The Journal of Eukaryotic Microbiology

ISOP Published by the International Society of Protistologists

Journal of Eukaryotic Microbiology ISSN 1066-5234

ORIGINAL ARTICLE

High Prevalence and Endemism of Trypanosomatids on a Small Caribbean Island

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Keywords
Blastocrithidia; conservation; endemic species; evolution; Heteroptera; island biogeography theory; *Leptomonas*; *Phytomonas*.

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Received: 22 October 2018; revised 13 November 2018; accepted November 16, 2018.

doi:10.1111/jeu.12704

ABSTRACT

We describe the monoxenous trypanosomatids parasitizing true bugs and flies on the island of Curaçao. Out of 248 examined true bugs belonging to 17 species, 93 individuals were found to be infected (overall 38% prevalence) by at least one trypanosomatid species (referred to as typing units; TUs). Out of 80 flies, six were infected. All detected trypanosomatids were compared based on their 18S rRNA sequences with TUs parasitizing bugs and flies described from mainland South America, allowing us to assess their diversity and distribution. Besides *Leptomonas pyrrhocoris* and *Leptomonas seymouri*, two known species of the subfamily Leishmaniinae, our analysis revealed six new TUs falling into the groups '*jaculum*', *Blastocrithidia* and *Herpetomonas*. Moreover, two new members of the genus *Phytomonas* and three new TUs belonging to the monophyletic group designated as 'new clade II' sensu *Mol. Phylogenet. Evol,* 69, 255 (2013) were isolated. The detected trypanosomatids were characterized by moderate diversity (13 TUs) species richness. Out of nine and four TUs from the heteropteran and dipteran hosts, respectively, 11 TUs have not been encountered before. Although a sampling bias may partially affect the comparison between trypanosomatid communities on Curaçao and the mainland, the high proportion of unique TUs from the former location suggests that the prominent role of islands in increasing the global diversity of macroscopic organisms may also extend to their protistan parasites.

SINCE the formulation of the theory of island biogeography (MacArthur and Wilson, 1967), islands have widely been considered to be model systems for studying fundamental principles in ecology, evolutionary biology and biogeography because island communities are relatively simple in terms of biodiversity and ecological interactions (Emerson 2002; Graham et al. 2017; Warren, et al. 2015). On islands, communities of macroscopic organisms show lower species diversity than in areas of similar size on the nearby mainland (Blondel 2000; Whittaker and Fernández-Palacios 2007). It is generally assumed that islands harbour endemic species and that the relative absence of predators creates (sub)species that would not survive elsewhere. Particularly very old and well isolated oceanic islands represent conditions resulting in higher levels of extant biodiversity and endemism of inhabiting organisms.

Oceanic islands are therefore often considered as important biodiversity hotspots (Myers et al. 2000).

However, the situation is different on small islands that are exposed to a strong influence from the mainland; due to their proximity to the continent, such islands are considered less conducive for the emergence of new and endemic species (Gray and Cavers 2014; Warren et al. 2015). Colonization from the mainland is very common, but the number of biotopes is often smaller on the island than on the mainland. In addition, extinction rates on continental islands are high due to the relative scarcity of suitable habitats and higher competition for limited resources (Losos and Ricklefs 2010). These principles underlying Island Biogeography Theory (IBT) were largely derived from studies of vertebrates, such as lizards, mammals and birds, and, to a lesser extent, from selected groups of insects and snails. However, very little is known about islandic microorganisms including parasitic ones.

Parasites can respond to the occurrence and distribution of their hosts. For example, human pathogens on oceanic islands follow the basic predictions of IBT, increasing in number with increasing size of oceanic islands when they lie closer to the mainland (Jean et al. 2016). Understanding the reasons why hosts lose, maintain or swap their parasites after colonizing new areas has long intrigued biogeographers. IBT predicts that continental hosts will harbour greater parasite richness than their island counterparts, retaining only a subset of the parasite species from the continental source pool (MacArthur and Wilson 1967). Parasite richness increases with host density and diversity and is therefore expected to decline on small and remote islands (Arneberg et al. 1998). Studies comparing differences in parasite diversity between the mainland and nearby island communities are scarce. To the best of our knowledge, pathogenic hemosporidians of birds are the only protist parasites whose communities have been compared between the mainland and nearby islands (Illera et al. 2015; Padilla et al. 2017; Pérez-Rodríguez et al. 2013).

