.	WIDE	ENDE	MG	M41_envi	MT174485	
Phytomonas sp. 1	WIDE	ENDE	MG	M23_envi	MT174479	
Phytomonas sp. 2	WIDE	WIDE	MG	Re25A_envi	MT174504	
Phytomonas sp. 3	WIDE	?	MG	Re06_envi	MT174500	

Table 2 continued

Trypanosomatid species	Genus	Species	Localization	Isolate for SSU	GB Acc.No.	Cult.
Phytomonas sp. 4	WIDE	SUBE	MG	Re03_envi	MT174499	
	WIDE	WIDE	MG			
Phytomonas sp. 5	WIDE	WIDE	MG	M42_envi	MT174486	
Herpetomonas muscarum			HG, MT	MMO-01_cult	KC205982	Y
Herpetomonas isaaci			?	MMO-02_cult	KC205992	Y
Herpetomonas puellarum			RA	MRe-01_envi	MT174494	
Herpetomonas sp. A			MG (?)	MMO-03_envi	MT174488	
Herpetomonas sp. B			HG	MMO-06_envi	MT174490	
Lafontella marileanei			MG	MMO-08_envi	MT174491	
Wallacemonas raviniae			HG	MMO-12_envi	MT174493	
Wallacemonas sp. 1			RA	MRe-04_cult	MT174496	Y
'new clade II' sp. A			MG	MMO-05_envi	MT174489	
Angomonas deanei			RA, HG	MMO-10_cult	MT174492	Y
Kentomonas sp. A			HG, RA	MRe-03_envi	MT174495	

Localization of the infection in the host intestine: *AMG* abdominal midgut, *HG* hindgut, *MG* midgut, *MT* Malpighian tubules, *RA* Rectal ampulla, *TMG* thoracic midgut

* TU newly detected in this study. Near full-length 18S rRNA gene (SSU) obtained from cells available in axenic culture (cult) or from DNA parasites originated from insect gut (envi). Distribution of species and genus of the insect host was sorted into the following categories: ENDE = taxon endemic for the particular island; SUBE = taxon subendemic, distributed in Madagascar, the Mascarene Islands, the Comoro Islands, and Seychelles; and WIDE = widely distributed taxa, including other biogeographic realms, such as the studied island(s) plus continental Africa, and/or beyond; ? = unknown distribution

et al. 2015; Králová et al. 2019), yet among the captured island bug families, along with Reduviidae, other six families (Belostomatidae, Geocoridae, Hydrometridae, Nabidae, Nepidae, and Stenocephalidae) were examined only scarcely and found to be negative. The same applies to 14, 16 and 19 parasite-free specimens belonging to Gerridae, Naucoridae, and Plataspidae, respectively.

In addition to the heteropteran hosts, 141 brachyceran flies were captured and examined. Unfortunately, due to logistic problems, a detailed taxonomic determination could have been carried out only on a limited number of specimens. Dissection and microscopic inspection revealed 13 and six trypanosomatid-infected individuals out of 85 and 56 flies captured in Madagascar and Reunion, respectively.

Determination and phylogenetic analysis of trypanosomatids

Out of 114 DNA samples extracted from the intestine of infected bugs (95 specimens) and flies (19 specimens) detected under light microscope, the 18S rRNA gene was successfully amplified from 111 individuals (98% efficiency) and in all but one case, we were able to obtain nearly full-length sequences. Based on these data, 39 different TUs, including 19 TUs not seen before, belonging to 11 trypanosomatid genera of three subfamilies were documented (Table 2; Fig. 1 and S1). Since in the current classification system, six
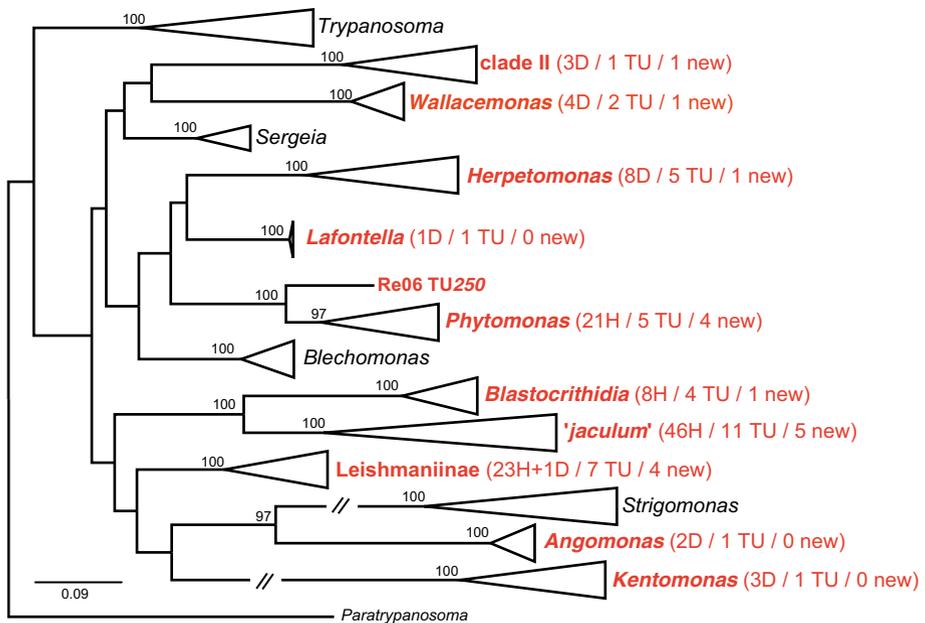
Fig. 1 Phylogenetic tree of trypanosomatids (the family Trypanosomatidae) based on the 18S rRNA gene sequences and reconstructed using the Maximum likelihood method. All genera are collapsed and their contents are shown in individual subtrees (Figs. 2, 3, 4, 5, 6). Numbers in parentheses show the total number of infected heteropterans (H) and dipterans (D), the total number of Typing Units (TU) detected in the insects captured in Madagascar, Reunion, and Mauritius, and the number of "endemic" TUs for a respective clade. The tree was rooted with the sequence of *Paratrypanosoma confusum*; the bootstrap values over 50% (1000 replicates) are shown at the nodes; the scale bar denotes the number of substitutions per site
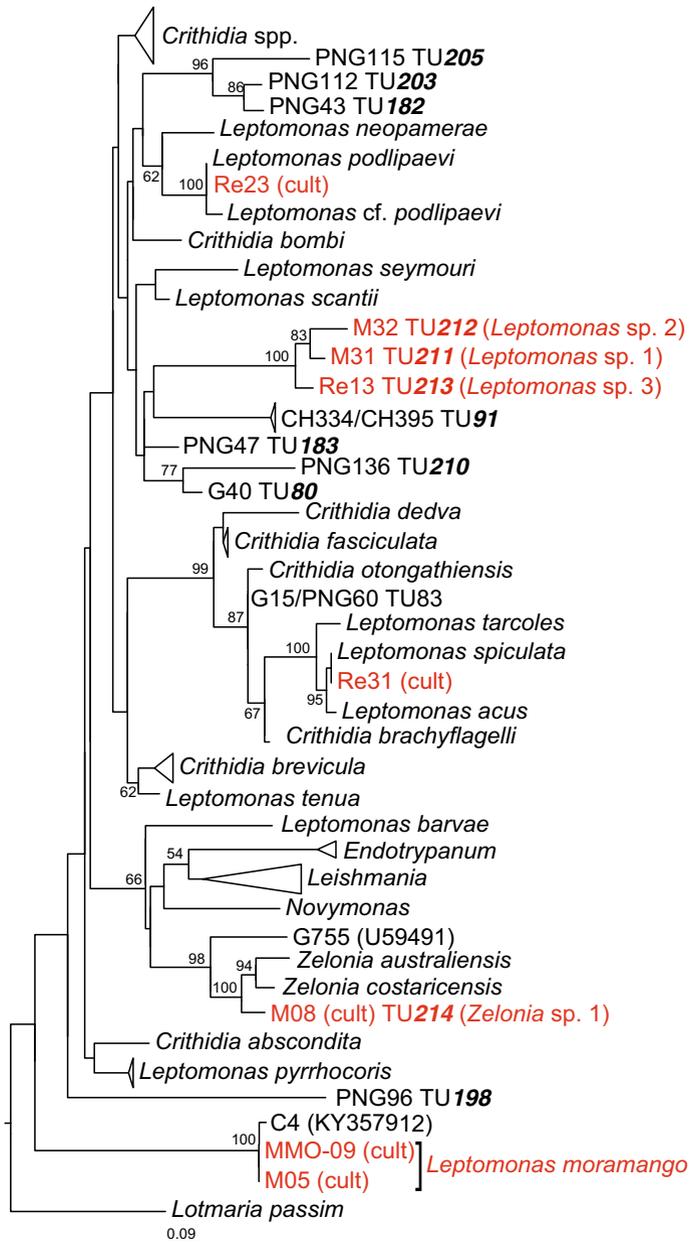
Fig. 2 Expanded subtree of the subfamily Leishmaniinae

formally recognized subfamilies and 22 genera are included in the family Trypanoso-matidae (Maslov et al. 2019), this study covers half of the known trypanosomatid diversity.

