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Probing into the diversity of trypanosomatid flagellates parasitizing insect hosts in South-West China reveals both endemism and global dispersal

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ABSTRACT

Flagellates of the class Kinetoplastea are known to frequently parasitize insects. We have collected 67 isolates from 407 Heteroptera hosts captured in several locations of South-West China. Their splice leader (SL) RNA gene repeats and small subunit (SSU) rRNA genes were PCR amplified from the infected tissue samples. In most cases, parasites were found in the midgut, rarely the infection was confined to the Malpighian tubes. Phylogenetic analysis of the obtained sequences has significantly expanded the known diversity of these monoxenous parasites. Fifteen typing units were found among these isolates including 11 potentially new species. Four typing units matched the previously known typing units from the Neotropics indicating a global distribution of the respective parasite species. At the same time, new clades appeared, testifying for a certain level of endemism. The host record of the parasites found indicated a variable specificity level of the host–parasite association including several cases of a very broad host range. Our results disprove the "one host – one parasite" paradigm and show that although the global diversity of monoxenous parasites is high, it is not as enormous as suggested earlier. Moreover, phylogenetic analysis revealed the presence, among the isolated strains, of a new *Phytomonas* species, which is the first documentation of this potentially pathogenic dixenous parasite of plants in China.

1. Introduction

The class Kinetoplastea includes mono- and bi-flagellated free-living, commensalic or parasitic protists (Vickerman, 1976). Since kinetoplastid flagellates include the most widespread parasites of humans and other vertebrates, it is not surprising that they belong to the best studied protists. The hallmark of this diverse and ubiquitous group is the presence of extensive mitochondrial DNA, localized in a mitochondrial subcompartment termed kinetoplast. A number of unusual molecular mechanisms found in these cells, such as RNA editing, extensive *trans*-splicing, and polycistronic transcription to name just the most prominent examples, were until recently considered unique to this group (Campbell et al., 2003; Lukeš et al., 2005), but may actually be present in other unrelated protists, albeit having had arisen independently (Lukeš et al., 2009). Expectedly, most of the interest was focused at the family Trypanosomatidae, and in particular at the genera *Trypanosoma* and *Leishmania*, the members of which cause many serious diseases in humans such as the African sleeping sickness, Chagas disease and leishmaniases. However, trypanosomatid species parasitizing only insects, such as *Crithidia fasciculata* or *Leptomonas collosoma*, are often used as convenient models in place of more fastidious pathogenic species (Kushnir et al., 2005). Therefore, it is highly relevant to study diversity of these flagellates and their relationships with well-studied trypanosomes and leishmanias.

The current view of the phylogenetic relationships among trypanosomatids is mainly based on the analyses of small subunit (SSU) rRNA genes (Lukeš et al., 1997; Hollar et al., 1998; Stevens et al., 1999; Merzlyak et al., 2001; Hamilton et al., 2004), and to a lesser extent glycosomal glyceraldehyde phosphate dehydrogenase (gGAPDH) genes (Hamilton et al., 2004; Yurchenko et al., 2006a,b; Svobodová et al., 2007). Although neither dataset has proven adequate for the resolution of these relationships at a satisfactory level, the main picture that has emerged from these works shows the genus *Trypanosoma* as a large monophyletic clade in a

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sister-group relationship with the rest of the family. The latter represents a vast assemblage of mingled monoxenous lineages, currently assigned in the genera *Blastocrithidia*, *Crithidia*, *Leptomonas*, *Sergeia* and *Wallaceina*, with the first three being speciose and polyphyletic, and the last two represented by a few species only. Emerging from the milieu of insect parasites are the dixenous flagellates of mammals or reptiles (*Leishmania*) (Yurchenko et al., 2006a,b) and plants (*Phytomonas*) (Hollar and Maslov, 1997), each represented by a monophyletic clade. The late emergence of *Leishmania* fully agrees with the classical hypotheses (Baker, 1963; Lainson and Shaw, 1987), which postulate that dixenous parasites emerged from primary parasites of insects during the acquisition of hematophagy. The finding of *Leishmania*-like protists among nucleated red blood cells of a cold blooded vertebrate in the phlebotomine sand fly trapped in ~120 million years old amber (Poinar, 2008) shows that even this relatively recent transition is, in fact, rather ancient. The origin of *Trypanosoma* is still obscure due to the very early emergence of this group, lending some credence to the alternative view, according to which dixenous species evolved from the primary parasites of vertebrates and switched to invertebrate vectors at a later time (discussed by Maslov and Simpson, 1995).

In any case, the phylogenies suggest that monoxenous parasites represent a significant, if not predominant, segment of the family's diversity. Despite their virtual omnipresence, especially in some host groups such as Heteroptera and Diptera, insect trypanosomatids remained largely neglected, and until the 1990s only sporadic descriptions, typically based on the "one host – one parasite" paradigm, were available (Podlipaev, 2001). This has to some extent changed after it was recognized that even monoxenous trypanosomatids have a potential to occasionally infect humans, in particular immunocompromised individuals (Morio et al., 2008; Barreto-de-Souza et al., 2008).

So far, two geographic areas (Costa Rica and Ecuador) were intensely sampled for insect trypanosomatids (Westenberger et al., 2004; Maslov et al., 2007). Increasing number of strains isolated almost invariably from heteropteran hosts in these biodiversity hotspots indeed revealed surprising diversity of these parasites. This is of particular interest, since in many instances the diversity among and within the individual branches exceeds that observed within the genus *Trypanosoma*, which is well-sampled at all continents (with the exception of Antarctica) and parasitizes vertebrates ranging from deep marine fishes thru reptiles and birds to mammals (Hamilton et al., 2005, 2007). Isolates from both countries were intermingled in the trees (Yurchenko et al., 2006a,b, 2008; Maslov et al., 2007), but that was not unexpected given the relatively limited distance and shared biotopes.

The observed genetic diversity is, however, not matched by morphology, as even very distantly related isolates often cannot be distinguished using features visible by light and electron microscopy. Moreover, morphological criteria used for decades in the taxonomy of insect trypanosomatids are rendered useless, as the various allegedly genus-specific characters and morphotypes are not monophyletic, but extensively spread throughout the trees (Hollar et al., 1998; Merzlyak et al., 2001; Yurchenko et al., 2008). Consequently, molecular phylogenetics represents the method of choice to map the diversity of insect trypanosomatids, with the ultimate aim to provide firm grounds for new taxonomy.