Monoxenous (= single-host) trypanosomatid flagellates are confined to insects and represent the bulk of known diversity of the family Trypanosomatidae, while a smaller fraction of trypanosomatids comprises dixenous (= two-hosts) species that circulate between a vertebrate/plant and an invertebrate host (Maslov et al. 2013; Podlipaev 2001). Phylogenetic studies strongly hint to the emergence of the dixenous flagellates that include members of the genera *Trypanosoma* and *Leishmania* and are responsible for numerous deadly diseases of humans and other mammals, from their monoxenous ancestors (Lukeš et al. 2014). Consequently, to improve our understanding of the evolution, diversity and pathogenicity of the well-studied trypanosomes and leishmanias, more information about the largely overlooked monoxenous parasites of insects is mandatory. Here, we examined the trypanosomatid flagellates parasitizing true bugs and flies on the small island of Curaçao and compared them with those from the nearby South America, making use of the extensive information that was recently gathered for this Neotropical region (Borghesan et al. 2013; Kozminsky et al. 2015; Maslov et al. 2007, 2010; Teixeira et al. 2011; Westenberger et al. 2004; Yurchenko et al. 2006).

MATERIALS AND METHODS

The Caribbean island of Curaçao (16N, 69W) is located approximately 66 km north of Venezuela, having a population of 160,000 on an area of 444 km^2. Although it is home to several endemic animals, its fauna and flora are highly similar to those of the nearby mainland. The island is very dry, has a tropical savannah climate and xeric scrublands with various forms of cacti and thorny shrubs as the prevailing vegetation (http://www.meteo.cw/climate.php).

Insects, predominantly true bugs (Heteroptera) and smaller number of flies (Diptera: Brachycera), were collected within two seasons in March and April 2015 and April 2016 by sweep netting in the vicinity of the Caribbean Research and Management of Biodiversity (Carmabi) research station in Piscadera Baai, and throughout a number of biotopes of Curaçao. Collected true bugs were identified to species using available keys and comparative collections, whereas flies were identified to the family level only. Host specimens were dry-mounted and deposited in the collections of Department of Entomology, National Museum, Prague, Czech Republic.

Within 24 h after capture, insects were killed and surface-sterilized with 70% ethanol, washed and dissected in 0.9% sterile saline solution and examined for parasites under a microscope as described previously (Votýpka et al. 2010, 2012a). To establish the primary cultures of trypanosomatids, contents of the insect intestines were cultivated in the Brain Heart Infusion as described elsewhere (Votýpka et al. 2014; Yurchenko et al. 2016). Total genomic DNA was isolated from the gut tissue, or in the case of successful cultivations, from 1-ml of axenically grown cultures, using a protocol described previously (Votýpka et al. 2014). For amplifying the trypanosomatid 18S rRNA gene, about 10 ng DNA was subjected to nested PCR described by Seward et al. (2017), and obtained PCR products were directly sequenced. The sequences were aligned using Geneious software (version 10.0.6, https://www.geneious.com), and phylogenetic trees were constructed using related sequences available in GenBank. Alignments for phylogenetic analysis were generated in Kalign, with ambiguously aligned positions in the trimmed alignment being removed manually in BioEdit. The final dataset contained 141 taxa and 2,957 characters. Analyses were performed in MrBayes and PhyML with model optimization in ModelTest (version 3.06). A general time-reversible substitution model with a mixed model for among site rate variation (GTR + Γ + I) was chosen as the best fitting model of sequence evolution. Bootstrap analyses involved heuristic searches with 1,000 replicates (Maximum likelihood). Bayesian inference was accomplished in MrBayes (version 3.2.2, Ronquist et al. 2012) with analysis run for five million generations with covarion and sampling every 100 generations. All other parameters were left in their default states.

RESULTS AND DISCUSSION

Heteropteran insects of 11 different families belonging to 16 genera and 17 species were examined for the presence of trypanosomatid parasites. Out of 246 dissected true bugs, 93 (37.8%) individuals were infected by trypanosomatids (Tables 1 and 2), a prevalence much higher than the average global one, which for these obligatory parasites or less frequently commensals varies between 5 and 25% for true bugs (Heteroptera) (Borghesan et al. 2018; Lukeš et al. 2018).