In 28 specimens (18 true bugs and 10 flies) a simultaneous infection by two different trypanosomatid species was documented based on the sequences obtained from either dissected gut samples or axenic cultures derived from the same specimens (Table S1 and
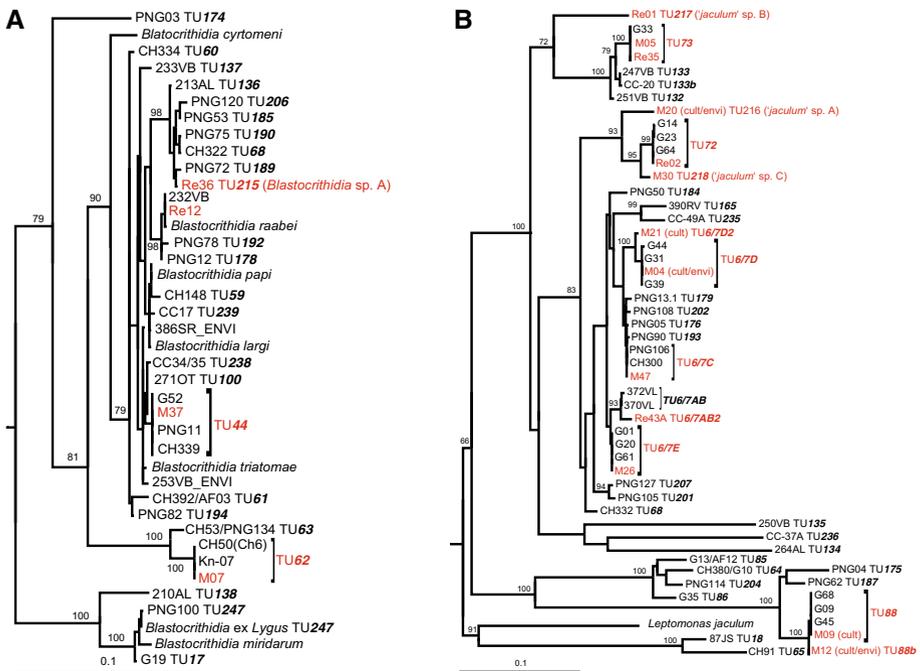
Fig. 3 Expanded subtree of the genus *Blastocrithidia* (**A**) and '*jaculum*' phylogroup (**B**)

see below). However, because PCR products were not cloned in the case of mixed infections detected in DNA samples extracted from the infected guts, the analysis of chromatograms resulted in most cases in the identification of only one species.

The increasing number of monoxenous trypanosomatid species (TUs) described to date allowed us to compare their phylogeny and distribution over a wide range of host taxa and geographic areas. It was obvious that several clades are associated with particular host groups, such as the "fly" genera (*Herpetomonas*, *Lafontella*, *Wallacemonas*, *Angomonas*, *Kentomonas*, and clade II. sensu Týč et al. 2013) or the "bug" genera (*Blastocrithidia* and '*jaculum*'). Reference sequences were selected for all major trypanosomatid clades; for the clades/genera comprising TUs found in the current dataset, all available 18S rRNA gene sequences were used. The resulting simplified tree topology (Fig. 1) is congruent with those published previously (Týč et al. 2013; Votýpka et al. 2013, 2019; Kostygov et al. 2016; Yurchenko et al. 2016; Ishemgulova et al. 2017; Králová et al. 2019). The phylogenetic analysis did not reveal any novel taxonomic group on the generic or family levels (Fig. 1; but see below).

Although due to the logistic reasons our collections of trypanosomatids from the studied islands significantly differed when the number of captured specimens and inspected localities are considered, there was a correlation between the amount of dissected insects and their trypanosomatid diversity. In Madagascar, 26 TUs were recorded in 438 dissected bugs belonging to 68 species, supplemented by 85 examined flies. Smaller collections from Reunion yielded 12 TUs from 56 flies and 173 bugs ranked into 22 species, while five TUs were derived from 49 bugs representing 10 species originating from Mauritius (Tables 1, 2).
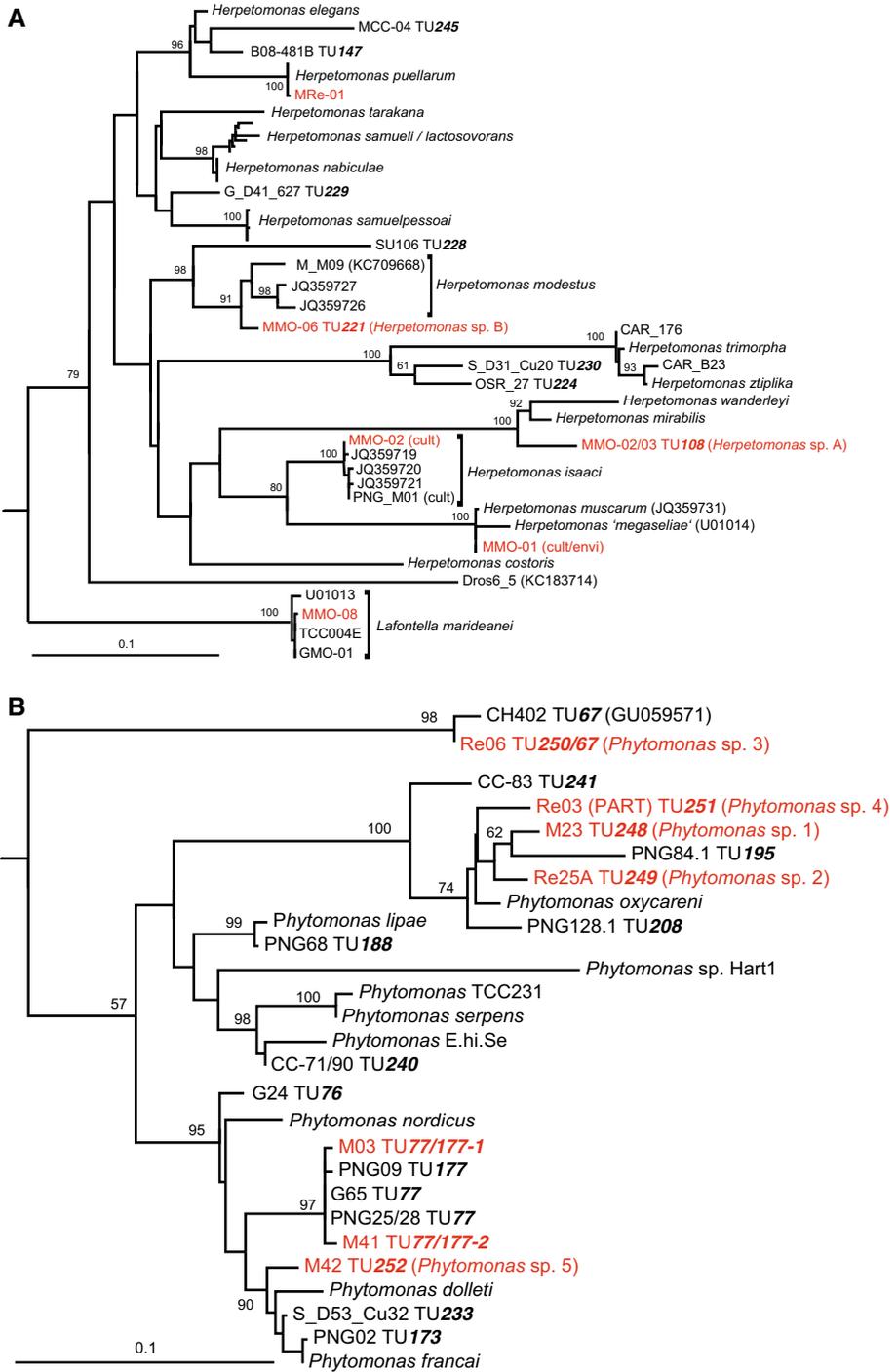
Fig. 4 Expanded subtree of the subfamily Phytomonadinae: *Herpetomonas* (**A**) and *Phytomonas* (**B**)
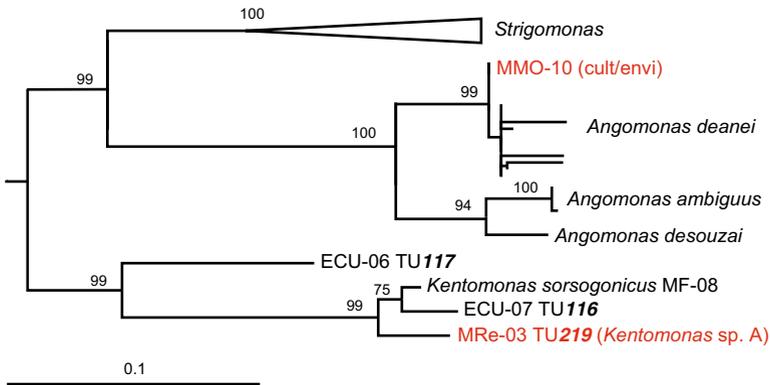
Fig. 5 Expanded subtree of the subfamily Strigomonadinae (*Angomonas*, *Strigomonas,* and *Kentomonas*)
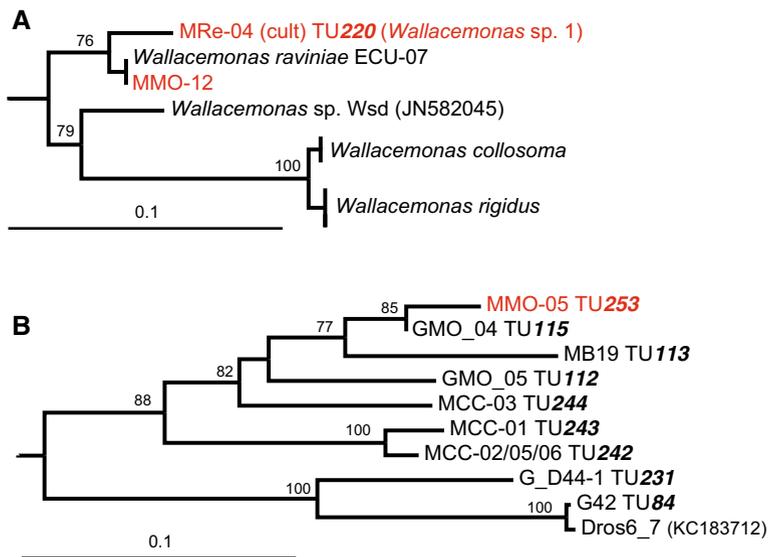
Fig. 6 Expanded subtree of the genus *Wallacemonas* (**A**) and clade II sensu Týč et al. 2013 (**B**)