At present, there are two major views concerning the global diversity and distribution of protists. These are the ubiquity (Fenchel and Finlay, 2004; Epstein and Lopez-Garcia, 2008) and the moderate endemism (Foissner, 2006) models. Although the debate about both views is far from over, there appears to be accumulating evidence, concerning free-living protists, supporting the latter view. We sought to address this and other issues by extensive sampling insect trypanosomatids in several locations in South-West China. To the best of our knowledge, prior to this study these organisms were investigated neither in China, nor in the rest of Asia. By applying a culture-independent approach, based on phylogenetic analysis of the genes PCR-amplified directly from infected hosts, we identified several putative new species. Most of these new species from China were found intermingled in the phylogenetic tree with the species from the Americas and Europe, generally suggesting the absence of a long and separate evolution for the monoxenous parasites that are separated geographically. Instead, the data suggest a high rate of species dispersal that is related to low host specificity and/or broad host distribution range. The data also suggest the intensive ongoing diversification, possibly in cases when the dispersal is limited. At the same time, the presence of well separated phylogenetic clades indicates the potential existence of endemic host–parasite associations.

2. Materials and methods

2.1. Field work

The geographic origin, date of isolation, host species, infected tissue, intensity of infection and cell type of parasites are shown in Table 2. Several ecologically different localities in the vicinity of Kunming City (25°05′N; 102°42′E), Da-Li City (25°42′N; 100°08′E), Da-Li – North-East of Erhai lake (25°50′N; 100°08′E), Jinhong City (22°00′N; 100°47′E), Jinhong – Sanchahe (22°01′N; 100°52′E) and Jinhong – Xishuangbanna (22°04′N; 102°42′E) in the Yunnan province, South-West China, were intensely sampled for heteropteran insects in June 2007. Heteropteran bugs were collected on vegetation by sweep-netting. Within 24 h after the capture, insects were killed with 96% ethanol, washed and dissected in 0.9% sterile saline solution under the binocular microscope, so that from each specimen, a piece of foregut, midgut, hindgut, and Malpighian tubes was placed on a separate slide. The tissue was squeezed by a cover slip and carefully examined for the presence of flagellates using 400× total magnification. When flagellates were detected, the infected gut material was transferred from the slide in 1 ml of 2% SDS, 100 mM EDTA solution and kept at the ambient temperature until the transfer to the laboratory (1–2 weeks), where it was kept at −80 °C until further use (Westenberger et al., 2004). The host material is deposited in the National Museum, Prague.

2.2. DNA isolation

The lysate of infected insect tissue, mostly gut content in the preservation solution, was used to isolate the total DNA using the PureLink™ Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's manual with minor modifications. The RNAse A treatment was omitted. The procedure involved utilization of 400 μl from the original DNA samples, the amounts of the reagents per the "Blood Lysate" protocol were scaled up twice, and multiple loading of the lysate on the same spin column were necessary during the DNA binding step. The DNA was eluted in 100 μl of the elution buffer.

2.3. PCR amplification, cloning and sequencing

PCR amplification of the SL RNA gene repeats with the primers M167 and M168 followed the procedures described previously (Westenberger et al., 2004; Maslov et al., 2007). The PCR products were gel-purified, cloned into pJET 1.2 (Fermentas, Glen Burnie, MD) or pT 7Blue (EMD Bioscience, San Diego, CA) vectors and sequenced as described in the aforementioned references. The SSU rRNA gene sequences were obtained after PCR amplification with

the primers S762 and S763 as described earlier (Maslov et al., 1996). The PCR products were gel-purified using QIAquick gel extraction kit (Qiagen, Valencia, CA) and sequenced directly using the amplification primers and a set of internal conserved primers S713, S714, S755 and S757 (Maslov et al., 1996) along both strands. The sequencing was performed at the UC Riverside IIGB Core Instrumentation facility. GenBank™ accession numbers are listed in Table 2.

2.4. Phylogenetic analysis

Prior to the SL RNA gene alignment, the sequences were edited by removing the amplification primer annealing sites (positions 21–42 in exon and intron of the SL RNA gene) and most of the intergenic region from the T-block (just downstream of the intron) to the −100 position upstream of the next exon, thereby leaving only the relatively conserved ~150 nt-long region from each repeat unit. The sequences were then aligned with Clustal X, version 2.0 (Larkin et al., 2007) (gap opening penalty of 12, gap extension penalty of 5). The neighbor-joining analysis, utilizing Kimura 2-parameter distances, was performed with PAUP* 4.0 beta version (Swofford, 1998). Bayesian analyses were performed using MrBayes 3.1.1 (Ronquist and Huelsenbeck, 2003). Base frequencies, rates for six different types of substitution, proportion of invariant sites and shape parameter of the gamma correction for the rate heterogeneity with four discrete categories were allowed to vary. The covarion model was used to allow the rate heterogeneity along the tree. The Markov chain Monte Carlo was run for 10×10^6 generations; the trees were sampled every 100th generation. The first 25,000 trees were discarded as burn-in. The best-fitting model of nucleotide substitution for maximum likelihood (GTR + I + Γ) was chosen with Modeltest 3.06 (Posada and Crandall, 1998). The neighbor-joining trees were bootstrapped in PAUP* and maximum likelihood trees were bootstrapped in PHYML (Guindon and Gascuel, 2003) with 1000 replicates.

The SSU alignment was generated with Clustal and then manually edited to remove the poorly alignable regions with MEGA 4 (Tamura et al., 2007). The best-fitting model of the sequence evolution was selection using the Akaike Information Criterion of MODELTEST, version 3.06 (Posada and Crandall, 1998). The maximum likelihood phylogenetic analysis with bootstrapping (100 replicates) was performed with PAUP (Swofford, 1998).

3. Results

3.1. Identification and barcoding of the trypanosomatid isolates

Seventy nine Heteroptera species out of 21 families were captured at six locations in the Yunnan province, which is considered as one of the biodiversity hotspots of China, and examined for the presence of trypanosomatid parasites (Tables 1 and 2). Out of more than 400 individual specimens examined, infections were found in approximately 16% of cases in 19 Heteroptera species representing 24% of all studied species. This value might be an underestimate of the actual prevalence because infections at low parasitemia levels might have been undetected. It should be mentioned that due to the limited duration of this study, the hosts analyzed, and hence the parasites found, must have represented the most common host species found on vegetation. Thus, the diversity revealed constitutes only a small fraction of the entire region's diversity. The infected tissues were preserved in the field using a strong detergent-chelator solution with the subsequent extraction of the total DNA.

As the first step towards the identification of the parasites in the infected hosts, we employed amplification, cloning and sequencing of spliced leader (SL) RNA gene repeats, trypanosomatid-specific markers that proved to be very useful for genotyping and barcoding (Westenberger et al., 2004; Maslov et al., 2007). Out of 67 samples collected, the amplification was successful in 51 cases (Fig. 1; Table 2). After excluding the apparently redundant PCR products (e.g. those having the same size and originating from the same host population), the remaining 32 samples were chosen for cloning and sequencing (Table 2). Among the obtained SL RNA repeat sequences, 15 different genotypes (typing units, each of which representing a separate species) were delineated using a threshold 90% identity rule employed previously (Maslov et al., 2007).

