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Cosmopolitan Distribution of a Trypanosomatid *Leptomonas pyrrhocoris*

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A trypanosomatid species, designated as Typing Unit 1 (TU*1*) by sequences of SL RNA gene repeats, has been found in the intestine of pyrrhocorids (Insecta: Heteroptera) in Europe, Mediterranean, Central America and some parts of Asia and Africa. Phylogenetic analysis of the SL repeat sequences has shown that the isolates group in the tree according to their geographic origin. The maximal sequence divergence was observed in parasites from Neotropics suggesting the origin within and subsequent migrations from this region. The global distribution of the parasite could have been facilitated by ubiquity of its hosts that include several genera of the family Pyrrhocoridae. In Europe the TU*1* flagellates frequently occur in *Pyrrhocoris apterus*, the host of *Leptomonas pyrrhocoris* Zotta, 1912, a species that had been insufficiently defined by host and light microscopy level morphology. Herein, the Zotta's species description has been amended to include the TU*1* SL RNA repeat, SSU rRNA, glycosomal GAPDH gene sequences, as well as ultrastructure. In addition, *Leptomonas scantii* n. sp. with an overlapping host range has been described. Moreover, 10 typing units of trypanosomatids found in the pyrrhocorid hosts demonstrate the extent of variability of trypanosomatids occurring in one host family.

Key words: Genotyping; *Leptomonas*; phylogeography; Pyrrhocoridae; Trypanosomatidae.

Introduction

The distinctive aspect of ecology of parasitic organisms, as compared to free-living ones, is that they dwell in a restricted type of 'environment' – the bodies of their macroscopic hosts. The bonding of parasites to a certain host defines the boundaries for the geographic distribution of parasites which, barring host switching, cannot extend beyond the host range. At the same time, mobility of hosts provides the means for dispersal and homogenization within the range. With respect to dixenous Trypanosomatidae with high specificity of parasite-vector associations these notions are illustrated by restriction of *Trypanosoma brucei* to the "*Glossina* belt" (Balmer et al. 2011; Brun et al. 2010; Krafsur 2009), *Trypanosoma cruzi* to the region occupied by Triatominae bugs (Coura et al. 2002; Sturm and

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Campbell 2010), and *Leishmania* species to the areas populated by appropriate species of phlebotomine sand flies (Lukeš et al. 2007; Sacks and Kamhawi 2001; Zemanová et al. 2004).

However, numerous trypanosomatids are monoxenous parasites of insects that are transmitted by contamination, predation, coprophagy and necrophagy (Wallace 1966). It can be anticipated that their dependence on specific host species is more relaxed compared to dixenous species that undergo a complex set of transformations (life cycle stages) within a vector in order to multiply and mature into an infective stage. Consequently, host switching is more likely to occur in monoxenous species since the adaptation mechanisms are simpler. Indeed, the occurrence of the same organism (defined in terms of specific genotypic traits) in hosts from different insect families was documented. Would the relaxed host specificity provide an efficient mechanism for global dispersal and homogenization, especially since the hosts themselves are mobile insects? And with the more efficient dispersal would the distribution of such organisms more resemble that of ubiquitous free-living protists (Fenchel and Finlay 2004; Finlay and Fenchel 2004) rather than that of dixenous flagellates? These questions are among those that we have been asking during the ongoing survey of trypanosomatids in Hemiptera (Heteroptera) in several areas of the Neotropics, as well as in Europe, Asia and Africa.

Our approach to genotyping and molecular barcoding of trypanosomatids is based on analysis of Spliced Leader RNA (SL RNA) gene repeats amplified from intestinal material of infected hosts (Maslov et al. 2007; Westenberger et al. 2004). In the trypanosomatid genome, SL RNA gene repeats form clusters composed of 150-200 tandemly arranged copies. Besides a conserved SL RNA mini-exon and more variable mini-intron, each repeat includes a fast-evolving intergenic region (Murthy et al. 1992). The intergenic region sequences are nearly identical within the same trypanosomatid species (or typing unit), but they are highly variable in size and sequence among different species (Thomas et al. 2005). Typing units are delineated using a 90% identity threshold level determined with pairwise comparisons of full-length repeats (Maslov et al. 2007).

We discovered a globally distributed trypanosomatid species, designated Typing Unit ***1*** (TU***1***) that invariably displays promastigote morphology and dwells in several host species from the family Pyrrhocoridae (Maslov et al. 2007). Up to now the only named species from this host group is *Leptomonas pyrrhocoris* (Zotta 1912, 1921) described in *Pyrrhocoris apterus* from Romania. Since only the basic morphology of this organism had been studied, and that analysis had been performed at the light microscopy level, it is nearly impossible to relate new isolates to the old species. We, therefore, re-describe this species using molecular criteria, as well as morphology.

The more recent analyses, including those presented in this work, show that this trypanosomatid species have a somewhat loose host-specificity, being found in *P. apterus, Pyrrhocoris marginatus* and *Scantius aegyptius* in the temperate and subtropical zones of Europe and Mediterranean basin and in various species of the genus *Dysdercus* elsewhere (Maslov et al. 2007; Votýpka et al. 2010). Moreover, in this work we have found that this cosmopolitan species shows a genotypic variation that correlates with its geographical distribution, suggesting independent evolution in different regions (vicariance) instead of global homogenization through multiple migrations. This finding is in line with the moderate endemism model proposed for terrestrial free-living protists (Foissner 2006). Although the sample size in some study areas was limited, the highest divergence was observed in the Neotropics, suggesting that this region was the geographical origin of this species.

In addition to *L. pyrrhocoris*, eleven trypanosomatid typing units belonging to rather diverse evolutionary lineages were found in Pyrrhocoridae hosts, including a new species (*L. scantii* n. sp.) that became available in culture and is also described herein.

Results

Trypanosomatids in Natural Populations of Pyrrhocoridae

Trypanosomatids are frequently found in the intestinal tract of pyrrhocorid hosts. In Europe, natural populations as well as reared colonies of a common host species *P. apterus* (firebug) are usually infected. The crowdedness of bug populations and propensity of this flightless species for cannibalism and coprophagy are likely to facilitate the spread of trypanosomatid infections. In the Neotropics, the populations of various species of the genus *Dysdercus* are often infected as well. These hosts are flight-capable, yet they usually form high density populations on the ground or vegetation and the nymphs often form large aggregations. The summary of the sampling data is presented in Table 1.