The degree of prevalence of infection by trypanosomatid parasites varied from very high to low: from *Dysdercus modestus* (81%; family Pyrrhocoridae) and *Catorhintha selector* and *Catorhintha guttula* (83 and 78%, respectively; family Coreidae), to *Neomegalotomus parvus* (73%; family Alydidae), *Niesthrea vincentii* (45%; family Rhopalidae) and *Mecidea longula* (9%; family Pentatomidae), to 11 species that were not infected (Table 1). Although in a few dissected specimens it was not possible to unambiguously determine exact localization within the digestive tract (usually because of a suboptimal dissection), most flagellates were found to occupy only the hindgut (38%), followed by only the midgut (23%), whereas in 37% of specimens infection was present in both hindgut and midgut (Table 2).

Due to the fact that some infections were caused by a very low number of parasites, we were able to amplify the target 18S rRNA gene of trypanosomatids from 78 dissected specimens only (83.9%). The obtained 18S rRNA sequences revealed that the true bugs examined hosted nine different trypanosomatid typing units (TUs, used as proxies of species; similar to the operational taxonomic units) (Tables 1 and 2; Fig. 1). While two TUs were identical to well known and widely distributed species of the genus *Leptomonas*, namely *L. pyrrhocoris* (Votýpka et al. 2012b) and *L. seymouri* (Kraeva et al. 2015), the other seven TUs were novel.

Leptomonas pyrrhocoris is globally distributed, infecting the midgut and hemocoel of several species of the family Pyrrhocoridae including *Pyrrhocoris apterus*, *P. marginatus*

Table 1. Summarized information about true bug (Heteroptera) hosts and their trypanosomatids, including the insect species distribution, the prevalence of parasites (including number of dissected vs. infected specimens) and the list of detected trypanosomatid species

Host species	Host family	Host species distribution	Dissected/infected*	Detected trypanosomatid species (No. of infected specimens)
Catorhintha guttula	Coreidae	USA to Argentina, Caribbean	18/14 (78%)	'*jaculum*' sp. 2 (12) *Phytomonas* sp. A (1)
Catorhintha selector	Coreidae	S USA to Venezuela, Caribbean	54/45 (83%)	'*jaculum*' sp. 2 (26) *Phytomonas* sp. A (4)
Cyptocephala antiguensis	Pentatomidae	S USA to northern S Am., Caribbean	3/0	
Dolichomiris linearis	Miridae	Cosmotropical	9/0	
Dysdercus modestus	Pyrrhocoridae	Curacao and Aruba, endemic	16/13 (81%)	*Leptomonas pyrrhocoris* (8) *Leptomonas seymouri* (1)
Geocoris punctipes	Geocoridae	S USA to Colombia, Caribbean	1/0	
Harmostes serratus	Rhopalidae	USA to S America, Caribbean	2/0	
Karnaviexallis johanseni	Coreidae	Venezuela	2/0	
Mecidea longula	Pentatomidae	Caribbean	33/3 (9%)	*Blastocrithidia* sp. 1 (3)
Nabis capsiformis	Nabidae	Cosmopolitan	2/0	
Neomegalotomus parvus	Alydidae: Alydinae	Mexico to Paraguay, Caribbean	37/27 (73%)	*Blastocrithidia* sp. 1 (1) *Blastocrithidia* sp. 2 (1) '*jaculum*' sp. 1 (2) '*jaculum*' sp. 2 (2) *Leptomonas pyrrhocoris* (1) *Leptomonas seymouri* (1) *Phytomonas* sp. B (10)
Neopamera bilobata	Rhyparochromidae	C + S America, Caribbean	2/0	
Neortholomus jamaicensis	Lygaeidae	C + S America, Caribbean	4/0	
Niesthrea vincentii	Rhopalidae	S America, Caribbean	11/5 (45%)	'*jaculum*' sp. 3 (2) *Phytomonas* sp. A (1)
Nysius raphanus	Lygaeidae	S USA to Mexico, Caribbean	3/0	
Trichocorixa orinocensis	Corixidae	Northern S America, Lesser Antilles	29/0	
Xyonysius californicus	Lygaeidae	S Canada to Argentina, Caribbean	19/0	

*The trypanosomatid species determination, based on 18S rRNA sequencing, was successful only for the subset of the infected true bugs.