With the aim of putting the trypanosomatids from China into a family-wide context, we compared them with the dataset of the SL RNA gene repeats generated previously that includes both the named species and the typing units (Westenberger et al., 2004; Maslov et al., 2007). The results of the neighbor-joining, maximum likelihood and Bayesian analyses based on the most conserved parts of the repeat (from the −100 position upstream of the exon to the start of the T-tract just downstream of the intron) are shown in Fig. 2. Although details of the trees obtained with these methods were different, the major conclusion derived from these analyses was the same. Based on the relationships with the other trypanosomatids, the species from China are divided into three categories as described below. The first of these are unique, well separated species, such as typing units Ch**8** and especially Ch**15** that have no close relatives identified so far (potential new genera). The second category includes the typing units that are associated with the previously discovered species and, therefore, represent new species of the previously established phylogenetic groups. This category includes typing units Ch**2**, Ch**3**, Ch**4**, Ch**6**, Ch**7** (associated with the *Blastocrithidia* clade), Ch**10** (associated with two described *Leptomonas* species), Ch**12** (associated with the *Phytomonas* clade), and Ch**9** and Ch**13** (associated with the Neotropical undescribed species represented by TU***18*** and TU***6/7***, respectively). Finally, the third category includes the few SL typing units that closely match sequences from the previous analyses, and therefore represent new isolates of the known species (described or undescribed). These are: Ch**1** (that matched Neotropical TU***44***), Ch**5** (Neotropical TU***14***), Ch**14** (Neotropical TU***6/7***) and Ch**11** (*Leptomonas pyrrhocoris* and several Neotropical isolates constituting TU***1***).

3.2. Phylogenetic affinities of the trypanosomatid isolates

With the only exception of Ch**11**, which is a member of the *L. pyrrhocoris* species, a previously established member of the 'SE' clade that also includes a score of *Leptomonas* and *Crithidia* species (Yurchenko et al., 2006a,b, 2008), no other isolate from China matched a species (described or undescribed) with a known phylogeny. It needs to be mentioned that the SL analysis, while very sensitive in detecting differences and similarities between sequences, does not produce a tree with a reliable deep branching order because even the most conserved regions in the SL repeat unit (~120 nt) cannot be meaningfully aligned across the entire family. It was therefore important to complement the results of the SL analysis with investigation of a more informative phylogenetic marker, such as the SSU rRNA gene. To this end, we selected a single member of each typing unit (Table 2) to amplify these genes directly from the infected gut DNA samples using trypanosomatid-specific primers (Maslov et al., 1996). Caution was taken to avoid those samples that contained more than a single trypanosomatid type (Yurchenko et al., 2009).

The set of reference species for the analysis also included a limited number of members of the major known trypanosomatid clades (Hollar et al., 1998; Merzlyak et al., 2001). The results of the analysis are shown in Fig. 3. The major tree topology is consistent with that obtained previously and, therefore, is not discussed herein. Remarkably, the phylogenetic associations of the Chinese

Table 1
Summary of the examined host families and species, also showing the number of inspected versus infected specimens (rate), along with the typing units (TU), to which the detected flagellates belong.

Host family	Host species	Rate	Typing unit
Acanthosomatidae	*Elasmostethus* sp.	2/0	
Alydidae	*Acestra yunnana*	3/0	
	Leptocorisa lepida	18/**3**	Ch**1**, Ch**14**
	Undetermined sp.	1/0	
Anthocoridae	*Orius* sp.	5/0	
Belostomatidae	*Diplonychus rusticus*	14/**2**	Ch**7**
Berytidae	*Yemma exilis*	1/0	
Coreidae	*Cletus punctulatus*	2/0	
	Cletus bipunctatus	2/**1**	Ch**13**
	Three *Cletus* spp.	46/0	
	Hydarella orientalis	15/0	
	Ochrochira sp.	5/**2**	Ch**7**
	Physomerus grossipes	1/0	
	Seven undetermined spp.	8/0	
Geocoridae	*Geocoris varius*	4/**2**	Ch**1**
	Geocoris sp.	1/0	
Gerridae	*Aquarius paludum paludum*	14/**6**	Ch**2**, Ch**7**, Ch**15**
	Gerris (*Macrogerris*) sp.	14/**3**	Ch**9**
	Three undetermined spp.	15/0	
Largidae	*Iphita limbata*	1/**1**	Ch**3**
	Macroheraia grandis	1/0	
	Physopelta quadriguttata	1/0	
Lygaeidae	*Nysius* sp.	28/0	
Miridae	*Stenodema* sp.	6/0	
	Four undetermined spp.	13/0	
Nabidae	*Himacerus* sp.	2/0	
	Nabis sp.	1/0	
Notonectidae	*Anisops* sp.	14/0	
Pentatomidae	*Arma* sp.	1/0	
	Bolaca unicolor	2/**1**	Ch**7**
	Carbula scutellata	1/0	
	Eysarcoris guttigerus	15/**13**	Ch**1**
	Eysarcoris montivagus	9/**1**	
	Euridema pulchra	6/0	
	Eysarcoris sp.	4/0	
	Homalogonia sp.	1/0	
	Hoplistocera sp.	1/0	
	Nezara antennata	1/0	
	Plautia sp.	5/0	
	Sarju taungyiana	16/**16**	Ch**6**, Ch**7**
	Two undetermined spp.	4/0	
Pyrrhocoridae	*Dysdercus poecilius*	14/**5**	Ch**11**
	Melamphacis faber	14/**5**	Ch**4**, Ch**10**
Reduviidae	*Euagoras plagiatus*	2/0	
	Isyndus reticulatus	1/0	
	Rhynocoris sp.	1/**1**	Ch**2**
	Scadra militaris	1/0	
	Tapirocoris cf. *limbatus*	3/**2**	Ch**6**
	Two undetermined spp.	2/0	
Rhopalidae	*Liorhyssus hyalinus*	1/0	
	Rhopalus sp.	2/0	
	Stictopleurus sp.	5/0	
Rhyparochromidae	*Dieuches pamelae*	1/**1**	Ch**8**
	Dieuches sp.	2/0	
	Elasmolomus sp.	1/0	
	Gyndes sp.	9/**1**	Ch**5**
	Metochus sp.	1/**1**	Ch**12**
	Primierus indicus	3/0	
	Paromius sp.	3/0	
	Poeantius sp.	1/0	
	Undetermined sp.	1/0	
Scutelleridae	*Hotea curculionoides*	1/0	
Tessarotomidae	*Eusthenes* sp.	19/0	
Tingidae	*Dictyla* sp.	15/0	
Total	79 species	**407**/**67**	Ch**1**–**15**

Table 2
List of infected host species, showing their geographic origin (Loc), developmental stage (Stage), infected tissue (Site), intensity of infection, cell type of the flagellate (Type), positivity (+)/negativity (–) for PCR of the SL RNA, and the SL RNA (SL) and SSU rRNA (SSU) typing units analyzed in this study.