Table 1. Trypanosomatid isolates and cultures obtained from Pyrrhocoridae.

Region	Host species	Intestinal DNA samples	Axenic cultures	Trypanosomatid species or Typing Unit	Infection frequency (infected/total)
Czech Rep. (Bohemia)	*Pyrrhocoris apterus*	H10–11, H18	H10, H11	*L. pyrrhocoris* (TU***1***)	6/21
		H21	N/A	TU***74***	1/21
Czech Rep. (Moravia)	*Pyrrhocoris marginatus*	P59–62, P64	P59	*L. pyrrhocoris* (TU***1***)	6/24
		P63	N/A	TU***44*** (= Ch**1**)	1/24
Slovakia	*Pyrrhocoris apterus*	SK01, SK03–10	SK01	*L. pyrrhocoris* (TU***1***)	4/11
		SK01–10	N/A	TU***59*** (= Ch**2**)	10/11
France	*Pyrrhocoris apterus*	F19, F20, F165	F19, F165	*L. pyrrhocoris* (TU***1***)	3/14
		F115	N/A	TU***73***	1/14
	Scantius aegyptius	F220–222	F221, F222	*L. scantii* (TU***71***)	3/13
Cyprus	*Pyrrhocoris apterus*	K10–K16	N/A	*L. pyrrhocoris* (TU***1***)	6/13
		K09, K12–K16	K12	*L. scantii* (TU***71***)	5/13
	Scantius aegyptius	K01–K03, K05–K08	K06	*L. pyrrhocoris* (TU***1***)	N/D
		K01–K05	K01	*L. scantii* (TU***71***)	N/D
Tunisia	*Scantius aegyptius*	Tun01	Tun01	*L. scantii* (TU***71***)	1/1
Ghana	*Dysdercus fasciatus*	G03–08, G58–59	G07, G58–59	*L. pyrrhocoris* (TU***1***)	8/9
		G07	N/A	TU***70***	1/9
		G14	N/A	TU***72***	1/9
Ecuador	*Dysdercus obscuratus flavopenius*	121–126AL	121AL–123AL, 125AL, 126AL	*L. pyrrhocoris* (TU***1***)	6/7
		124AL	124AL	TU***58***	1/7
Costa Rica	*Dysdercus obliquus*	10VL, 12VL, 57VL	10VL, 12VL, 57VL	*L. pyrrhocoris* (TU***1***)	3/6
	Dysdercus lunulatus	13BT, 14BT	14BT	*L. pyrrhocoris* (TU***1***)	2/4
	Dysdercus sp. (nymph)	25–32EC	25EC–32EC	*L. pyrrhocoris* (TU***1***)	9/20
	Dysdercus flavolimbatus flavolimbatus	79MV, 80MV	N/A	*L. pyrrhocoris* (TU***1***)	2/2
		80MV	N/A	TU***34***	1/2
	Dysdercus chriquinis	156–169CRT	N/A	N/D	14/30
	Dysdercus obscuratus flavopenius	322–326RV	324RV–326RV	*L. pyrrhocoris* (TU***1***)	5/18
	Dysdercus mimulis	329MV	329MV	*L. pyrrhocoris* (TU***1***)	1/17
	Dysdercus bimaculatus	330MV	N/A	TU***34***	1/1
China	*Dysdercus poecilus*	278JI2, 282JI2	278JI2, 282JI2	*L. pyrrhocoris* (Ch**11**)	5/14
	Melamphaus faber	391JI1	N/A	TU***66*** (= Ch**10**)	2/14
		392JI1	N/A	TU***61*** (= Ch**4**)	3/14

Eight out of ten investigated populations of Pyrrhocoridae in Costa Rica and one out of two populations in Ecuador were found to be infected with trypanosomatids. In China two out of three investigated populations were positive and in Ghana both studied populations were infected. The occurrence frequencies of infected hosts within their populations varied from a single specimen up to more than a half of the tested individuals. Distribution of the parasite in European host species (*P. apterus, P. marginatus* and *S. aegyptius*) is much broader and all investigated populations (five in the Czech Republic and Slovakia, three in France and two in Cyprus) were infected. Prevalence of infection in adult bugs in different populations varied between 65 to 100%. Because the firebug *P. apterus* is a very popular laboratory model, we investigated also several laboratory colonies of the bug originating from independent sources. All studied populations were found to be infected by *L. pyrrhocoris* (data not shown). We conclude that trypanosomatid infections are widespread in natural populations of pyrrhocorids and at least most of the species encountered are susceptible.

The intestinal DNA samples from infected hosts were used to amplify SL RNA gene repeats, the sizes of which were shown to be species-specific and useful for the initial identification of the flagellates (Maslov et al. 2007; Westenberger et al. 2004). An example of this analysis is shown in Figure 1A. The gel shows the presence of several different products among the amplified SL RNA repeats, including a ~1.1 kb DNA fragment (testifying to the presence of *L. pyrrhocoris*, see below) amplified from most isolates, as well as the bands of 0.5 kb in isolates K12 and K14 and the 0.8 kb band in isolates SK02. The 1.1 and 0.5 kb products were amplified from the sample K14. As was shown previously, each type of the PCR products represents a typing unit (molecular species) of trypanosomatids (Maslov et al. 2007; Westenberger et al. 2004). The example, therefore, illustrates the presence of one species in the samples K10, G04, SK01 (SL RNA repeat size of 1.1 kb), of another in K12 (0.5 kb repeat), a third in SK02 (0.8 kb repeat), and a mixed infection in the samples K14 and G07. This assessment was further verified by gel-isolation of the bands of different sizes and their cloning and sequencing. Overall, the molecular analyses of pyrrhocorid parasites, including the new intestinal samples described herein as well as those published previously (Maslov et al. 2007; Votýpka et al. 2010; Westenberger et al. 2004), included 27 samples from continental Europe (Czech Republic, Slovakia and France),