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

Trypanosomatid species	Host species (no. of infected specimens)	Localization in host*	18S rRNA GeneBank acc. no.	Isolate voucher	Culture
Heteroptera					
Leptomonas pyrrhocoris (TU***1***)	*Dysdercus modestus* (8) *Neomegalotomus parvus* (1)	MG (HG)	MK056191	CC-03	Yes
Leptomonas seymouri (TU***234***)	*Dysdercus modestus* (1) *Neomegalotomus parvus* (1)	MG (HG)	MK056192	CC-04	Yes
'*jaculum*' sp. 1 (TU***235***)	*Neomegalotomus parvus* (2)	HG/MG	MK056198	CC-49	Yes
'*jaculum*' sp. 2 (TU***236***)	*Catorhintha selector* (28) *Catorhintha guttula* (12) *Neomegalotomus parvus* (2)	HG (MG)	MK056199	CC-37	Yes
'*jaculum*' sp. 3 (TU***237***)	*Niesthrea vincentii* (2)	HG	MK056200	CC-20	
Blastocrithidia sp. 1 (TU***238***)	*Mecidea longula* (3) *Neomegalotomus parvus* (1)	MG (HG)	MK056188	CC-34	
Blastocrithidia sp. 2 (TU***239***)	*Neomegalotomus parvus* (1)	MG	MK056189	CC-17	
Phytomonas sp. A (TU***240***)	*Catorhintha selector* (4) *Catorhintha guttula* (1) *Niesthrea vincentii* (1)	MG, HG	MK056193	CC-71	
Phytomonas sp. B (TU***241***)	*Neomegalotomus parvus* (10)	HG/MG	MK056194	CC-83	
Diptera: Brachycera					
'new clade II' sp. 1 (TU***242***)	*Drosophila* sp. (3)	MG	MK056195	MCC-02	
'new clade II' sp. 2 (TU***243***)	*Drosophila* sp. (1)	MG	MK056196	MCC-01	
'new clade II' sp. 3 (TU***244***)	Drosophilidae sp. (1)	MG	MK056197	MCC-03	
Herpetomonas sp. A (TU***245***)	Drosophilidae sp. (1)	HG	MK056190	MCC-04	

*HG, hindgut; MG, midgut; MG/HG, both parts of the intestine infected; less infected part is in brackets.

and *Scantius aegyptius* in Europe and the Mediterranean and several species of *Dysdercus* in the Neotropics, Africa and China (Kozminsky et al. 2015; Votýpka et al. 2010, 2012a,b). The genome of this species was sequenced from 13 isolates from different localities around the world, demonstrating their correlation with biogeography (Flegontov et al. 2016). This trypanosomatid was recorded almost exclusively from the family Pyrrhocoridae, the exception being one infected specimen of *Neolethaeus* cf. *cantrelli* collected in Papua New Guinea from the related family Rhyparochromidae (Grybchuk-Ieremenko et al., unpublished data). In our collection from the island of Curaçao, *L. pyrrhocoris* was detected mainly in the pyrrhocorid *D. modestus.* However, we found this parasite also in one specimen of *N. parvus* (Table 1), a representative of the phytophagous family Alydidae, which is closely related to the Pyrrhocoroidea. As a phytophage with seed preference but with high willingness to feed on the bodies of dead organisms, *N. parvus* feeds on leguminous plants and has a life style similar to pyrrhocorid bugs (Schaefer and Panizzi 2000). Therefore, feeding on dead infected *Dysdercus* bugs inhabiting the same habitats cannot be ruled out, which would explain the low degree of infection as a result of the transfer of parasites to *N. parvus* from its accidental food.

The life strategy of *N. parvus*, an occasional necrophage, could also explain the very high diversity of trypanosomatids found in this heteropteran host, that is seven trypanosomatid TUs out of a total of nine recorded in all sampled true bugs in this study (Table 1). *Phytomonas* sp. B occurs in this true bug species regularly and exclusively (Table 1) and it can be assumed that *N. parvus* is its main host/vector. On the other hand, other trypanosomatid species found in *N. parvus* occur sporadically (Table 1), likely originating from feeding by *N. parvus* on dead bodies of other true bugs.