Host species	No.	Loc[a]	Stage	Site[b]	Intensity	Type[c]	SL PCR	SL TU GenBank No.	SSU TU GenBank No.
Leptocorisa lepida	300	JI2	Adult	MG	+	M	+	Ch**14** GU063788 GU063789	Ch**14** GU059563
	304	JI2	Adult	MG	++	M	+		
	306	JI2	Adult	MG	++	M	+	Ch**1** GU063790	
Diplonychus rusticus	152	DA1	Adult	MG	+	L	+	Ch**7** GU063780	
	154	DA1	Adult	MG	+	L	–		
Cletus bipunctatus	332	JI2	Adult	MG	+	S	+	Ch**13** GU063792-GU063794	Ch**13** GU059565
Ochrochira sp.	53	DA1	Adult	MG	++++	S	+	Ch**7** GU063760	Ch**7** GU059559
	76	DA2	Adult	MG	+++	M	+	Ch**7** GU063761	
Geocoris varius	411	JI1	Adult	MG	+	S	+		
	412	JI1	Adult	MG	+	S	+	Ch**1** GU063805	
Aquarius p. paludum	230	JI3	Adult	MG	+	M	–		
	231	JI3	Adult	MG	+++	MIX	+	Ch**2,7,15** GU063781-GU063783	Ch**15** GU059562
	232	JI3	Adult	MG	++	MIX	–		
	404	JI1	Adult	MG	++	L	+	Ch**15** GU063803 GU063804	Ch**15** GU059572
	405	JI1	Adult	MG	+	L	–		
	406	JI1	Adult	MG	+	MIX	–		
Gerris (*Macrogerris*) sp.	89	DA2	Adult	MG	+++	L	+		
	91	DA2	Adult	MG + HG	+++	MIX	+	Ch**9** GU063775 GU063776	Ch**9** GU059560
	134	DA1	Adult	MG	++	MIX	–		
Iphita limbata	334	JI2	Nymph	MG	++	M	+	Ch**3** GU063795	Ch**3** GU059566
Bolaca unicolor	37	DA1	Adult	MG	+	L	+	Ch**7** GU063758	
Eysarcoris montivagus	246	JI3	Adult	MG	++++	L	+		
Eysarcoris guttigerus	249	JI3	Adult	MG	++++	L	+	Ch**1** GU063784 GU063785	
	338	JI2	Adult	MG	+++	M	–		
	339	JI2	Adult	MG	+++	M	+	Ch**1** GU063796	Ch**1** GU059567
	340	JI2	Adult	MG	++	M	+		
	341	JI2	Adult	MG	+++	M	+		
	342	JI2	Adult	MG	+++	M	+		
	343	JI2	Adult	MG	+++	M	–		
	344	JI2	Adult	MG	+++	M	–		
	345	JI2	Adult	MG	+++	M	–		
	346	JI2	Adult	MG	+++	M	–		
	347	JI2	Adult	MG	+++	M	–		
	348	JI2	Adult	MG	+++	M	–		
	349	JI2	Adult	MG	+++	M	–		
Sarju taungyiana	48	DA1	Adult	MG	+++	M	+		
	49	DA1	Adult	MG	+++	M	+		
	50	DA1	Adult	MG	++++	M	+	Ch**6** GU063759	Ch**6** GU059558
	51	DA1	Adult	MG	++++	M	+		
	77	DA2	Adult	MG	++	M	+		
	78	DA2	Adult	MG	+++	M	+	Ch**7** GU063762GU063763	
	79	DA2	Adult	MG	++	M	+	Ch**6** GU063764-GU063767	
	80	DA2	Adult	MG + HG	++	M	–		
	81	DA2	Nymph	MG	++	M	+	Ch**6** GU063768	
	82	DA2	Adult	MG	+++	M	+		

(*continued on next page*)

Table 2 (*continued*)

Host species	No.	Loc[a]	Stage	Site[b]	Intensity	Type[c]	SL PCR	SL TU GenBank No.	SSU TU GenBank No.
	83	DA2	Adult	MG	+++	M	+	Ch**6** GU063769 GU063770	
	84	DA2	Adult	MG	+++	M	+		
	85	DA2	Adult	MG	+++	M	+	Ch**6** GU063771 GU063772	
	86	DA2	Adult	MG	+++	M	+		
	87	DA2	Adult	MG	+++	M	+	Ch**7** GU063773 GU063774	
	88	DA2	Adult	MG	+++	M	+		
Dysdercus poecilus	278	JI2	Nymph	MG	+	M	+	Ch**11** GU063786	
	279	JI2	Nymph	MG	++	M	+		
	280	JI2	Nymph	MG	+	M	+		
	281	JI2	Nymph	MG	+	M	+		
	282	JI2	Nymph	MG	++	M	+	Ch**11** GU063787	
Melamphaus faber	387	JI1	Adult	MG	+	L	+		
	390	JI1	Adult	MG	+	L	+		
	391	JI1	Adult	MG	++	MIX	+	Ch**10** GU063798	Ch**10** GU059569
	392	JI1	Adult	MG	+	L	+	Ch**4** GU063799	Ch**4** GU059570
	395	JI1	Nymph	MG	+	S	–		
Rhynocoris sp.	148	DA1	Adult	MG	+++	M	+	Ch**2** GU063779	Ch**2** GU059561
Tapirocoris cf. *limbatus*	111	DA2	Adult	MG	++++	S	+	Ch**6** GU063777	
	112	DA2	Adult	MG	++	S	+	Ch**6** GU063778	
Dieuches pamelae	380	JI1	Adult	MG	++	M	+	Ch**8** GU063797	Ch**8** GU059568
Gyndes sp.	322	JI2	Adult	MT	+++	M	+	Ch**5** GU063791	Ch**5** GU059564
Metochus sp.	402	JI1	Adult	MT	++	L	+	Ch**12** GU063800-GU063802	Ch**12** GU059571

[a] Locality: DA1 – Da-Li City; DA2 – Da-Li Erhai lake; JI1 – Jinhong City; JI2 – Jinhong Sanchahe; JI3 – Jinhong Xishuangbanna.
[b] Site of infection: MG – midgut; HG – hindgut; MT – Malpighian tubes.
[c] Flagellate cell type: S – small and short; M – medium size; L – large and long cells.

isolates that had been identified as members of the 'SE', *Blastocrithidia* and *Phytomonas* clades, were confirmed by the SSU data. Also confirmed was a separate position of the Ch**15** lineage. A novel aspect of the SSU analysis is the identification of a new clade, composed of a parasite from the intestine of the water scorpion (*Nepa cinerea*), *Leptomonas jaculum* from North-West Russia (Kostygov and Frolov, 2007), and the typing units Ch**9**, Ch**13** and Ch**14**. The additional China typing unit Ch**8** is a sister lineage to this clade. These relationships are well supported by the bootstrap analysis.