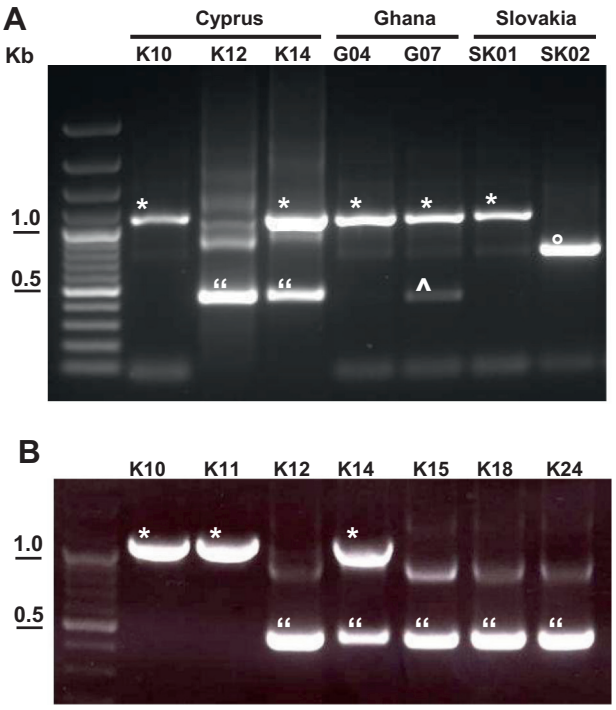

Figure 1. PCR amplification of Spliced Leader RNA gene repeats from a panel of selected intestinal DNA samples (**A**) and from axenic cultures (**B**). The geographic origin and designation of isolates are shown above the lanes in **A**; all cultures in **B** were from Cyprus. Individual bands of PCR products were cloned, sequenced and compared by multiple sequence alignment with the database of reference sequences representing the previously defined typing units. Based on this analysis the PCR products marked with asterisks were determined to represent TU***1*** (= *L. pyrrhocoris*) and those with quotation mark represent *L. scantii* sp. nov. The product marked with a small circle represents TU***59*** (= Ch2) and that marked with a caret represents TU***70***. Lower intensity 1 kb and larger bands in isolate K12 (Fig. 1A) and in isolates K12, K15, K18 and K24 (Fig. 1B) represent dimers of the monomeric 0.5 kb repeat. GeneRulerTM 100 bp DNA Ladder Plus (Fermentas) is shown to the left of each panel.

17 from the Mediterranean (Cyprus and Tunisia), 9 from Ghana, 4 from China and 44 from the Neotropics (Costa Rica and Ecuador).

To facilitate the comparison of the sequences to the database of previously determined SL RNA gene repeats, only the most conserved regions were used for multiple sequence alignment followed by clustering of the sequences with neighbor-joining (NJ) (Westenberger et al. 2004). It needs to be stressed that SL repeats from distantly related trypanosomatids still could not be aligned

unambiguously; therefore, the main and only purpose of this analysis was to identify groups of closely related sequences, which aligned reliably and appeared as terminal clusters on the NJ dendrogram. This was followed by pairwise comparisons of the respective full length repeats and the 90% identity applied to determine if the respective trypanosomatids represent the same or different typing units (molecular species). Based on these analyses (Supplementary Fig. S1), the sequences found in Pyrrhocoridae were categorized into several typing units. The most notable of them was the previously established TU***1*** (= *L. pyrrhocoris*, see also below) that corresponds to the 1.1 kb PCR product illustrated in Figure 1A. The TU***1*** trypanosomatids were the most frequently occurring flagellates in the infected host populations including Europe, Asia and Africa, along with the previous finding of this group in Central and South America and China. The other PCR products represented different additional typing units (including some new ones), which are described below.

Cultivation of trypanosomatids from infected hosts was successful in most cases attempted. Judging by the sizes of the SL RNA gene products, most of the obtained axenic cultures faithfully represented the initial infections. These analyses are illustrated by comparison of Figure 1B, which shows cultures derived from the infected pyrrhocorids found in Cyprus, to respective intestinal samples in Figure 1A: the K10 culture shows the same 1.1 kb band as the intestinal sample K10 (both of them representing TU***1***), the K12 culture shows the 0.5 kb band same as the intestinal sample K12 (TU***71***), and the K14 culture shows two bands (a mix of TU***1*** and TU***71***). In a few notable exceptions, organisms in the obtained cultures represented a trypanosomatid species that differed from those in natural infections. Such cases are further addressed below. The axenic cultures obtained are listed in Table 1.

Phylogenetic Position of *L. pyrrhocoris*

In order to evaluate phylogenetic relationships of trypanosomatids from pyrrhocorids with other members of the family Trypanosomatidae, the small subunit ribosomal RNA (SSU rRNA) and glycosomal glyceraldehyde phosphate dehydrogenase (gGAPDH) genes were amplified from all obtained axenic cultures of TU***1***. Each individual gene sequence was nearly identical (uncorrected *p*-distances were less than 1%) to its counterparts from other TU***1*** cultures. Therefore, a single sequence of each gene from the sample H10 was chosen to represent the entire group. The SSU rRNA and gGAPDH sequences were concatenated and used to infer the phylogenetic tree shown in Figure 2. The analysis showed that TU***1*** trypanosomatids represent a member of the "SE" clade, which also includes numerous monoxenous species, as well as the dixenous genus *Leishmania* (Hollar et al. 1998; Merzlyak et al. 2001). The position of the TU***1*** trypanosomatids matched that of the organism previously isolated from *P. apterus* in Southern Russia and (somewhat arbitrarily) referred to as "*Leptomonas pyrrhocoris*" (Frolov 1987; Yurchenko et al. 2006a). This, along with the match of the SL RNA sequences (Supplementary Fig. S1), indicates that TU***1*** and "*L. pyrrhocoris*" are the same species. As shown by the tree, this organism is closely related to several monoxenous species ascribed to the genus *Leptomonas*. Since the identity of the original species *L. pyrrhocoris* Zotta 1912 remains obscure (see above), and it is likely that it would match the ubiquitous TU***1***, we re-describe *L. pyrrhocoris* using a TU***1*** (isolate H10) as hapantotype.

Since deep branching order is particularly poorly resolved with the dataset used and since the main purpose of this analysis is to ascertain the identity of the analyzed trypanosomatid isolates rather than investigate the family's phylogeny, the general tree topology is not discussed herein. It is only worth mentioning that a similar branching order was observed previously with the gGAPDH-based analyses (Hamilton et al. 2004; Yurchenko et al. 2006a).