Most of the 39 recorded TUs were found only in a single host species and only on one island (Table 2). However, several exceptions deserve to be mentioned, as they testify of a wider distribution of trypanosomatids across the studied islands and insect host species. Lower host specificity is clearly a common feature of the following 9 species—*Leptomonas moramango* [TU*105*], *Leptomonas podlipaevi* [TU*48A*], TU*6/7C*, TU*44*, TU*72*, TU*77/177*, TU*108*, and novel TU*218* and TU*251*—captured in two host species on the same island. In Madagascar, three heteropteran host species were found to be infected by TU*6/7D*, and five host species accommodate TU*88*/TU*88b*. *Leptomonas spiculata* (TU*32*) was found in three and two host species in Reunion and Mauritius, respectively. Different insect hosts captured on different islands were detected also for already described TU*73*

(Madagascar and Mauritius) and the previously unknown TU*253* (Madagascar and Reunion). Finally, TU*72* parasitized the same host species on Reunion and Mauritius (Table 2).

Trypanosomatids from heteropteran bugs

Out of the 39 documented TUs, heteropteran bugs exclusively hosted 27 TUs of five genera including 15 (56%) new ones, while 11 TUs of seven genera were confined to dipteran flies, of which four (36%) were new (Table 2). Only a single species, *L. moramango* (TU*105*), was found in both unrelated groups of insects. Phylogenetic analysis based on the PCR-amplified 18S rRNA sequences from the microscopically-positive heteropteran bugs revealed the presence of a high number of trypanosomatid species and genera (Table 2; Fig. 1).

In our dataset, the genus *Leptomonas* is represented by three novel species (TU*211*, TU*212*, and TU*213*) and three already known ones (Table 2; Fig. 2). While *L. podlipaevi* (TU*48A*) (Kozminsky et al. 2015) and *L. spiculata* (TU*32*) (Maslov et al. 2007; Kozminsky et al. 2015) were previously identified in the Neotropics, *L. moramango* (TU*105*) is a special case. It was already encountered previously (Týč et al. 2013; Yurchenko et al. 2014); however, until now it was found exclusively in Madagascar and, unlike the previous two American leptomonads, represents a species with a limited geographical range. However, the 18S rRNA gene (KY357912) of the trypanosomatid strain C4 isolated in 1987 from the water strider *Limnoporus* (formerly *Gerris*) *rufoscutellatus* (Gerridae) captured in Leningrad region, Russia (Podlipaev et al. 2004) differs in only three nucleotides from *L. moramango*.

The newly detected TU*214* fell into the genus *Zelonia*, which so far accommodates only two species, namely *Z. costaricensis* from the reduviid bugs captured in Costa Rica, Brazil, and Panama (Yurchenko et al. 2006; Espinosa et al. 2018) and *Z. australiensis* isolated from the dipteran blackfly *Simulium dycei* in Australia (Barratt et al. 2017a, b). Both recently erected genera *Novymonas* and *Zelonia* are basal to all dixenous Leishmaniinae (*Leishmania* s.l. plus *Paraleishmania*) (Yurchenko et al. 2006; Kostygov et al. 2016; Barratt et al. 2017a, b; Kostygov and Yurchenko 2017; Espinosa et al. 2018), and presumably represent the closest ancestors of parasites that transitioned from a monoxenous to a dixenous life cycle (Jirku et al. 2012; Lukeš et al. 2014; Kostygov and Yurchenko 2017). Phylogenetic analyses suggested that the South American *Z. costaricensis* and the Australian *Z. australiensis* diverged when these continents completely separated (Barratt et al. 2017a, b). As a new member of the genus *Zelonia*, the Madagascan TU*214* nicely fills the gap in the distribution of these flagellates over the former Gondwana, a supercontinent that existed from the Neoproterozoic (about 550 MYR) until the Jurassic (about 180 MYR). Indeed, our finding further supports the Gondwanan origin of the dixenous parasitism in Leishmaniinae (Barratt et al. 2017a, b).

The genus *Blastocrithidia*, characterized by its unique non-canonical genetic code (Záhonová et al. 2016; Bianchi et al. 2019), is represented by a single new typing unit, TU*215* and three previously encountered species (Table 2; Fig. 3A). *Blastocrithidia raabei*, detected here in the sub-endemic pentatomid bug *Antestiella mauritii* collected in Reunion, was originally described from the intestine and hemolymph of the dock bug *Coreus marginatus* (Heteroptera: Coreidae) in Poland (Lipa 1966) and later from the same host in Russia, Kazakhstan, Tajikistan, and Armenia as the subspecies *B. raabei rostrata* (Podlipaev 1988). However, the previously mentioned possibility of a hemolymph infection (Lipa 1966) was recently refuted, as the parasite was repeatedly found only in the host

midgut (Frolov et al. 2020), which also corresponds with our findings of the elongated cells having a morphotype described previously (Lipa 1966; Podlipaev 1988; Frolov et al. 2020). It is worth noting that the digestive tract of phytophagous bugs, such as *C. marginatus*, has a very unusual organization, with its anterior and posterior parts being effectively isolated from each other by two intermediate segments impassable for food fluids and microorganisms (Kikuchi et al. 2011; Ohbayashi et al. 2015). Consequently, it was speculated that because of this unusual organization of the intestinal tract, the flagellates must overcome barriers, which are otherwise refractory to microbial infections (Frolov et al. 2020). However, *A. mauritii* probably lacks this anatomic peculiarity (Miyamoto 1961).

Another member of the genus *Blastocrithidia*, TU*44*, was found in a wide range of heteropteran families collected in the Neotropics (Maslov et al. 2007; Kozminsky et al. 2015), China (Votýpka et al. 2010), and Ghana (Votýpka et al. 2012a), so its current documentation in two pentatomid species from Madagascar further supports its cosmopolitan distribution. The detection of TU*62*, also clearly affiliated with the genus *Blastocrithidia*, in the pentatomid bugs nicely correlates with its host's occurrence in China (Votýpka et al. 2010) and Kenya (Votýpka et al. 2012a).

The '*jaculum*' group (Fig. 3B), which is a well-defined, although not yet formally described genus closely related to *Blastocrithidia*, contained 11 TUs from the studied islands, out of which six were described previously (TU*6/7C*, TU*6/7D*, TU*6/7E*, TU*72*, TU*73*, and TU*88* [split into two different genotypes TU*88* and TU*88b*]), and three that can be justifiably considered as novel (TU*216*, TU*217*, and TU*218*). With respect to the two remaining detected genotypes (TU*6/7D2* and TU*6/7AB2*) we cannot currently distinguish between them being members of the existing variable species groups TU*6/7D* and TU*6/7AB* or new species (Fig. 3B). Indeed, the TU*6/7* species complex is composed of several closely related taxa poorly resolved by the 18S rRNA marker. However, an alternative marker, the spliced leader (SL) RNA sequence (Maslov et al. 2013), provides a finer resolution. TU*6/7AB* was previously recorded in two families, Alydidae and Reduviidae, in the Neotropics (Westenberger et al. 2004; Kozminsky et al. 2015) and Ghana (Votýpka et al. 2012a), while other two members of the TU*6/7* complex (TU*6/7D* and TU*6/7E*) were detected in Alydidae from Ghana (Votýpka et al. 2012a) and TU*6/7E* was also found in the families Gerridae and Miridae from Europe (our unpubl. data; Maslov et al. 2013). Previously, TU*72* was detected in Ghana in several bug families (Votýpka et al. 2012a, b), and in a sarcophagid fly (Týč et al. 2013), while TU*73* was found in a pyrrhocorid bug captured in France (Votýpka et al. 2012b) and bugs from Ghana, with the latter country also hosting TU*88* (Votýpka et al. 2012a).