The isolate 402 (Ch**12**) is clearly a new *Phytomonas* species, and being potentially a plant pathogen, asks for particular attention. Its insect host (*Metochus* sp.) is a rhyparochromid bug (Rhyparochromidae, Amyot and Serville 1843, formerly a subfamily of Lygaeidae), representing a group with a variety of feeding habits, including phytophagy. Localization of the parasite exclusively in the Malpighian tubes is highly unusual. Vectors of plant-parasiting *Phytomonas* are known in only a few cases, with pentatomids being the best documented vectors of phloem-restricted (Dollet, 2001) and fruit-dwelling phytomonads in Neotropics (Jankevicius et al., 1989), and lygaieds also being potential vectors among other phytophagous families (Camargo, 1999). In the SL-based tree, the isolate 402 (Ch**12**) forms a new long branch within the *Phytomonas* spp. cluster, of which the Hart1 strain is the earliest branch (Fig. 2). In the well-supported *Phytomonas* (P) clade of the SSU tree, the Chinese isolate forms a sister branch to the Hart1 strain, isolated from the cocos plant, succumbing to the hartrot disease in Guyana. However, the plant host of this, supposedly dixenous, trypanosomatid remains unknown.

3.3. Host–parasite specificity

The host record of the 15 different trypanosomatid species identified in this study (Tables 1 and 2), along with the data collected in the Neotropics previously (Westenberger et al., 2004; Maslov et al., 2007), suggests a generally loose specificity of the host–parasite associations, at least for the studied parasites of Heteroptera. For example, during this study, typing unit Ch**1** is found in the hosts from three host families (Alydidae, Geocoridae and Pentatomidae), while the matching Neotropical TU***44*** was previously found in Coreidae (Maslov et al., 2007). Moreover, Ch**7** is found in Belostomatidae, Coreidae, Gerridae and Pentatomidae. In a few cases, but not all, the occurrence of a trypanosomatid in more than one host species may be explained by the host predator–prey relationship (e.g. Ch**6** in Reduviidae and Pentatomidae or Ch**7** in Gerridae and three other families), at least hypothetically. Overall, these findings demonstrate that one particular trypanosomatid species may have a broad range of hosts.

Viewing these associations from the host perspective brings a similar conclusion. One host species, in some cases one particular

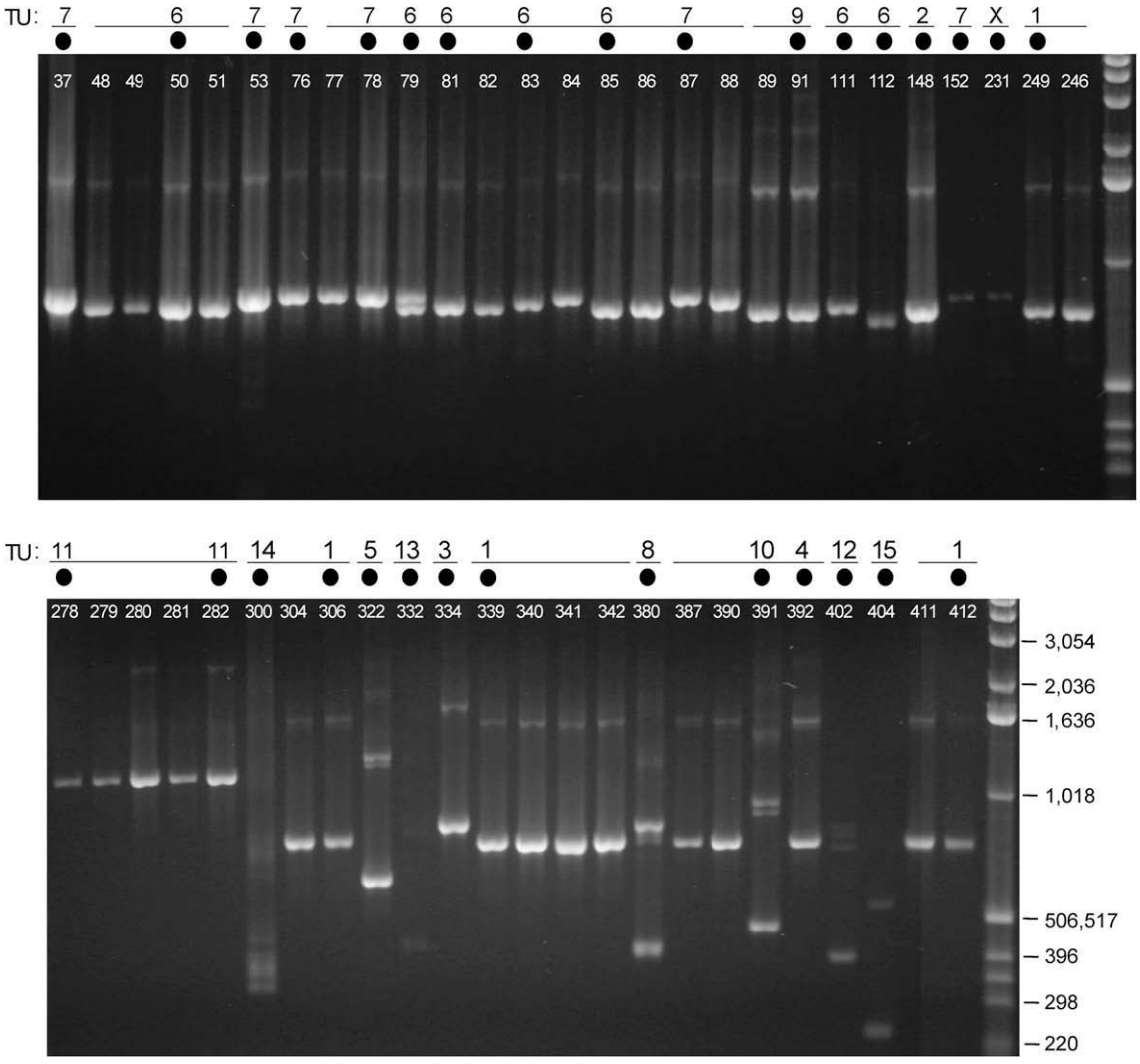

Fig. 1. PCR amplification of SL RNA gene repeats from a panel of gut samples derived from Heteroptera hosts infected with trypanosomatids. Only the samples that produced specific amplification products are shown. The amplification products were resolved in 1% agarose gels. White numbers denoting the lanes represents the respective sample ID's. Lines above adjacent lanes indicate sets of samples derived from same host populations. Black dots indicate the samples that were sequenced. Numbers above the lanes identify typing units (TU). Letter X above sample 231 indicates that the PCR products obtained from these samples belong to typing units 2, 7 and 15 (indicated in the text as Ch**2**, Ch**7** and Ch**15**). The sizes of the marker bands (1 kb ladder, invitrogen) are shown to the right of the bottom panel.