Two TUs belonging to the genus *Blastocrithidia* likely represent new species (Fig. 1). One of them, *Blastocrithidia* sp. 1 (TU*238*; represented by the isolate CC-34), is closely related (6–10 differences per 2,100 nucleotides [nt]) to *Blastocrithidia triatomae* infecting kissing bugs (subfamily Triatominae) in the Neotropical regions (Cerisola et al. 1971) and several other unnamed TUs isolated from true bugs in Ecuador (253VB; Kozminsky et al. 2015), Ghana (G36, G47, G48, G52, G54, G55, G63; Votýpka et al. 2012a) and China (CH336; Votýpka et al. 2010). The second, *Blastocrithidia* sp. 2 (TU*239*; represented by the isolate CC-17), is closely related (four differences per 2,100 nt) to *Blastocrithidia papi* (isolates 531Py and Pa3) (Frolov et al. 2017) and *Blastocrithidia largi* (Maslov et al. 2010). In the case of the two *Blastocrithidia* TUs, new species status remains unclear given the very high similarity to their closest relatives.

The situation in the '*jaculum*' clade is much clearer (Fig. 1). The sequence similarity of the three new species to their closest relatives ranges between 80 and 84% in '*jaculum*' sp. 2 (TU*236*; represented by the isolate CC-17) vs. isolate 250VB and 264AL (TU*134* and TU*135*; from the