The only dixenous trypanosomatids in the current islands-derived dataset belong to the genus *Phytomonas*, which is capable of parasitizing over 20 plant families (Jaskowska et al. 2015). It is interesting to note that the type species of this genus, *Phytomonas davidi*, was described more than 100 years ago from the latex of *Euphorbia* spp. in Mauritius (Lafont 1909, 1910). Later on, Lafont (1911) and França (1920) demonstrated its transmission by the phytophagous bugs *Nysius euphorbiae* (Lygaeidae) and *Stenocephalus agilis* (Stenocephalidae), respectively. While none of the detected *Phytomonas* spp. is from Mauritius, several TUs were found in Reunion and Madagascar. Whereas the family Lygaeidae is represented in our collection by 95 specimens parasitized by several TUs, including one new *Phytomonas* species (TU*251*), the family Stenocephalidae was represented by a single parasite-free specimen.

While only one TU (TU*77/177*) has a sequence virtually identical to 18S rRNA published previously, the other five detected phytomonads (TU*248*, TU*249*, TU*250*, TU*251*,

and TU*252*) clearly qualify as new species (Table 2; Fig. 4B). Despite their economic importance as serious pathogens of plants (Schwelm et al. 2018), our knowledge of the diversity and biogeography of phytomonads remains rather fragmented. Since only about one-quarter of all described species comes from Europe, Asia, Africa, and Australia/ Oceania, while the majority is of American origin (Jankowska et al. 2015; Frolov et al. 2019), five new *Phytomonas* TUs encountered herein represent a significant contribution.

One of them, *Phytomonas* sp. 3 (TU*250*; environmental isolate Re06) from a bug belonging to the genus *Horridipamera* (Rhyparochromidae) captured in Reunion deserves special attention. Despite significant efforts, only a partial 18S rRNA sequence of an almost identical TU*67* (isolate CH402; Acc. No. GU059571) from Chinese *Metochus* sp. (belonging to the same family Rhyparochromidae) is available (Votýpka et al. 2010) and forms an unstable branch within the *Phytomonas* clade (Votýpka et al. 2010, 2012a). However, the newly obtained full-size 18S rRNA sequence of TU*250* (Re06_envi) provides sufficient information for a thorough phylogenetic analysis (Fig. 4B). In both maximum likelihood (Fig. 1) and Bayesian analyses (data not shown), TU*250/67* appeared as the basal branch within the genus *Phytomonas* and replaced *P. lipae* and *P. oxycareni* in this position, supported by phylogenies based on the 18S rRNA, glycosomal glyceralde-hyde dehydrogenase and heat shock protein 83 sequences (Seward et al. 2017; Frolov et al. 2019). Although our attempts to amplify protein-coding genes of TU*250* from the intestine of *Horridipamera* sp. failed, we consider our data robust enough to claim that this flagellate is so distant from all known members of the genus *Phytomonas* (Figs. 1, 4B) that it may qualify as a member of a new genus, although more sequence data will be needed for such an assignment.

The only previously recorded *Phytomonas* (TU*77/177*) in our dataset comes in two slightly different genotypes: genotype 1 was found in two specimens of the pentatomid *Aspavia longispina*, while genotype 2 originated from another pentatomid, *Sciocoris wolffi* (Fig. 4B). TU*77* was found in several bug families (Reduviidae, Alydinae, Pentatomidae, Coreidae, and Lygaeidae) in Ghana (Votýpka et al. 2012a) and both TU*77* and TU*177* parasitize pentatomids in Papua New Guinea (Králová et al. 2019). Due to only two to three nucleotide differences in the 18S rRNA gene, we assume that TU*77*, TU*177*, and two new Madagascan genotypes represent a single *Phytomonas* species (TU*77/177*) distributed in Africa, the adjacent islands, Papua New Guinea and, likely, beyond.

Trypanosomatids from dipteran flies

Except for the above-mentioned *L. moramango* (TU*105*), in the dissected flies we have recorded only trypanosomatid genera typical for dipteran hosts (Table 2; Fig. 1). From the endosymbiont-containing subfamily Strigomonadinae (Votýpka et al. 2014; Maslov et al. 2019), two genera were encountered (Table 2). The well-studied *Angomonas deanei* with cosmopolitan distribution (Teixeira et al. 2011; Týč et al. 2013) was detected in two flies from Madagascar, while the closely related genus *Kentomonas* (Votýpka et al. 2014) was represented by new TU*219* (*Kentomonas* sp. A), which is closely related to *Kentomonas sorsogonicus* (Fig. 5). The finding of *Wallacemonas raviniae* (TU*110*), a representative of the genus *Wallacemonas* (Kostygov et al. 2014; Yurchenko et al. 2014), makes it a cosmopolitan flagellate, since it was previously detected in a sarcophagid fly from Ecuador (Týč et al. 2013). Moreover, TU*220* (*Wallacemonas* sp. 1) also clearly falls into this genus (Table 2; Fig. 6A). The 'clade II sensu Týč et al. 2013', which clearly qualifies for a new genus, is represented by a novel TU*253* (Table 2, Fig. 6B). This group appears to be

restricted to flies (Diptera) (Týč et al. 2013; Chandler and James 2013; Votýpka et al. 2018, 2019), although it was at least once found in a weakly mixed infection in the Ghanaian reduviid bug *Nagusta* cf. *puncticollis* (Votýpka et al. 2012a), which might have acquired it from its fly prey.

The genus *Herpetomonas*, also typically found in flies, appeared in our dataset as five species, with three of them previously described and probably having a cosmopolitan distribution (Table 2; Fig. 4A). The well-known *Herpetomonas muscarum*, the type species of the genus *Herpetomonas*, has been confirmed in the USA and Brazil using sequence data (Teixeira et al. 1997; Borghesan et al. 2013), while based on morphology, it has been encountered all over the world (Podlipaev 1990). *Herpetomonas puellarum* was previously detected in Ghana, Czechia, Brazil, and Guinea Bissau (Borghesan et al. 2013; Týč et al. 2013) and *Herpetomonas isaaci* (TU*107*) is known from Papua New Guinea, Brazil, and Guinea Bissau (Borghesan et al. 2013; Týč et al. 2013). *Herpetomonas* sp. A (TU*108*; Týč et al. 2013) was found exclusively on Madagascar and, unlike the previous three cosmopolitan herpetomonads, has a limited geographic range. The novel *Herpetomonas* sp. B (TU*221*) is closely related to *Herpetomonas modestus*, so far confirmed from Brazil (Borghesan et al. 2013) and Mongolia (Týč et al. 2013). Finally, the monotypic *Lafontella*, formerly part of the genus *Herpetomonas* (Yurchenko et al. 2016), is represented by *Lafontella mariadeanei* (Table 2; Fig. 4A) found in the fly *Chrysomya putoria* (Calliphoridae) captured in Madagascar. This genetically slightly polymorphic species was reported from Brazil (Borghesan et al. 2013) and Ghana (Týč et al. 2013).

Endemic versus wide distribution

Based on the distribution of studied trypanosomatids in heteropterans, the hosts were sorted into the following three categories: (i) endemic (ENDE) to either Madagascar, Reunion, or Mauritius; (ii) sub-endemically (SUBE) distributed in Madagascar, the Mascarenes, Comoro, and Seychelles (usually only some of them); (iii) and widespread (WIDE), which means found at least on one island plus continental Africa and (possibly) beyond (Tables 1, 2, and S1; Fig. S1). Since none of the genera of true bugs that were found to be infected is (sub)endemic to the studied islands, the mutual comparison could not be made at the generic level. However, the analysis was informative at the host species level, with the following ratio based on the known geographic distribution: (i) endemic 32/8(25%)/8/2(25%) meaning that out of 32 endemic host species, 8 (= 25%) were infected by 8 different TUs, and out of these two (= 25%) were detected for the first time. Consequently, they are labeled as endemic for the particular island; (ii) sub-endemic 12/3(25%)/6/1(17%), and (iii) widespread 36/18(50%)/16/8(50%). Since it is unclear whether TU*6/7AB2* from the widely distributed *Stenocoris annulicornis* is novel or not (Fig. 3B), it has been excluded from the analysis.