individual, may be infected with several distantly related parasites, as exemplified in this study by the one specimen of *Aquarius p. paludum* (Gerridae) found to harbor three species of trypanosomatids (Ch**2**, Ch**7** and Ch**15**). With this, and our earlier findings (Maslov et al., 2007; Yurchenko et al., 2009), mixed infections of a single host with several species of trypanosomatids appear almost a rule, rather than exception, particularly in predatory species.

The remaining cases in which a single parasite species was found per one host species cannot be taken as an evidence of strongly specific associations due to a limited sampling size. The only potential exception might be a consistent association of the parasites of the *L. pyrrhocoris* species group (including Ch**11** typing unit, and the previously described Neotropical TU***1***) with the *Dysdercus* hosts (and the related *Pyrrhocoris* species, family Pyrrhocoridae) (Westenberger et al., 2004; Maslov et al., 2007). However, one species of *Dysdercus* was also found to carry an unrelated parasite species (TU***34***) (Maslov et al., 2007), as well as one specimen of the Old World *Pyrrhocoris apterus* was infected by a parasite clearly dissimilar to *L. pyrrhocoris* (J.V., unpubl. results). In conclusion, these data show that the "one host – one parasite" paradigm might be tenable just in a few cases.

3.4. Tissue specificity

Localization of the trypanosomatid infection within the insect host is only rarely provided in the literature (Sbravate et al., 1989; Godoi et al., 2002), and if so, the flagellates are predominantly located in the intestine. In this study, we have dissected all 407 heteropteran specimens with the intention to establish the exact site of infection, which is relevant to the transmission of these parasites. Indeed, a large majority of infections was present in the intestine, yet it was mostly confined only to the midgut, predominantly its abdominal part (97% infections), while other parts of the intestinal tract were invaded rather exceptionally. Infections of the Malpighian tubes were very rare (isolates 322 (Ch**5**) and 402 (Ch**12**)), but in both cases the flagellates were localized exclusively in this organ. However, not all tubes were occupied, with at least one of them remaining parasite-free. The only *Phytomonas* species found in our dataset belongs to this group, while unusually big flagellates were present in the Malpighian tubes of the *Metochus* host.

In this context it is worth mentioning that 8 out of 62 nymphs at different developmental stages were infected (13%), which cor-