Regional Intrapopulation Variability of *L. pyrrhocoris*

Because of the global dispersal of *L. pyrrhocoris*, our next goal was to investigate the existence of biogeographic patterns in its distribution. As the first step, a regional variability was investigated by RAPD using genomic DNA extracted from cells in the axenic cultures. The RAPD patterns obtained for the group of European isolates from three species of pyrrhocorid bugs (*P. apterus, P. marginatus* and *S. aegyptius*; Table 1) were almost identical (Fig. 3 A, B), testifying to the high level of genetic homogeneity of *L. pyrrhocoris* in this region. On the other hand, a comparatively higher diversity of RAPD patterns was observed among the isolates from the Costarican Heteroptera (Fig. 3B).

Biogeographic Patterns of *L. pyrrhocoris*

Variability of the *L. pyrrhocoris* isolates in relation to their geographic origin was further addressed

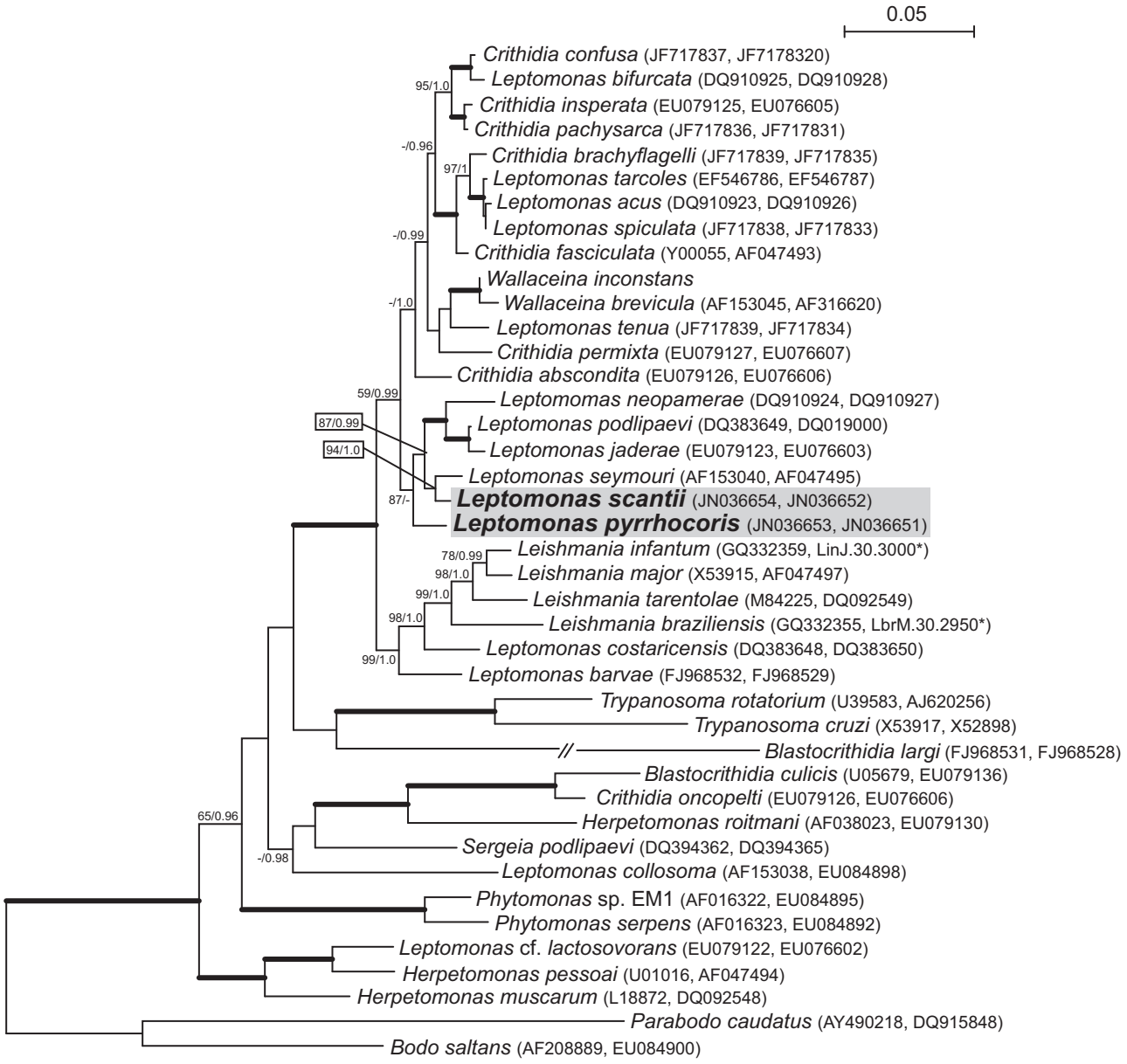

Figure 2. Maximum likelihood tree of Trypanosomatidae inferred from the SSU rRNA + gGAPDH concatenated gene dataset with emphasis on positions of *Leptomonas pyrrhocoris* sp. emend. and *Leptomonas scantii* n. sp. The best tree shown (with *Ln*-likelihood of -19158.366335) was derived using a heuristic search under the GTP + Γ model with α-shape parameter of 0.306768. The bootstrap values and BI posterior probabilities are shown at select nodes. Dashes indicate that the clades were not recovered or support was poor. The thick branches of the tree indicate 100/1.0 support. The tree was rooted using the sequences of *Bodo saltans* and *Parabodo caudatus*. The SSU rRNA and gGAPDH sequences of *L. pyrrhocoris* H10 determined in this work were deposited under the GenBank™ accession numbers JN036653 and JN036651, and those of *L. scantii* F221 were JN036654 and JN036652, respectively. The accession numbers of the reference SSU rRNA and gGAPDH gene sequences (respectively) were retrieved from GenBank™. The scale bar denotes the number of substitutions per site. The extremely long branch of *B. largi* was arbitrarily shortened.