At first glance, widespread host species are more frequently infected by trypanosomatids and also more often host endemic (i.e. newly detected) TUs. Altogether, only two newly detected TUs were found exclusively in the (sub)endemic hosts: TU*218* in *Cletus presignus* and TU*248* in *Clavigralla madagascariensis*; TU*251* was found not only in the sub-endemic *Nysius albipennis/euphorbiae*, but also in the widely distributed *Taylorilygus apicalis* (Tables 2 and S1). However, it is necessary to take into consideration that, at least in some cases, the widespread true bug species are more common in the inspected biotopes and therefore possibly over-represented in our collection. Due to the odds of a given parasite being detected in a particular host significantly increasing with the number of the

dissected specimens, it is not surprising that the more the host species is encountered, the higher the frequency of its detected parasites.

Out of 39 identified TUs, 21 were exclusive to the studied islands and are, at least provisionally, labeled as endemic. Out of them, 19 TUs were detected for the first time, the remaining two TUs, namely *L. moramango* (TU*105*) and *Herpetomonas* sp. A (TU*108*) have been mentioned previously (Týč et al. 2013; Yurchenko et al. 2014); however, they were found only in Madagascar in the dipterid flies *Chrysomya putoria, Musca* sp. (TU*108*), and *Pachycerina* cf. *vaga* and the heteropteran bug *Stictopleurus scutellaris coquerelii* (TU*105*).

Insects captured in Madagascar were found to host 12 endemic TUs. In true bugs, it was three species of the '*jaculum*' group (TU*6/7D2*, TU*216*, and TU*218*), three *Leptomonas* species (*L. moramango* (TU*105*), TU*211*, and TU*212*), and one and two members of the genera *Zelonia* (TU*214*) and *Phytomonas* (TU*248* and TU*252*), respectively. Additional three likely endemic TUs, two from the genus *Herpetomonas* (TU*108* and new TU*221*) and the other new typing unit (TU*253*) assigned to the unnamed genus 'clade II. sensu Týč et al. 2013' were found in the dissected flies (Table 2). In Reunion, 8 newly detected TUs were found; five in heteropterans and three in dipterans. True bugs carried one new '*jaculum*' (TU*217*), one *Leptomonas* (TU*213*), and three *Phytomonas* species (TU*249*, TU*250*, and TU*251*). This collection was complemented with one new species each from the genera *Wallacemonas* (TU*220*) and *Kentomonas* (TU*219*), both detected in flies. The above-mentioned (sub)endemic TU*253* found in flies represents the only newly described TU occurring on more than one studied island. For comparison, only three previously described TUs (TU*32*, TU*72*, and TU*73*) were detected on more than one studied island (see above). Finally, only two new TUs affiliated with the provisional genus '*jaculum*' (TU*6/7AB2*) and the genus *Blastocrithidia* (TU*215*) originated from heteropteran hosts captured in Mauritius.

The finding of 21 novel *a.k.a.* endemic TUs represent more than half (56%) of all the identified trypanosomatids found in heteropterans and dipterans, demonstrating very high endemism of these parasites in the island ecosystem, almost equal for both host insect groups. This clearly shows that endemism affects not only animals including insect hosts, but also their parasites. However, there is a considerable discrepancy between the incidence of endemic TUs in (sub)endemic vs. widespread heteropterans. Intuitively, one would assume that an endemic TU will preferentially parasitize an endemic host species, yet this is not the case, as the majority (17 out of 21) of novel TUs were found in the geographically widely distributed hosts. This intriguing observation may be best explained by the fact that these trypanosomatids have lower host specificity. Hence, they are endemic for a specific island(s), but in addition to its original, although still unknown endemic host(s), they also infect other host species. Alternatively, these TUs are de facto not endemic to the islands and share a wide geographic distribution with their hosts. We think that the real picture covers both possibilities, namely that some of the newly discovered TUs are genuinely endemic, occurring only on islands of the south-western Indian Ocean, whereas the other newly detected TUs may also occupy other geographic areas.

Madagascar is a continental island and thus the Madagascar Plate (Madagascar block) was once attached to the Gondwana supercontinent (breaking apart from Africa about 115–120 million years ago), and later the Indo-Australian Plate (split from India between 84 and 95 million years ago). The fauna of Madagascar is quite different from the one of the adjacent continents. Madagascar hosts a species-rich and highly endemic fauna of Heteroptera, but due to the lack of any comprehensive monograph or catalog, more precise estimates could be provided only for a few taxonomic groups, such as Tingidae (with 135

species of which 61% are endemic; Guilbert 2020), Rhyparochromidae (62/66%; Kment et al. 2016), Reduviidae: Ectrichodiinae (73/100%), and Pentatomidae: Halyini (40/100%). Most of the Madagascan taxa have apparent relationships with the fauna of continental Africa, but there are also taxa with Oriental affinities (Kment 2013; Forthman et al. 2016); for example the still undescribed representatives of the otherwise south-east Asian family Urostylididae (Zhou and Rédei 2018). On the other hand, Mauritius and Reunion are oceanic islands of volcanic origin not older than 10 and 2 million years, respectively. Their fauna of Heteroptera is not very rich, mostly shared with the African continent and Madagascar, and with the considerably lower endemism than in Madagascar; more detailed data are available only for Reunion, hosting 108 species of which 11 (10.2%) are endemic (Legros et al. 2016).

Axenic cultures, environmental samples, and mixed infections

Under the laboratory conditions, axenic cultures from 37 true bugs and dipteran flies were successfully established, and the cryopreserved cells are available for further studies (Tables 2 and S1). The success rate of establishing axenic flagellates was relatively high; out of 114 dissected trypanosomatid-positive specimens, we have tried to obtain the culture from 96 individuals and were successful in 37 cases (114/96(82%)/37(39%)). Being applied on all three islands, this approach yielded the following consistent results: Madagascan true bugs 52/42(81%)/16(38%) and flies 13/10(78%)/4(40%), Reunion true bugs 33/25(76%)/9(36%) and flies 6/6(100%)/3(50%), and Mauritius true bugs 10/10(100%)/5(50%).

Subsequent 18S rRNA-based sequence analysis (Figs. 1, 2, 3, 4, 5, 6; Tables 2 and S1) assigned 37 axenic cultures to 15 species: one *Angomonas*, *Blastocrithidia*, *Wallacemonas,* and *Zelonia* each, two *Herpetomonas*, four *Leptomonas* and five 'jaculum'. Out of them, five (33%) are new species: *Leptomonas* sp. 2 (TU**212**), 'jaculum' sp. C (TU**218**), 'jaculum' TU**6/7D2**, *Wallacemonas* sp. 1 (TU**220**), and *Zelonia* sp. 1 (TU**214**). Out of 37 well-established cultures, dissection of true bugs originating from Madagascar resulted in 16 axenic cultures representing a single *Blastocrithidia* and *Zelonia*, two *Leptomonas*, and five 'jaculum'. Flies from the same island yielded four cultures represented by one *Leptomonas* and *Angomonas* each and two *Herpetomonas* spp. All 9 stabilized cultures obtained from the dissected true bugs captured in Reunion are just two *Leptomonas* species, while three cultures isolated there from flies belong to a single *Wallacemonas* species. Finally, all five cultures established from bugs captured in Mauritius were identified as a single *Leptomonas* species.

In any case, a comparison of trypanosomatids that propagated in the axenic cultures with those detected only by their 18S rRNA sequences amplified from the primary material, which was almost exclusively the insect intestine, is quite informative. In 8 cases a difference between the culture and PCR-detected parasites was noticed (Table S1). In five of them, the species detected in DNA extracted from the true bug intestine and belonging to the genus 'jaculum' (TU**73**, TU**88**, and TU**216**) or *Phytomonas* (TU**248**) were in the cultures overgrown by *L. moramango* (TU**105**), *Zelonia* sp. 1 (TU**214**), and 'jaculum' (TU**218** and TU**6/7** complex). In three samples from flies captured in Reunion, *H. pullarum* and TU**253** (clade II sensu Týč et al. 2013) were under the axenic conditions suppressed by *Wallacemonas* sp. 1 (TU**220**). This comparison of primary material from the insect hosts with the established cultures clearly demonstrates that: i) mixed infections are relatively common, ii) trypanosomatids obtained by axenic cultivation may not be the dominant

species within the mixed infection in vivo, and iii) only a subset has the capacity to flourish in media and under the conditions provided in our experimental setup (Lukeš and Votýpka 2020).

Tissue localization and host-parasite specificity

Out of 11 trypanosomatid genera detected in this study, four (*Blastocrithidia*, '*jaculum*', *Phytomonas*, and *Zelonia*) were exclusive for bugs and six (*Herpetomonas*, *Lafontella*, *Wallacemonas*, *Angomonas*, *Kentomonas*, and clade II. sensu Týč et al. 2013) for flies. These host preferences correspond nicely with similar studies performed in a range of geographic locations (Wallace 1966; Podlipaev 1990; Texeira et al. 1997, 2011; Votýpka et al. 2010, 2012a, b, 2019; Wilfert et al. 2011; Borghesan et al. 2013; Týč et al. 2013; Kozminsky et al. 2015; Schoener et al. 2018; Králová et al. 2019) and further confirm very high host specificity of the trypanosomatid genera. Only the genus *Leptomonas* was found in both host groups.