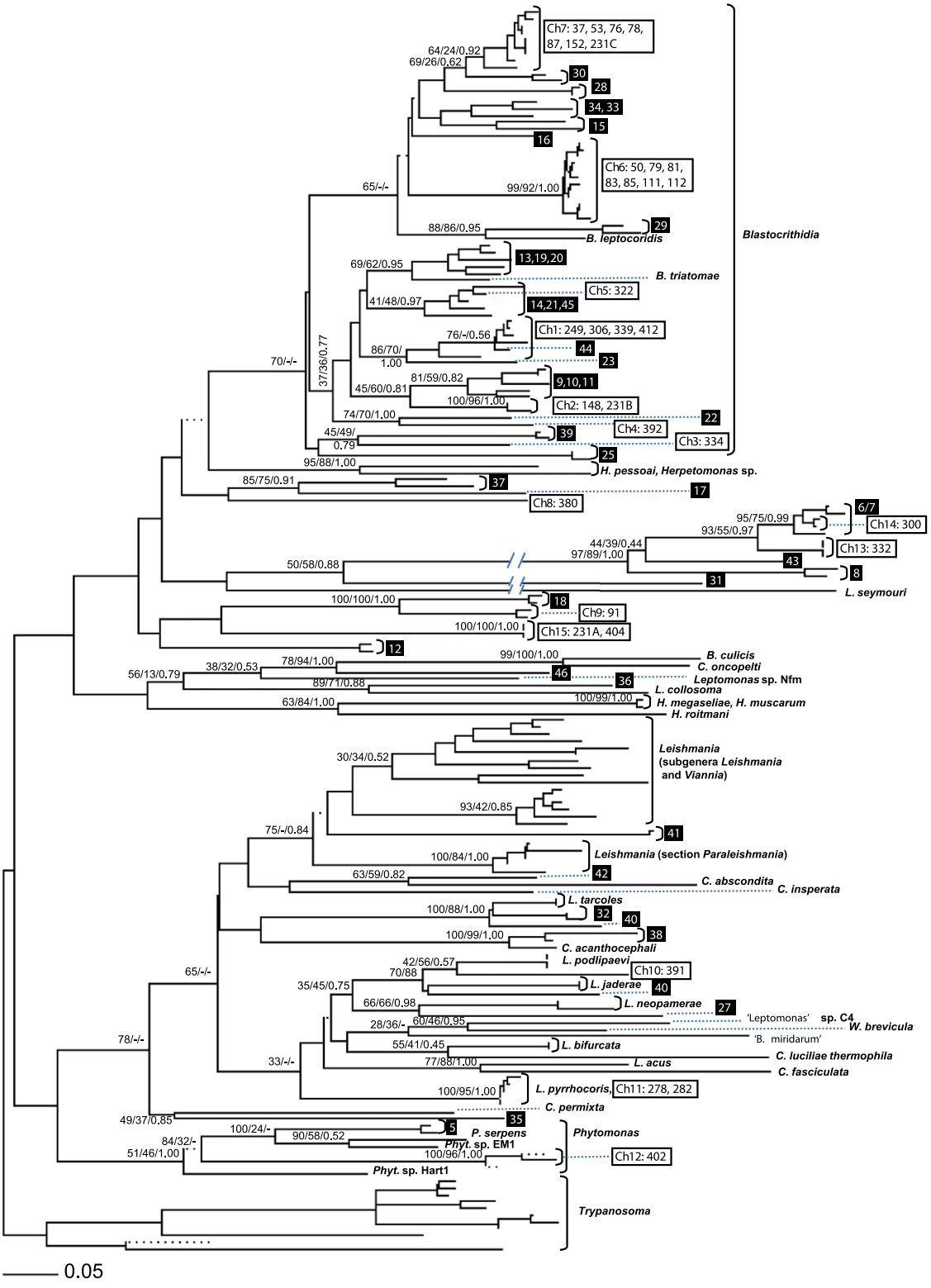

Fig. 2. Neighbor-joining (NJ), maximum likelihood (ML) and Bayesian analyses of the SL RNA gene repeats of Trypanosomatidae. The most conserved sections of the sequences (from −100 position upstream of the exon to the 3′ end of the intron, excluding the amplification primer sequences) were aligned with Clustal X, version 2.0 (Larkin et al., 2007). For the NJ tree shown, Kimura 2-parameter distances were calculated and the tree inferred using PAUP* 4.0 beta version (Swofford, 1998). ML and Bayesian analyses were performed as described in Materials and Methods. Boostrap values shown at the best supported clades correspond to NJ followed by ML. The third value in a set represents Bayesian support. Dashes indicate that clade were not recovered or poorly supported with a particular method. No values are shown for the clades that were not supported with all the methods. Besides most described species of monoxenous trypanosomatids and a limited set of dixenous parasites (the genera *Leishmania*, *Phytomonas* and *Trypanosoma*), the taxa used also included the set of Neotropical typing units (numbers in black boxes). The isolates from China are shown in frames, whence a designation of the typing unit is followed by the individual isolates. The scale bar below the tree represents substitutions per site. Taxa shown is quotation marks represent misnomers as discussed previously (Yurchenko et al., 2008, 2009).

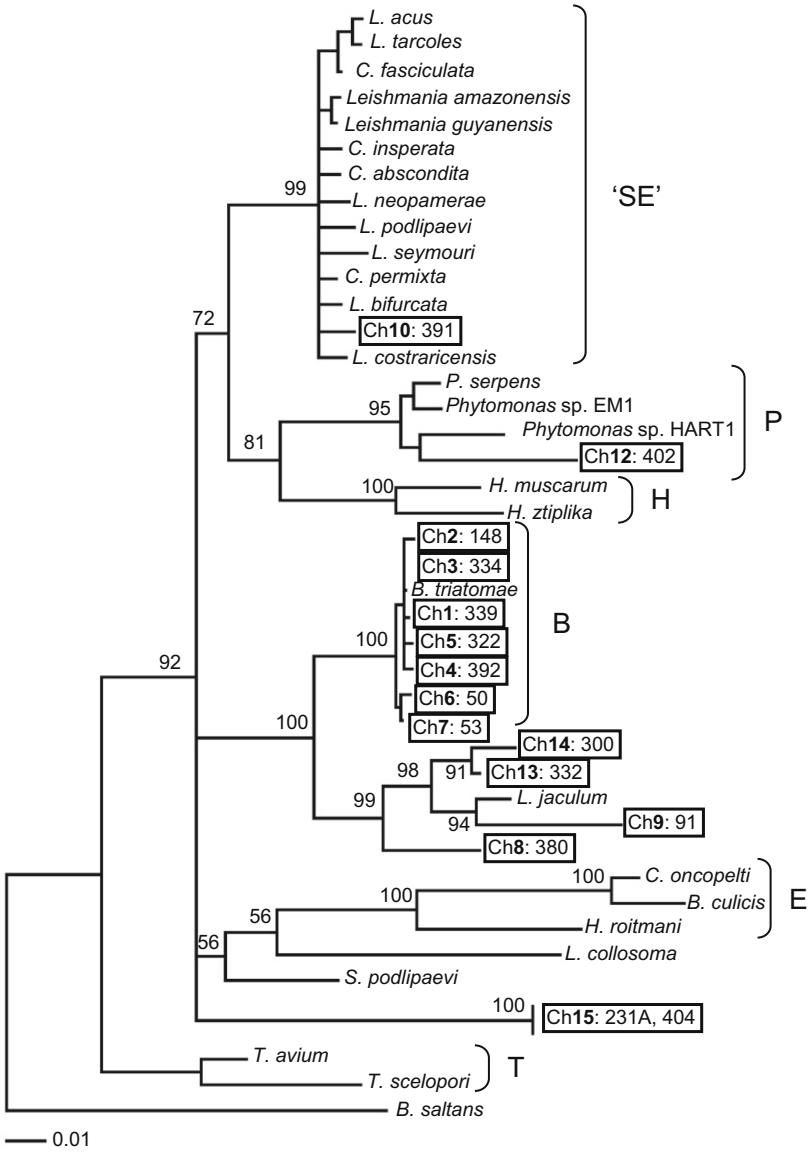

Fig. 3. Maximum likelihood analysis of the SSU rRNA gene sequences from the new trypanosomatids species described in this study. A single member of each new typing unit (shown by the designations in frames) was selected and the SSU sequences amplified directly from the respective gut DNA sample. The dataset also included a limited number of reference species and strains representing the major lineages and phylogenetic clades of the Trypanosomatidae: 'SE' – 'slowly-evolving' (the name referring exclusively to the relative rate of the SSU sequence divergence), P – dixenous plant parasites of the genus of *Phytomonas*, H – primarily apoendosymbiontic *Herpetomonas* species, B – the emerging clade of *Blastocrithidia triatomae* and closely related organisms, E – morphologically diverse group containing endosymbiont-bearing trypanosomatids and their apoendosymbiontic derivatives, T – the genus *Trypanosoma*. The alignment, produced with Clustal X with the manual removal of the ambiguous regions, was 1835 characters long. The tree was rooted with the bodonid *Bodo saltans*, the closest known relative of the Trypanosomatidae. The best-fitting model of the sequence evolution (GTR, with $I = 0.5507$ and $\Gamma = 0.6005$) was selected using AIC test of MODELTEST, version 3.06 (Posada and Crandall, 1998). The best tree ($-\mathrm{Ln} = 9620.63586$, not shown) was searched for by heuristic search. It showed T-clade as branching off first, followed the clade ((('SE', P), (H, *L. collosoma*)), (E, *Sergeia podlipaevi*)) in sister-group relationship with the clade including the remaining taxa, most of which from China. The shown majority consensus tree ($-\mathrm{Ln} = 9695.94594$) was derived after 100 replicates, with the bootstrap support values as shown. The scale bar shows the number of substitutions per site.

responds with infection rate in the adult bugs (17%; 59 out of 345). Almost invariably, the infections in nymphs were mild.

In an attempt to estimate the intensity of diagnosed infections, each of them was scored on a scale ranging from a very mild infection (+) with only a few flagellates observed, to very heavy infections (++++), which were manifested by tens of thousands of parasites, usually obstructing the intestinal tract, mainly in the area of abdominal midgut. Cell types of observed parasites were cursorily divided into three categories: S – small, short and often stilliform cells, M – medium size and typical promastigote-shaped cells and, finally, L – large, long and slender cells. As shown in Table 2, due to a limited dataset, it is difficult to convincingly demonstrate a correlation between the infected host species/families on one side, and the intensity of infection and morphotype of a given parasite on the other side. However, hosts belonging to the families Pentatomidae, Coreidae and Reduviidae generally carry heavier infections than bugs from the other families.