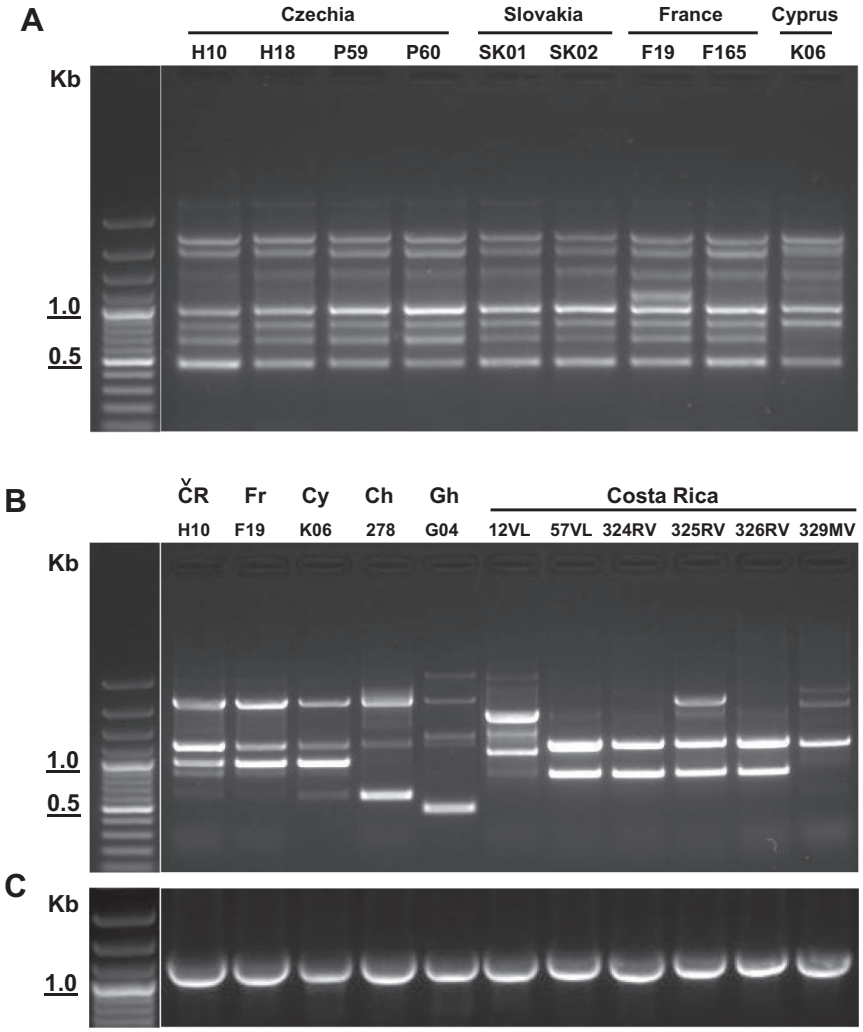

Figure 3. RAPD analysis of selected *L. pyrrhocoris* isolates available in culture. Primers used for the analysis were OPE12 (**A**) or OPE5 (**B**). The bottom panel (**C**) shows PCR amplification of SL repeats from the axenic cultures used for the RAPD. The origin of isolates is shown above the panels: ČR – Czech Republic, Ch – South-West China, Cy – Cyprus, Gh – Ghana, Fr – France.

by the analysis of the complete SL RNA gene sequences isolated from the field samples and cultures, in combination with the TU***1*** sequences determined in the previous works (Maslov et al. 2007; Votýpka et al. 2010; Westenberger et al. 2004). Pairwise comparisons of individual SL RNA repeats have revealed a variable degree of sequence identity with the lowest (93.8%) level observed between the intestinal sample 80MV-int2 and the culture sample 14BT-cul3, originating from two *Dysdercus* species in Costa Rica. Remarkably, that level was lower than the levels observed between Costa Rican and most of non-Neotropical samples, indicating a high heterogeneity within the former. The existence of a biogeographic divergence pattern was then investigated phylogenetically. The high levels of sequence similarity allowed for an unambiguous alignment of the entire repeats excluding a few positions with gaps. The multiple sequence alignment was then used to infer an unrooted phylogenetic tree (Fig. 4). A remarkable feature of the tree is clustering of the Old World (Eurasia and Africa) in relation to their geographical origin. Thus, the isolates from the temperate zone of Europe formed a separate highly supported group. Similarly, the groups of the Mediterranean, Chinese, and African isolates were separated from each other and the temperate European clade.

On the other hand, the Neotropical isolates formed a rather unstructured assembly in spite of