The observed intensity of infection ranged from very mild (in some cases only solitary flagellates were observed and consequently PCR has occasionally failed) to very heavy (Tables 2 and S1). In the latter cases, the infected part of the intestine was literally packed with the parasites. Although in a small fraction of dissected specimens it was not possible to unambiguously determine the exact location of the infection within the digestive tract, the flagellates were most frequently found in the midgut (79%), followed by the hindgut (29%), and the Malpighian tubules (5%) (note that in some cases multiple infections of midgut and hindgut occur).

Most detected members of the subfamily Leishmaniinae (*L. moramango*, *L. spiculata*, *L. podlipaevi*, *Leptomonas* sp. 2 [TU*212*], *Leptomonas* sp. 3 [TU*213*], and *Zelonia* sp. 1 [TU*214*]) were confined to the middle of the intestine (mesenteron) of their heteropteran hosts (Tables 2 and S1), which is characteristic for most leptomonads. *Leptomonas* sp. 1 (TU*211*) was an exception, as it infects exclusively the Malpighian tubules, which is the preferred organ of only about 2% of monoxenous trypanosomatids (Lukeš et al. 2018). The situation related to tissue localization and host specificity is somewhat unusual in the case of *L. moramango*. In its heteropteran host it occupies simultaneously midgut, hindgut, and the Malpighian tubules while in dipterans, the flagellates were observed exclusively in the hindgut. The axenic culture of *L. moramango* was previously obtained from the fly *Pachycerina* cf. *vaga* (Týč et al. 2013), which is therefore listed as its type host (Yurchenko et al. 2014). However, the heteropteran *Stictopleurus scutellaris* may as well be the primary host that might have acquired this trypanosomatid through its accidental necrophagous and/or coprophagous behavior, and one may speculate that *L. moramango* has a very broad ecological valence and can parasitize a wide range of hosts. Although it is difficult to discriminate between the specific and non-specific infections in trypanosomatids with a wide range of known hosts, based on their localization, very low prevalence, and host specificity of other *Leptomonas* species, we believe that the heteropteran bug is the primary host.

A good correlation with the available data is also apparent when members of the genera *Blastocrithidia* and '*jaculum*' are concerned (Tables 2 and S1). They usually parasitize the midgut of true bugs (Votýpka et al. 2010, 2012a, b; Králová et al. 2019), but some species are predominantly found in the hindgut (Votýpka et al. 2019). All detected TUs belonging to the "fly" genera (*Herpetomonas*, *Lafontella*, *Wallacemonas*, *Angomonas*, *Kentomonas*, and clade II. sensu Týč et al. 2013) were usually found in the hindgut, more specifically in

the rectal ampulla, and quite rarely also in the midgut (Tables 2 and S1), which is in agreement with previous studies (Teixeira et al. 1997, 2011; Borghesan et al. 2013; Týč et al. 2013; Votýpka et al. 2019).

The only encountered genus with a dixenous life cycle is *Phytomonas*, here well represented by 7 different TUs (Tables 2; Fig. 4). We have found them in six families of sap-sucking bugs (Pentatomidae, Coreidae, Rhopalidae, Rhyparochromidae, Lygaeidae, and Miridae), which obviously became infected from plants (Camargo et al. 1990). Although we have found *Phytomonas* spp. invariably in the midgut (Tables 2 and S1), based on the available data, this location shall only be transient, as they are transmitted to plants throughout the bite and are therefore typically located in the salivary glands of their insect vectors (Jaskowska et al. 2015; Frolov et al. 2016; Seward et al. 2017). We are fully aware that the dissection and examination focused exclusively on the intestinal tract can be a source of bias, yet the field conditions do not allow satisfactory inspection of the salivary glands. In addition to this, despite all our efforts, the vast majority of *Phytomonas* TUs could not be introduced into culture (our unpubl. data; Seward et al. 2017).

Conclusions

In this work, we surveyed trypanosomatids from the heteropteran and dipteran hosts collected in Madagascar, Reunion, and Mauritius. These islands are famous as biodiversity hotspots with many endemic macro-organisms. Based on two short-term field expeditions we documented a very high diversity of insect trypanosomatids. Although the proportion of endemic TUs (56%) was not as high as in our previous study in Papua New Guinea (83%), the newly detected species certainly represent a significant contribution to the increasing biodiversity of trypanosomatids known so far and demonstrate that our knowledge is still far from being comprehensive. In line with previous reports (Teixeira et al. 1997, 2011; Votýpka et al. 2010, 2012a, 2019; Borghesan et al. 2013; Týč et al. 2013; Kozminsky et al. 2015; Králová et al. 2019), our study highlights the association between the insect host families and parasites and supports the cosmopolitan distribution of a subset of species. On the other hand, it demonstrates for the first time that the high endemism of macro-organisms on the remote islands is inherent to parasitic protists as well. Based on these findings we conclude that any conservation activities should take into account not only the protection of animals themselves, but also consider the protection of their symbionts, including parasites and, thus, protect a chiefly neglected segment of biodiversity, which is potentially comparable with the "visible" biodiversity of their hosts. Although our analysis has not revealed any new clades on the generic level, indicating possible saturation in this respect, the newly detected *Phytomonas* sp. 3 (TU*250*; isolate Re06-envi) is remarkable in that it constitutes the basal and highly diverged branch. Moreover, five newly detected TUs (including two belonging to the enigmatic '*jaculum*' clade) are available in axenic cultures and are worthy of further investigation.

References

Barratt J, Ellis J, Stark D, Barratt J (2017a) The evolution of trypanosomatid taxonomy. Parasit Vector 10:287

Barratt J, Kaufer A, Peters B, Craig D, Lawrence A, Roberts T, Lee R, McAuliffe G, Stark D, Ellis J (2017b) Isolation of novel trypanosomatid, *Zelonia australiensis* sp. nov (Kinetoplastida: Trypanosomatidae) provides support for a Gondwanan origin of dixenous parasitism in the Leishmaniinae. PLoS Negl Trop Dis 11:e0005215

Bianchi C, Kostygov AY, Kraeva N, Záhonová K, Horáková E, Sobotka R, Lukeš J, Yurchenko V (2019) An enigmatic catalase of *Blastocrithidia*. Mol Biochem Parasitol 232:111199

Borghesan TC, Ferreira RC, Takata CSA, Campaner M, Borda CC, Paiva F, Milder RV, Teixeira MMG, Camargo EP (2013) Molecular phylogenetic redefinition of *Herpetomonas* (Kinetoplastea, Trypanosomatidae), a genus of insect parasites associated with flies. Protist 164:129–152

Buckley TR, Palma RL, Johns PM, Gleeson DM, Heath ACG, Hitchmough RA, Stringer IAN (2012) The conservation status of small or less well known groups of New Zealand terrestrial invertebrates. NZ Entomol 35:137–143

Camargo EP, Kastelein P, Roitman I (1990) Trypanosomatid parasites of plants (*Phytomonas*). Parasitol Today 6:22–25

Carlson CJ, Phillips AJ, Dallas TA, Alexander LW, Phelan A, Bansal S (2020) What would it take to describe the global diversity of parasites? Proc Natl Acad Sci USA. https://doi.org/10.1101/815902

Chandler JA, James PM (2013) Discovery of trypanosomatid parasites in globally distributed *Drosophila* species. PLoS ONE 8:e61937

d'Avila-Levy C, Boucinha C, Kostygov A, Santos HLC, Morelli KA, Grybchuk-Ieremenko A, Duval L, Votýpka J, Yurchenko V, Grellier P, Lukeš J (2015) Exploring environmental diversity of kinetoplastid flagellates in the high-throughput DNA sequencing era (Review). Mem Inst Oswaldo Cruz 110:956–965

Dheilly NM, Martínez-Martínez J, Rosario K, Brindley PJ, Fichorova RN, Kaye JZ, Kohl K, Knoll LJ, Lukeš J, Perkins SL, Poulin R, Schriml L, Thompson LR (2019) Parasite microbiome project: grand challenges. PLoS Pathog 15:e1008028

Dobson A, Lafferty KD, Kuris AM, Hechinger RF, Jetz W (2008) Homage to Linnaeus: how many parasites? How many hosts? Proc Natl Acad Sci USA 105:11482–11489

Dougherty ER, Carlson CJ, Bueno VM, Burgio KR, Cizauskas CA, Clements CF, Seidel DP, Harris NC (2015) Paradigms for parasite conservation. Conserv Biol 30:724–733

Emerson BC (2002) Evolution on oceanic islands: molecular phylogenetic approaches to understanding pattern and process. Mol Ecol 11:951–966

Espinosa OA, Camargo EP, Teixeira MMG, Shaw JJ (2018) An appraisal of the taxonomy and nomenclature of trypanosomatids presently classified as *Leishmania* and *Endotrypanum*. Parasitology 145:430–442