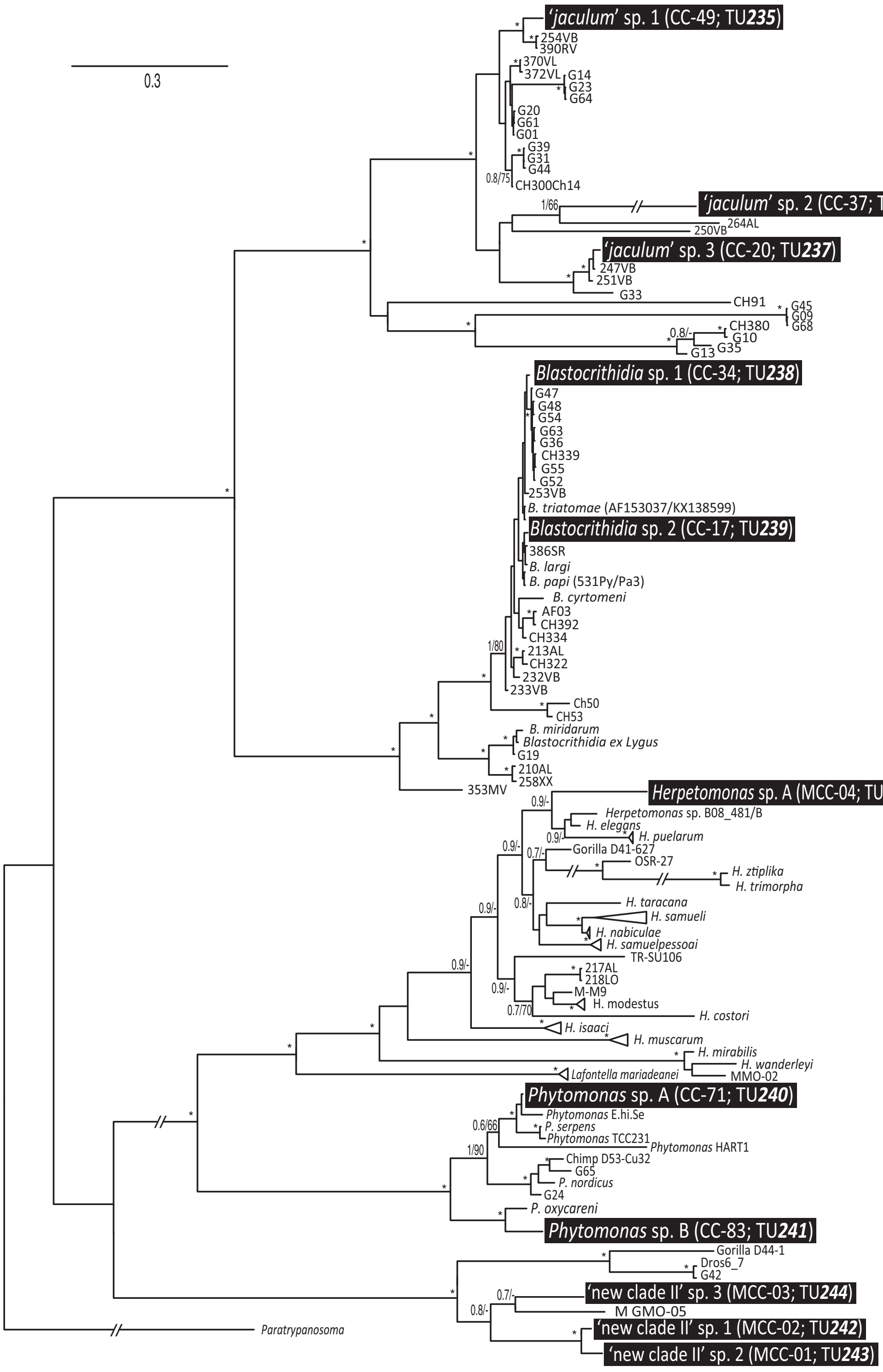

Figure 1 An 18S rRNA-based Bayesian phylogenetic tree of trypanosomatid sequences (~2.0 kb) obtained from true bugs and flies collected in Curaçao. Only phylogenetic relationships of clades, which contain new typing units (TUs) are shown. They fall into the following groups: '*jaculum*' (three TUs), *Blastocrithidia* (two TUs), *Herpetomonas* (one TU), *Phytomonas* (two TUs) and 'new clade II' (three TUs). Bootstrap values from Bayesian posterior probabilities (MrBayes; five million generations) and maximum likelihood analysis (PhyML; 1,000 replicates) are shown at the nodes; dashes indicate < 50% bootstrap support or different topology; asterisks mark branches with maximal statistical support; double crossed branches are 50% of the original length. The tree was rooted with *Paratrypanosoma*, the closest relative of the family Trypanosomatidae. Names of species or strains are given; the branch lengths are drawn proportionally to sequence differences (see scale bar).

true bug of the family Coreidae captured in Ecuador) (Kozminsky et al. 2015) to 97% in '*jaculum*' sp. 1 (TU*235*; represented by the isolate CC-49) vs. isolates 254VB and 390RV (TU*165*; from the true bug of the family Coreidae captured in Ecuador and Costa Rica) (Kozminsky et al. 2015). On the other hand, '*jaculum*' sp. 3 (TU*237*; represented by the isolate CC-20), is closely related to isolates 247VB (TU*133*; two differences per 2,100 nt) and 251VB (TU*132*; 28 differences per 2,100 nt), both originating from the true bug of the family Rhopalidae captured in Ecuador (Kozminsky et al. 2015). Mixed infection of two or more species was found in five cases (7%) (Table 2). We successfully established axenic cultures of both *Leptomonas* species and two new members of the '*jaculum*' clade, namely '*jaculum*' sp. 1 and '*jaculum*' sp. 2.

Two additional TUs are likely new species of the genus *Phytomonas* (Fig. 1). *Phytomonas* sp. A (TU*240*; represented by the isolate CC-71) is closely related (99%) to the isolate E. hi. Se (originated from a plant host *Euphorbia hirta*, Senegal), while *Phytomonas* sp. B (TU*241*; represented by the isolate CC-83) has 98% sequence similarity with *Phytomonas oxycareni* isolated from a true bug *Oxycarenus lavaterae* captured in Czechia (Seward et al. 2017). *Phytomonas* sp. B occurs exclusively in *Neomegalotomus parvus*, a true bug feeding preferentially on seeds of leguminous plants. In contrast, *Phytomonas* sp. A was detected in three species (Table 2) of the families Coreidae and Rhopalidae (Table 1), phytophagous true bugs associated with several plant families including *Nyctaginaceae*, *Sterculiaceae* and *Malvaceae*. This low host specificity associated with a broad and diverse diet may also be responsible for a relatively low number of specimens infected by *Phytomonas* sp. A, a parasite which probably occurs only in a restricted number of host plants. Phytomonads are the causative agents of a range of serious diseases of plants (Jaskowska et al. 2015), so it is likely that they also exercise some pathogenic effect on plants in Curaçao.