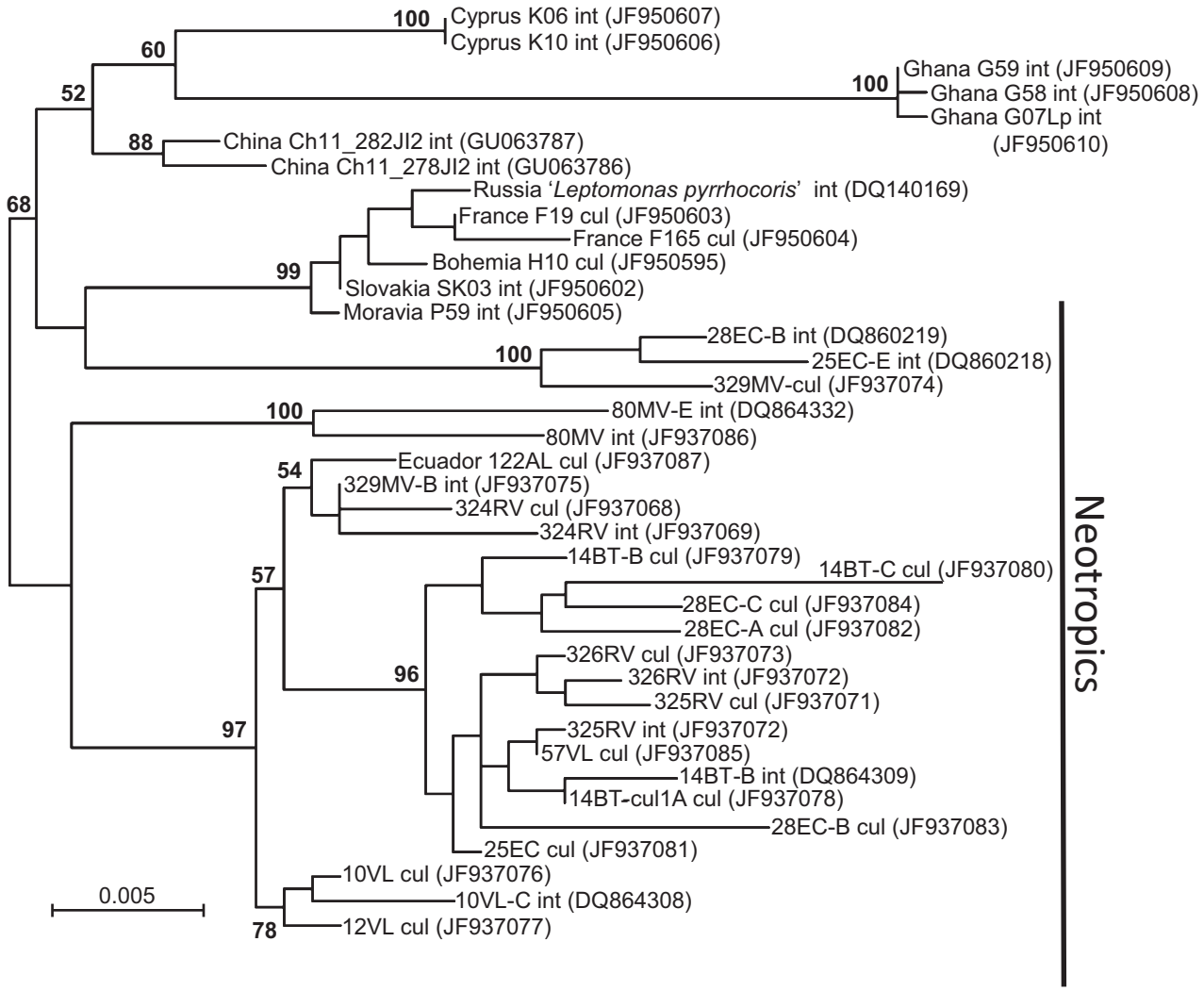

Figure 4. Biogeographic pattern of the intraspecies variability of *L. pyrrhocoris* lineages. The best maximum likelihood tree of full-length SL RNA repeats shown (*Ln*-likelihood of -3036.454069) was derived using a heuristic search under the GTP + Γ model with α-shape parameter of 0.063833. Bootstrap support values are shown at select nodes. The tree is unrooted. The bar represents number of substitutions per site. The sequences determined in this work were deposited in GenBank™ with the accession numbers listed below according to their geographic origin (country), followed by the isolate code and indication whether the sequence was obtained from an intestinal sample (int) or from axenic culture (cul). Bohemia (Czech Rep.) H10 cul (JF950595); Costa Rica 10VL cul (JF937076); Costa Rica 12VL cul (JF937077); Costa Rica 14BT-A cul (JF937078); Costa Rica 14BT-B cul (JF937079); Costa Rica 14BT-C cul (JF937080); Costa Rica 25EC cul (JF937081); Costa Rica 28EC-A cul (JF937082); Costa Rica 28EC-B cul (JF937083); Costa Rica 28EC-C cul (JF937084); Costa Rica 57VL cul (JF937085); Costa Rica 80MV int –(JF937086); Costa Rica 324RV cul (JF937068); Costa Rica 324RV int (JF937069); Costa Rica 325RV int (JF937070); Costa Rica 325RV cul (JF937071); Costa Rica 326RV int (JF937072); Costa Rica 326RV cul (JF937073); Costa Rica 329MV cul (JF937074); Costa Rica 329MV-B int (JF937075); Cyprus K06 int (JF950607); Cyprus K10 int (JF950606); Ecuador 122AL cul (JF937087); France F19 cul (JF950603); France F165 cul (JF950604); Ghana G07Lp int (JF950610); Ghana G58 int (JF950608); Ghana G59 int (JF950609); Moravia (Czech Rep.) P59 int (JF950605); Slovakia SK03 int (JF950602). In addition, several previously determined SL RNA repeat sequences, all derived from intestinal samples, were retrieved from the GenBank™.

the fact that most of them originate from a relatively small region – Costa Rica. In the presented tree these isolates appear as a paraphyletic group but in other analyses they were recovered as a monophyletic group that was a sister clade to the Old World assembly. The bootstrap support for either of these topologies was low. Moreover, the Costa Rican lineages are positioned in the tree regardless of their host and locale. This is especially well illustrated by the sequences obtained from the intestinal samples 25EC and 28EC and respective cultures: the two sequences (25EC-int and 28EC-int, along with the culture sequence 329MV-cul originating from a different host species and collection site; Table 1) were clustered together, while the corresponding culture sequences were scattered throughout the tree mingling with the sequences of other origins. The pairwise identity levels between individual sequences within the 25EC or 28EC groups were at the 95.2–95.7% levels, which was close to (or often lower than) the levels observed in most comparisons of Costa Rican and non-Neotropical samples (94.8–97.4%). Thus, the diversity of SL RNA repeats observed within a single Costa Rican host can be of the same magnitude as the diversity observed globally.

Other Trypanosomatid Species in Pyrrhocorid Hosts

In addition to *L. pyrrhocoris*, the natural populations of pyrrhocorid hosts were often found infected with other trypanosomatid species. Overall, eleven additional typing units detected in the pyrrhocorids worldwide represent the first detailed study of trypanosomatids in one host family and demonstrate broad spectrum of these parasites able to infect pyrrhocorid bugs. Two of these typing units were recovered in culture and one of them (TU***71***) is described below as a new species.

From *S. aegyptius* captured in southern France we obtained an isolate (F221) that according to the sequences of SL RNA repeats amplified from the host and from culture represented a separate typing unit (TU***71***; a candidate new species). In Cyprus, trypanosomatid from this species was found in *S. aegyptius* as well as in *P. apterus*, and in Tunisia it was found in *S. aegyptius* (Fig. 1B and Table 1). As shown in Figure 1A and B, this species (with SL repeats of 0.5 kb) and *L. pyrrhocoris* (with 1.1 kb repeats) were found infecting the same populations of *P. apterus* and *S. aegyptius* including co-inhabiting individual hosts. This organism was easily cultivable and the SSU rRNA and gGAPDH sequences were obtained from its cultures. Phylogenetic analysis of the concatenated dataset (Fig. 2) confirmed that it represented a new species, which is closely related to several other *Leptomonas* species and is described below under the name *Leptomonas scantii* sp. nov.