França C (1920) La flagellose des euphorbes II. Ann l'Inst Pasteur, Paris 34:432–465

Forthman M, Chłond D, Weirauch C (2016) Taxonomic monograph of the endemic millipede assassin bug fauna of Madagascar (Hemiptera: Reduviidae: Ectrichodiinae). Bull Am Mus Nat Hist 400:1–152

Frolov AO, Malysheva MN, Yurchenko V, Kostygov AY (2016) Back to monoxeny: *Phytomonas nordicus* descended from dixe-nous plant parasites. Europ J Protistol 52:1–10

Frolov AO, Malysheva MN, Ganyukova AI, Spodareva VV, Yurchenko V, Kostygov AY (2019) Development of *Phytomonas lipae* sp. n. (Kinetoplastea: Trypanosomatidae) in the true bug *Coreus marginatus* (Heteroptera: Coreidae) and insights into the evolution of life cycles in the genus Phytomonas. PLoS ONE 14:e0214484

Frolov AO, Malysheva MN, Ganyukova AI, Spodareva VV, Králová J, Yurchenko V, Kostygov AY (2020) If host is refractory, insistent parasite goes berserk: trypanosomatid *Blastocrithidia raabei* in the true bug *Coreus marginatus*. PLoS ONE 15:e0227832

Gómez A, Nichols E (2013) Neglected wild life: parasitic biodiversity as a conservation target. Int J Parasitol Parasites Wildl 2:222–227

Graham N, Gruner D, Lim J, Gillespie RG (2017) Island ecology and evolution: challenges in the Anthropocene. Environ Conserv. https://doi.org/10.1017/S0376892917000315

Grybchuk D, Akopyants NS, Kostygov AY, Konovalovas A, Lye L-F, Dobson DE, Zangger H, Fasel N, Butenko A, Frolov AO, Votýpka J, Avila-Levy CM, Kulich P, Moravcová J, Plevka P, Rogozin IB,

Serva S, Lukeš J, Beverley SM, Yurchenko V (2018) Viral discovery and diversity in trypanosomatid protozoa with a focus on relatives of the human parasite *Leishmania*. Proc Natl Acad Sci USA 115:E506–E515

Grybchuk-Ieremenko A, Losev A, Kostygov A, Lukeš J, Yurchenko V (2014) High prevalence of trypanosome co-infections in freshwater fishes. Folia Parasitol 61:495–504

Guilbert E (2020) New species of Tingidae (Insecta: Heteroptera) from Madagascar. Zootaxa 4759(3):391–404

Ishemgulova A, Butenko A, Kortišová L, Boucinha C, Grybchuk-Ieremenko A, Morelli KA, Tesařová M, Kraeva N, Grybchuk D, Pánek T, Flegontov P, Lukeš J, Votýpka J, Pavan MG, Opperdoes FR, Spodareva V, d'Avila-Levy CM, Kostygov AY, Yurchenko V (2017) Molecular mechanisms of thermal resistance of the insect trypanosomatid *Crithidia thermophila*. PLoS ONE 12:e0174165

Jaskowska E, Butler C, Preston G, Kelly S (2015) *Phytomonas*: trypanosomatids adapted to plant environments. PLOS Pathog 11:e1004484

Jirků M, Yurchenko VY, Lukeš J, Maslov DA (2012) New species of insect trypanosomatids from Costa Rica and the proposal for a new subfamily within the Trypanosomatidae. J Eukaryot Microbiol 59:537–547

Kikuchi Y, Hosokawa T, Fukatsu T (2011) An ancient but promiscuous host-symbiont association between *Burkholderia* gut symbionts and their heteropteran hosts. ISME J 5:446–460

Kment P (2013) *Carduelicoris stehliki*, a new genus and species of Pentatomidae (Hemiptera: Heteroptera) from Madagascar Acta Mus Moraviae. Sci Biol 98:415–432

Kment P, Hemala V, Baňař P (2016) *Rhyparoclava pyrrhocoroides*, a new genus and species of autapomorphic Rhyparochromidae with clavate antennae from Madagascar (Hemiptera: Heteroptera). Acta Entomol Mus Nat Pragae 56:517–545

Kostygov A, Dobáková E, Grybchuk-Ieremenko A, Váhala D, Maslov DA, Votýpka J, Lukeš J, Yurchenko V (2016) Novel trypanosomatid-bacterium association: evolution of endosymbiosis in action. mBio 7:e01985–e2015

Kostygov A, Grybchuk-Ieremenko A, Malysheva M, Frolov A, Yurchenko V (2014) Molecular revision of the genus *Wallaceina*. Protist 165:594–604

Kostygov A, Yurchenko V (2017) Revised classification of the subfamily Leishmaniinae (Trypanosomatidae). Folia Parasitol 64:020

Kozminsky E, Kraeva N, Ishemgulova A, Dobakova E, Lukeš J, Kment P, Yurchenko V, Votypka J, Maslov DA (2015) Host-specificity of monoxenous typanosomatids: statistical analysis of the distribution and transmission patterns of the parasites from Neotropical Heteroptera. Protist 166:551–568

Králová J, Grybchuk-Ieremenko A, Votýpka J, Novotný V, Kment P, Lukeš J, Yurchenko V, Kostygov AY (2019) Insect trypanosomatids in Papua New Guinea: high endemism and diversity. Int J Parasitol 49:1075–1086

Lafont A (1909) Sur la présence d'un parasite de la classe des flagellés dans le latex de l'*Euphorbia pilulifera*. CR Séances Soc Biol Ses Fil 66:1011–1013

Lafont A (1910) Sur la présence d'un *Leptomonas*, parasite de la classe des Flagellés, dans le latex de trois Euphorbiacées. Ann l'Inst Pasteur Paris 24:205–219

Lafont A (1911) Sur la transmission du *Leptomonas davidi* des euphorbes par un hémiptère, *Nysius euphorbiae*. CR S Soc Biol Ses Fil 70:58–59

Legros V, Gasnier S, Rochat J (2016) First general inventory of the terrestrial Heteroptera fauna and its specificity on the oceanic island of Reunion. Ann Soc Entomol Fr (N.S.) 52:311–342

Lipa JJ (1966) *Blastocrithidia raabei* sp. n., a flagellate parasite of *Mesocerus marginatus* L. (Hemiptera: Coreidae). Acta Protozool 4:19–23

Lukeš J, Votýpka J (2020) Field isolation and cultivation of trypanosomatids from insects. In: Michels P, Ginger M, Zilberstein D (eds) Trypanosomatids methods in molecular biology. Humana, New York, pp 3–21

Lukeš J, Butenko A, Hashimi H, Maslov DA, Votýpka J, Yurchenko V (2018) Trypanosomatids are much more than just trypanosomes: clues from the expanded family tree. Trends Parasitol 34:466–480

Lukeš J, Skalický T, Týč J, Votýpka J, Yurchenko V (2014) Evolution of parasitism in kinetoplastid flagellates. Mol Biochem Parasit 195:115–122

MacArthur RH, Wilson EO (1967) The theory of island biogeography. Princeton University Press, Princeton, p 203

Maslov DA, 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 Parasitol 75:197–205

Maslov DA, Westenberger SJ, Xu X, Campbell DA, Sturm NR (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 DA, Votýpka J, Yurchenko V, Lukeš J (2013) Diversity and phylogeny of insect trypanosomatids: all that is hidden shall be revealed. Trends Parasitol 29:43–52

Maslov DA, Opperdoes FR, Kostygov AY, Hashimi H, Lukeš J, Yurchenko V (2019) Recent advances in trypanosomatid research: genome organization, expression, metabolism, taxonomy and evolution. Parasitol 146:1–27

Miyamoto S (1961) Comparative morphology of alimentary organs of Heteroptera, with phylogenetic consideration. Sieboldia 2:197–259

Mihalca AD, Gherman CM, Cozma V (2011) Coendangered hard-ticks: threatened or threatening? Parasit Vector 4:71

Novotný V, Drozd P, Miller SE, Kulfan M, Janda M, Basset Y, Weiblen GD (2006) Why are there so many species of herbivorous insects in tropical rainforests? Science 313:1115–1118

Ohbayashi T, Takeshita K, Kitagawa W, Nikoh N, Koga R, Meng XY, Tago K, Hori T, Hayatsu M, Asano K, Kamagata Y, Lee BL, Fukatsu T, Kikuchi Z (2015) Insect's intestinal organ for symbiont sorting. Proc Natl Acad Sci USA 112:E5179–E5188

Okamura B, Hartigan A, Naldoni J (2018) Extensive uncharted biodiversity: the parasite dimension. Integr Comp Biol 58:1132–1145