Out of 25 dissected brachyceran flies, trypanosomatids were detected in the gut of six specimens. In five of them, flagellates were confined to the midgut. The remaining case was unusual in that the protists occupied a region where the Malpighian tubules exit into the hindgut, although they were not observed in the tubule's lumen. The detected trypanosomatids fall into four new TUs (Table 1; Fig. 1). Three TUs belong to a monophyletic group only known from dipteran hosts, designated as the 'new clade 2' (Týč et al. 2013; Votýpka et al. 2018). These three closely related TUs form a monophyletic (sub)clade with the isolate GMO-05 (TU*112*) from a fly determined only to the genus *Musca,* captured in Ghana (Votýpka et al. 2010): 94% sequence similarity supports the establishment of the 'new clade 2' sp. 1 (TU*242*; represented by the isolate MCC-02), sp. 2 (TU*243*; represented by the isolate MCC-01) and sp. 3 (TU*244*; represented by the isolate MCC-03) (96.5% sequence similarity). The remaining TU represents a new species of the genus *Herpetomonas*. *Herpetomonas* sp. A (TU*245*; represented by the isolate MCC-04) is related to the isolate B08-481/B (TU*147*) (97% sequence similarity) originated from the flea *Monopsyllus sciurorum* captured in Czech Republic (Votýpka et al. 2013).

In this work we have characterized trypanosomatids from heteropteran and brachyceran hosts collected on the small island of Curaçao. To the best of our knowledge, it is the first time that the diversity of protistan parasites was analyzed in an island just a few dozen kilometers from the mainland. The total number of nine detected TUs in heteropteran insects appears rather low, which could be explained by homogenized biotopes and limited habitats on this populated and very dry island. The fact that the majority of trypanosomatids encountered in the tropical rainforests of Papua New Guinea are novel (Grybchuk-Ieremenko et al., unpublished data) was anticipated due to its isolation and generally extremely high biodiversity. However, our finding that in the heteropterans of Curaçao 7 out of 9 (78%) trypanosomatid TUs were not previously described was unexpected, even if we take into account a possible bias caused by limited sampling and the disputable inclusion of two new TUs of the genus *Blastocrithidia*. This finding is even more surprising given that the diversity of South American trypanosomatids has been extensively studied (Borghesan et al. 2013, 2018; Jirků et al. 2012; Kozminsky et al. 2015; Maslov et al. 2007, 2010; Teixeira et al. 2011; Westenberger et al. 2004; Yurchenko et al. 2006), especially as compared to their Asian, African and Australian counterparts (Barratt et al. 2017; Votýpka et al. 2010). Consequently, we expected the probability of finding a new TU in the Neotropics to be rather low.

Due to the scarcity of data on trypanosomatid infections in dipteran flies (Borghesan et al. 2013, 2018; Hamilton et al. 2015; Teixeira et al. 2011; Týč et al. 2013; Wilfert et al. 2011), only limited conclusions can be drawn for this group. Out of four TUs found in fly hosts that represent new species, three (TU*242*-4) belong to an unnamed genus designated as the 'new clade 2' sensu Týč et al. (2013), and deserve a special commentary. All members of this clade were obtained from the brachyceran flies, with the single exception of strain G42 (representing TU*84*), which was isolated from the true bug *Nagusta* cf. *punctaticollis* (Reduviidae) captured in Ghana. Since this is a predatory heteropteran bug, one can assume that these flagellates passively move from the prey, probably a brachyceran fly, to the predator. This hypothesis would explain the low level of infection we observed for this trypanosomatid in the intestine of dissected bugs (Votýpka et al. 2012a).

Species richness is a fundamental metric used to validate studies aiming to address why some areas abound with species diversity, while it is lacking in others. Both oceanic and continental islands are particularly interesting for studies of ecology, evolutionary biology and biogeography. Despite the rather inhospitable dry climate of the island of Curaçao, and a limited diversity of habitats, we found a relatively high number of trypanosomatid species (alias TUs) in sampled insects. The moderate diversity and high level of endemicity of trypanosomatids documented in Curaçao suggests a potentially important role of small islands in the preservation of microorganismal biodiversity, even those situated near the continental mainland.

ACKNOWLEDGMENTS

We thank Forest Rohwer (San Diego State University, USA) for his invaluable help in organizing the research trips. We would also like to thank our colleagues from Carmabi and to M.Tkoč (National Museum, Prague) for identification of brachyceran flies. This work was supported by the Canadian Institute for Advanced Research to P.J.K. and J.L., the ERC CZ project LL1601, Czech Grant Agency (No. 18-15962S) and the ERD Funds (OPVVV 16_019/0000759) to J.L.

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