In Ecuador a population of *Dysdercus obscuratus* was found that was infected with *L. pyrrhocoris* (Table 1) (Maslov et al. 2007). Surprisingly, axenic culture 124AL did not match *L. pyrrhocoris* but represented a separate typing unit, TU***58***. This organism apparently represented a minor component of the trypanosomatid infection of the original host that was dominated by *L. pyrrhocoris*. Two additional typing units, TU***74*** and TU***73***, were found in European pyrrhocorid populations, TU***59*** (=Ch**2**) was encountered in Europe and earlier in China, TU***44*** (=Ch**1**) was common to Europe, China and Costa Rica, and, finally, TU***70*** and TU***72*** were confined to Ghana. Unfortunately, the corresponding organisms could not be recovered in culture.

Besides those, typing units TU***34*** was found in a Central American species of *Dysdercus* (Table 1) (Maslov et al. 2007; Westenberger et al. 2004). TU***34*** is a member of the *Blastocrithidia* clade that includes *B. triatoma*. Two additional typing units were reported in pyrrhocorids from China (Votýpka et al. 2010). One of them, TU***61***(=Ch**4**), is a *Blastocrithidia* and the other, TU***66***(=Ch**10**), is a member of the "SE" clade. Thus, the total of twelve species of trypanosomatid parasites with broad phylogenetic affinities has been discovered in Pyrrhocoridae so far. However, unlike *L. pyrrhocoris* that appears to be limited to this family, some of the other organisms have a broader species range: the trypanosomatids that constitute TU***44*** (=Ch**1**) were also found in Alydidae, Geocoridae, Pentatomidae from China (Ch**1**) and in Coreidae from Costa Rica (TU***44***); TU***59*** (=Ch**2**) is found in Gerridae and Reduviidae in China; and TU***34*** is also present in Costa Rican Largidae (Maslov et al. 2007; Votýpka et al. 2010).

Morphological Characterization of *L. pyrrhocoris* in Hosts and Culture

The original description of *L. pyrrocoris* by Zotta (1912) included only a light microscopy examination of parasites found in *P. apterus* in Romania. The organism was originally ascribed to the genus *Herpetomonas* but was later renamed as *Leptomonas* (Zotta 1921). Several authors subsequently referred to *L. pyrrhocoris* in various parts of Europe as typical promastigotes, 12–17 μm in length, typically with a twice-as-long flagellum (Wallace 1966). However, since a positive identification

of trypanosomatids by morphometry alone is unreliable, the true identity of the trypanosomatid species described by Zotta remains unknown. We have shown in this work that the isolates from *P. apterus* and *P. marginatus* of the Eastern (Russia), Central (Bohemia, Moravia, Slovakia), Western (France) and Southern (Cyprus) European origin are phylogenetically closely related to each other on one hand, and well separated from other trypanosomatids on the other. It is highly likely that this ubiquitous organism does match the species described by Zotta. We, therefore, re-describe *L. pyrrhocoris* using the European isolate H10 from Prague (Czech Republic) as the type.

Cells of *L. pyrrhocoris* are invariably promastigotes in host and culture (Fig. 5). Cell dimensions in the host were relatively uniform, while in culture a highly heterogeneous cell population was observed, varying from oval cells of $7.3 \pm 2.6\,\mu m$ in length (with the lower boundary $3.3\,\mu m$) with an equally short flagellum, to long slender promastigotes of $13.1 \pm 2.1\,\mu m$ in length (with the upper boundary $24.6\,\mu m$) with a long flagellum (Fig. 5A-D).

All features observed by transmission (Fig. 5E-G) and scanning electron microscopy (Fig. 5H and I) are characteristic for all trypanosomatids, such as single reticulated mitochondrion on the periphery of the cell, an oval nucleus, glycosomes, acidocalcisomes, the rod-shaped kinetoplast disc, as well as the missing paraflagellar rod within the flagellar pocket. Longitudinal sections of the prolonged promastigotes reveal a deep flagellar pocket. Remarkably, the basal body of the flagellum and the closely associated kinetoplast disc are not positioned at the very bottom of the flagellar pocket, as commonly seen in promastigotes, but near its side wall at some distance off the bottom.

It should be mentioned that other investigated cultures (F19, H18, KYPR06, 10VL and 14BT) showed high variability of cell dimensions and flagellum length ($p < 0.001$, one-way ANOVA) with no clear correlation to their geographic origin (data not shown). An analysis of the intraspecies morphological variability will be presented elsewhere.

Morphology and Ultrastructure of *L. scantii* n. sp. Cells in Host and Culture

The majority of cells in gut smears were long slender promastigotes with some cells being oval shaped (Fig. 6A-C). Morphology in culture was rather diverse including a continuum of sizes and lengths from typical slender promastigotes (Fig. 6A) to cells of oval shapes (Fig. 6I) and even amastigotes (data not shown).

The slender to pear-shape promastigotes have a deep flagellar pocket with a rather thin kinetoplast disk located at its bottom (Fig. 6D) and positioned between the basal body of the flagellum and the oval nucleus. Although the cell shape varied substantially, most of them had furrows on the surface that became prominent towards the anterior end of the cell (Fig. 6H-J). These furrows are well visible in cross-sectioned promastigotes (Fig. 6H), whence a full corset of subpellicular microtubules is also evident. Flagellum of this species is equipped with a rather small paraflagellar rod (Fig. 6F).

Diagnosis

Phylum Euglenozoa Cavalier-Smith, 1981
Class Kinetoplastea Honigberg, 1963, emend. Vickerman, 1976
Subclass Metakinetoplastina Vickerman, 2004
Order Trypanosomatida Kent, 1880, stat. nov. Hollande, 1952
Family Trypanosomatidae Doflein, 1951

Leptomonas pyrrhocoris (Zotta 1912) emend. Votýpka, Lukeš and Maslov

Generic assignment: This is done in accordance with the taxonomic system of Trypanosomatidae designed by Hoare and Wallace according to which the genus *Leptomonas* Kent 1880 is defined by the presence of a single morphotype (promastigote) and monoxenous (single invertebrate host) life cycle (Hoare and Wallace 1966; Wallace 1966).