Pawlowski J, Audic S, Adl S, Bass D, Belbahri L, Berney C, Bowser SS, Čepička I, Decelle J, Dunthorn M, Fiore-Donno AM, Gile GH, Holzmann M, Jahn R, Jirku M, Keeling PJ, Kostka M, Kudryavtsev A, Lara E, Lukeš J, Mann DG, Mitchell EAD, Nitsche F, Romeralo M, Saunders GW, Simpson AGB, Smirnov AV, Spouge JL, Stern RF, StoeckT ZJ, Schindel D (2012) CBOL protist working group: barcoding eukaryotic richness beyond the animal, plant, and fungal kingdoms. PLoS Biol 10:e1001419

Podlipaev S (1988) *Blastocrithidia raabei rostrata* subsp. n. (Mastigophora, Trypanosomamonadida) parasite of a bug *Coreus marginatus*. Zool Zh 67:1407–1411 **(in Russian)**

Podlipaev SA (1990) Catalogue of world fauna of Trypanosomatidae (Protozoa). Proc Zool Inst, Leningrad 144:1–178 **(in Russian)**

Podlipaev SA, Sturm NR, Fiala I, Fernandes O, Westenberger SJ, Dollet M, Campbell DA, Lukeš J (2004) Diversity of insect trypanosomatids assessed from the spliced leader RNA and 5S rRNA genes and intergenic regions. J Eukaryot Microbiol 51:283–290

Poulin R (2014) Parasite biodiversity revisited: frontiers and constraints. Int J Parasitol 44:581–589

Rocha CFD, Bergallo HG, Bittencourt EB (2016) More than just invisible inhabitants: parasites are important but neglected components of the biodiversity. Zoologia 33:e20150198

Rózsa LA, Vas Z (2015) Co-extinct and critically co-endangered species of parasitic lice, and conservation-induced extinction: should lice be reintroduced to their hosts? Oryx 49:107–110

Seward EA, Votýpka J, Kment P, Lukeš J, Kelly S (2017) Description of *Phytomonas oxycareni* n. sp. from the salivary glands of *Oxycarenus lavaterae*. Protist 168:71–79

Schoener E, Uebleis SS, Cuk C, Nawratil M, Obwaller AG, Zechmeister T, Lebl K, Rádrová J, Zittra C, Votýpka J, Fuehrer H-P (2018) Trypanosomatid parasites in Austrian mosquitoes. PLoS ONE 13:e0196052

Schwelm A, Badstöber J, Bulman S, Desoignies N, Etemadi M, Falloon RE, Gachon CMM, Legreve A, Lukeš J, Merz U, Nenarokova A, Strittmatter M, Sullivan BK, Neuhauser S (2018) Not in your usual Top 10: protists that infect plants and algae. Mol Plant Pathol 19:1029–1044

Stork NE, Lyal CHC (1993) Extinction or "co-extinction" rates? Nature 366:307

Strona G, Fattorini S (2014) Parasitic worms: how many really? Int J Parasitol 44:269–272

Strona G (2015) Past, present and future of host–parasite coextinctions. Int J Parasitol Parasites Wildl 4:431–441

Teixeira MMG, Borghesan TC, Ferreira RC, Santos MA, Takata CSA, Campaner M, Nunes VLB, Milder RV, de Souza W, Camargo EP (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 MMG, Takata CSA, Conchon I, Campaner M, Camargo EP (1997) Ribosomal and kDNA markers distinguish two subgroups of *Herpetomonas* among old species and new trypanosomatids isolated from flies. J Parasitol 83:58–65

Turvey ST, Cheke AS (2008) Dead as a dodo: the fortuitous rise to fame of an extinction icon. Hist Biol 20:149–163

Týč J, Votýpka J, Klepetková H, Šuláková H, Jirků M, Lukeš J (2013) Growing diversity of trypanosomatid parasites of flies (Diptera: Brachycera): frequent cosmopolitism and moderate host specificity. Mol Phylogenet Evol 69:255–264

Votýpka J, Maslov 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

Votýpka J, Klepetková H, Jirků M, Kment P, Lukeš J (2012a) Phylogenetic relationships of trypanosomatids parasitizing true bugs (Insecta: Heteroptera) in sub-Saharan Africa. Int J Parasitol 42:489–500

Votýpka J, Klepetková H, Yurchenko V, Horák A, Lukeš J, Maslov A (2012b) Cosmopolitan distribution of a trypanosomatid *Leptomonas pyrrhocoris*. Protist 163:616–631

Votýpka J, Suková E, Kraeva N, Ishemgulova A, Duž* I, Lukeš J, Yurchenko V (2013) Diversity of Trypanosomatids (Kinetoplastea: Trypanosomatidae) parasitizing fleas (Insecta: Siphonaptera) and description of a new genus *Blechomonas* gen. n. Protist 164:763–781

Votýpka J, Kostygov AY, Kraeva N, Grybchuk-Ieremenko A, Tesařová M, Grybchuk D, Lukeš J, Yurchenko V (2014) *Kentomonas* gen. g., a new genus of endosymbiont-containing Trypanosomatids of Strigomonadinae subfam. n. Protist 165:825–838

Votýpka J, d'Avila-Levy CM, Grellier P, Maslov DA, Lukeš J, Yurchenko V (2015) New approaches to systematics of Trypanosomatidae: criteria for taxonomic (re)description. Trends Parasitol 31:460–469

Votýpka J, Pafčo B, Modrý D, Mbohli D, Tagg N, Petrželková KJ (2018) An unexpected diversity of trypanosomatids in fecal samples of great apes. Int J Parasitol Parasites Wildl 7:322–325

Votýpka J, Kment P, Kriegová E, Vermeij MJA, Keeling PJ, Yurchenko V, Lukeš J (2019) High prevalence and endemism of trypanosomatids on a small Caribbean island. J Eukaryot Microbiol 66:600–607

Wallace FG (1966) The trypanosomatid parasites of insects and arachnids. Exp Parasitol 18:124–193

Warren BH, Simberloff D, Ricklefs RE, Aguilée R, Condamine FL, Gravel D, Morlon H, Mouquet N, Rosindell J, Casquet J, Conti E, Cornuault J, Fernández-Palacios JM, Hengl T, Norder SJ, Rijsdijk KF, Sanmartín I, Strasberg D, Triantis KA, Valente LM, Whittaker RJ, Gillespie RG, Emerson BC, Thébaud C (2015) Islands as model systems in ecology and evolution: prospects fifty years after MacArthur–Wilson. Ecol Lett 18:200–217

Westenberger SJ, Sturm NR, Yanega D, Podlipaev SA, Zeledon R, Campbell DA, Maslov DA (2004) Trypanosomatid biodiversity in Costa Rica: genotyping of parasites from Heteroptera using the Spliced Leader RNA gene. Parasitology 129:537–547

Wilfert L, Longdon B, Ferreira AG, Bayer F, Jiggins FM (2011) Trypanosomatids are common and diverse parasites of *Drosophila*. Parasitology 138:585–865

Windsor DA (1995) Equal rights for parasites. Conserv Biol 9:1–2

Windsor DA (1998) Most of species on Earth are parasites. Int J Parasitol 28:1939–1941

Yurchenko V, Kostygov A, Havlová J, Grybchuk-Ieremenko A, Ševčíková T, Lukeš J, Ševčík J, Votýpka J (2016) Diversity of Trypanosomatids in cockroaches and the description of *Herpetomonas tarakana* sp. n. J Eukaryot Microbiol 63:198–209

Yurchenko V, Votýpka J, Tesařová M, Klepetková H, Kraeva N, Jirků M, Lukeš J (2014) Ultrastructure and molecular phylogeny of four new species of monoxenous trypanosomatids from flies (Diptera: Brachycera) with redefinition of the genus *Wallaceina*. Folia Parasitol 61:97–112

Yurchenko VY, Lukeš J, Jirků M, Zeledon R, Maslov DA (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, Lukeš J (2018) Parasites and their (endo)symbiotic microbes. Parasitology 145:1261–1264

Záhonová K, Kostygov AY, Ševčíková T, Yurchenko V, Eliáš M (2016) An unprecedented non-canonical nuclear genetic code with all three termination codons reassigned as sense codons. Curr Biol 26:2364–2369

Zhou Y-Y, Rédei D (2018) A new synonymy in East Asian Urostylididae (Hemiptera: Heteroptera). Zootaxa 4504:145–150

Affiliations

Jan Votýpka[1,2] · **Petr Kment[3]** · **Vyacheslav Yurchenko[4,5]** · **Julius Lukeš[2,6]**

✉ Jan Votýpka
jan.votypka@natur.cuni.cz

✉ Julius Lukeš
   jula@paru.cas.cz

1 Department of Parasitology, Faculty of Science, Charles University, Prague, Czech Republic

2 Institute of Parasitology, Biology Centre, Czech Academy of Sciences,
   České Budějovice (Budweis), Czech Republic

3 Department of Entomology, National Museum, Prague, Czech Republic

4 Faculty of Science, University of Ostrava, Ostrava, Czech Republic

5 Martsinovsky Institute of Medical Parasitology, Sechenov University, Moscow, Russia

6 Faculty of Sciences, University of South Bohemia, České Budějovice (Budweis), Czech Republic