4. Discussion

In this work we present first phylogenetic analysis of insect trypanosomatids from Asia, which allows us to address issues concerning their prevalence, diversity, geographic distribution, and various aspects of host–parasite relationships. Our data further

expand the emerging complex view of their distribution and specificity, further deconstructing the "one host – one parasite" paradigm, that had been used as a theoretical basis for new species descriptions for the last century (Podlipaev, 1990) until the recent applications of molecular techniques for identification of the parasites have finally began to challenge this view (Podlipaev, 2003; Podlipaev et al., 2004). We repeatedly find adult heteropterans infected with two and even more species, and at the same time, genetically identical isolates are often found in hosts from different families (this work and Westenberger et al., 2004; Maslov et al., 2007). It is also remarkable that to the 51 new species/typing units (some of which have been already described but most of which are undescribed) from Neotropics (Westenberger et al., 2004; Yurchenko et al., 2006a,b; Maslov et al., 2007; Yurchenko et al., 2008), we have added 14 additional ones from China, and three out of these matched the respective Neotropical species. Only one typing unit (Asian Ch**11** and Neotropical TU***1***) can be assigned to *Leptomonas pyrrhocoris*, originally described from Europe. While our results support the predictions of high, although still mostly hidden, global species richness of the heteropteran Trypanosomatidae (Podlipaev, 2000), they also indicate that the final number is not really enormous, making the task of describing a global diversity practical, at least with respect to the most common parasite–host associations. A significant benefit of this work is that a wealth of information about the phylogeny of trypanosomatids can be obtained solely by a culture-independent approach.

A molecular survey, which operates with typing units (TUs) as synonyms of species, significantly expands available dataset of the discovered trypanosomatid genotypes and allows for testing of the hypotheses addressing the group's biogeographic patterns. For free-living protists, most of the recent data lend a tentative support for the view favoring their global distribution (Fenchel and Finlay, 2004; Epstein and Lopez-Garcia, 2008). The situation with parasitic organisms might be different due strict adherence of some parasites to their hosts and a narrow biogeographic range of the hosts. The limited distribution scenario, for instance, is apparently prevailing for the dixenous species with complex life cycles and specific vector-parasite interactions, such as the pathogenic species of *Leishmania* or *Trypanosoma* (Stevens et al., 2001; Lukeš et al., 2007; Waki et al., 2007). However, the factors limiting the distribution of the dixenous parasites might not be so strong for the monoxenous species. Indeed, this conclusion is derived from the observed cases of loose specificity of host–parasite associations, discussed above. Thus, a *Blastocrithidia* represented by Ch**1** is found in three host families (Alydidae, Geocoridae, Pentatomidae) in China and the matching TU***44*** is found in Coreidae in Neotropics. Apparently, this is an example of a parasite species with a low host specificity that achieves a wide geographic distribution by colonizing a broad variety of hosts.

Another mechanism to achieve a global distribution, even for parasites with relatively narrow host specificity, is through the global host distribution. An example of this kind is TU Ch**14** (= Neotropical TU**6/7**). This species has been found only in the family Alydidae, however, all four isolates of it were derived from different species of this family, indicating that this parasite achieves a broad distribution by crossing the host species boundaries within a globally distributed host family. Another example of this kind is Ch**11** (= Neotropical TU***1*** and *L. pyrrhocoris* from Europe). This species is predominantly found in members of the genus *Dysdercus* (Pyrrhocoridae) and therefore displays a relatively high level of host specificity. Nonetheless, *Dysdercus* spp. are very broadly distributed, and so is their parasite.

The question of endemism is more difficult to assess, especially at this early stage of a global survey. It is obvious that a parasite species may appear to be endemic for a certain region only due to an insufficient sampling in other regions. From a general standpoint, the conditions for endemism include a relatively new emergence of the parasite. With respect to the recently evolved parasites, it is remarkable that the *Blastocrithidia* species from Ch**7**, represented in this work by multiple isolates and therefore quite common in the sampled areas in China, have never been observed in the Neotropics. This organism demonstrates a rather broad host range (found in four different host families) and hence its apparent absence in the Neotropics is likely due to the insufficient time for its dispersal. This conclusion is consistent with a relatively short branches of the Ch**7** lineage (Fig. 2) indicating a recent divergence from the closely related Neotropical species TU***30***. On the other hand, for long established parasites, the conditions for endemism include a high level of host specificity and a limited host distribution range. Apparently, there must be parasites and hosts satisfying these conditions, but further work is still needed to demonstrate the existence of endemic host–parasite associations. Yet, it appears that a large number of genetically unique isolates were identified among the typing units both in China and the Neotropics, with a potential for endemism. At present one of the best candidates is a *Blastocrithidia* from Ch**6**. This trypanosomatid was found with high prevalence in one species of pentatomids and also in the predatory reduviids, both in the same locality, and the Ch**6** cluster (Fig. 2) is well separated from other members of the large *Blastocrithidia* clade.

The close sister-group relationships between the species from China and the Neotropics are quite commonly observed in Fig. 2. In addition to the Ch**7**/TU***30*** pair mentioned earlier, we notice the Ch**2**/TU***9***–***11***, Ch**9**/TU***18***, Ch**10**/*L. podlipaevi* closely related pairs. These appear to be the cases of allopatric speciation, which is due to existing geographic barriers. The exact timing and driving force of these divergences is of interest and may be related to the factors defining the diversification of pathogenic *Leishmania*.

This study is among the few that address the localization of a large set of monoxenous trypanosomatids in their insect hosts. Infections in the gut can be best explained by contaminative transmission, which is indeed generally considered to be the prevalent route in cycling of these parasites (Wallace, 1966; Tieszen and Molyneux, 1989). However, along with the previous works (Sbravate et al., 1989; Godoi et al., 2002) we have also found a few cases, in which the flagellates were confined to the Malpighian tubes. Finding the same TUs in various heteropteran families including Reduviidae and Gerridae (Ch**6** and Ch**7**) imply alternative transmission routes, in particular possible transfer to another host by feeding or predatory behavior.

Among dozens of monoxenous trypanosomatids, we have found one isolate that, based on the phylogenetic analysis, clearly belongs to the dixenous genus *Phytomonas*. To our knowledge, this is the first documentation of this potential plant pathogen from China. The found isolate is a new species that forms a rather long branch interspersed between the *Phytomonas* strains Hart1 and EM1 responsible for the "hartrot" disease of coconuts and "marchitez sorpresiva" of oil palm, known to cause economic losses in South and Central America (Dollet, 2001). In the case of *Phytomonas* sp. from our dataset, the host plant remains unknown.

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