Species diagnosis: The species is identified by molecular phylogenetic analysis of the SL RNA, SSU rRNA and gGAPDH gene sequences that are to cluster with the reference sequences from isolate H10. The GenBank™ accession numbers of the sequences from isolate H10 are JN036653 (SSU rRNA), JN036651 (gGAPDH) and JF950595 (SL RNA gene repeats). The intraspecies variability of 1062-1080 bp SL RNA gene repeat sequences has been observed, but repeats from different isolates did not show identity levels below 90%. Cell morphology is that of a typical promastigote with highly variable length, width and flagellum length, and no unique features. Kinetoplast is typically positioned off the bottom of the flagellar pocket along one of its walls, with the pocket extending further almost to the nucleus.

Type host: Intestine (midgut) of *Pyrrhocoris apterus* Linnaeus, 1758 (Heteroptera: Pyrrhocoridae).

Type locality: Prague (50° 04′ 05″ N, 14° 25′ 35″ W), Bohemia, the Czech Republic.

Additional hosts and localities: Intestine (midgut) and haemocoel of several species of Pyrrhocoridae (Insecta, Heteroptera) including *P. apterus, P. marginatus* and *S. aegyptius* in Europe and Mediterranean, *Dysdercus poecilius* in China, and several species of *Dysdercus* in the Neotropics.

Type material: The designated hapantotype is the axenic culture of isolate H10 deposited in the collection of the Department of Parasitology, Faculty of Science, Charles University, Prague, Czech Republic, and in the collection of the Czech

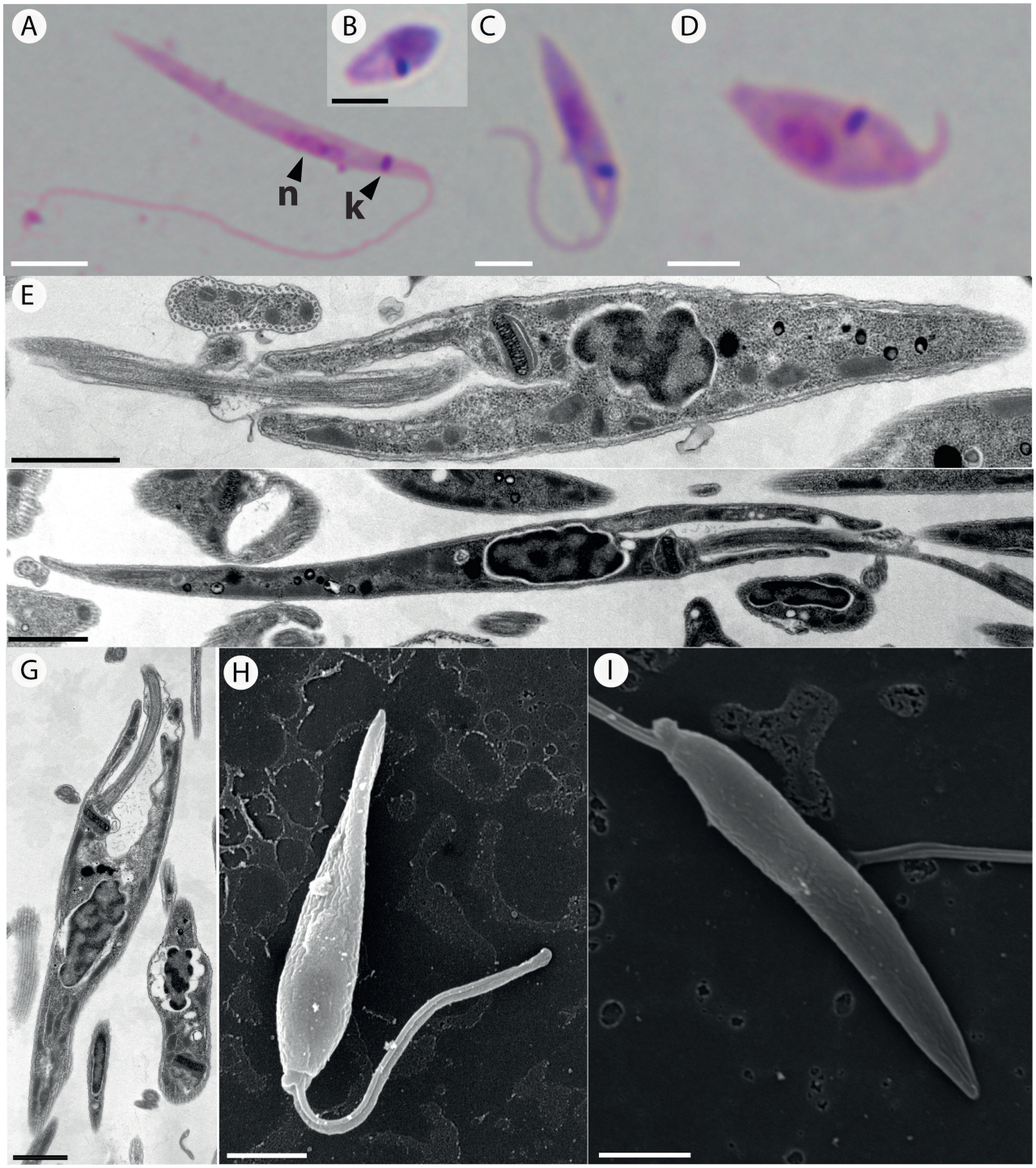

Figure 5. Morphology and ultrastructure of *Leptomonas pyrrhocoris* (Zotta 1912) emend. sp. Light microscopy images of Giemsa-stained culture form of cells (**A-D**) showing variability of cell shape and length from a slender promastigotes (**A**) with a long flagellum, nucleus (n) and kinetoplast (k) shown by arrowheads via intermediate size cells (**C** and **D**) to an oval shaped amastigote (**B**). Transmission electron microscopy (**E-G**) images of longitudinal sections of cells which show variability of cell width, a deep flagellar pocket and the kDNA disk located next to the basal body of the flagellum along the lateral wall of the flagellar pocket at some distance off its the bottom. Scanning electron microscopy (**H, I**) of typical promastigotes. Scale bar corresponds to 2 μm (**A** – **D**, **H**, **I**) and 500 nm (**E** – **G**).

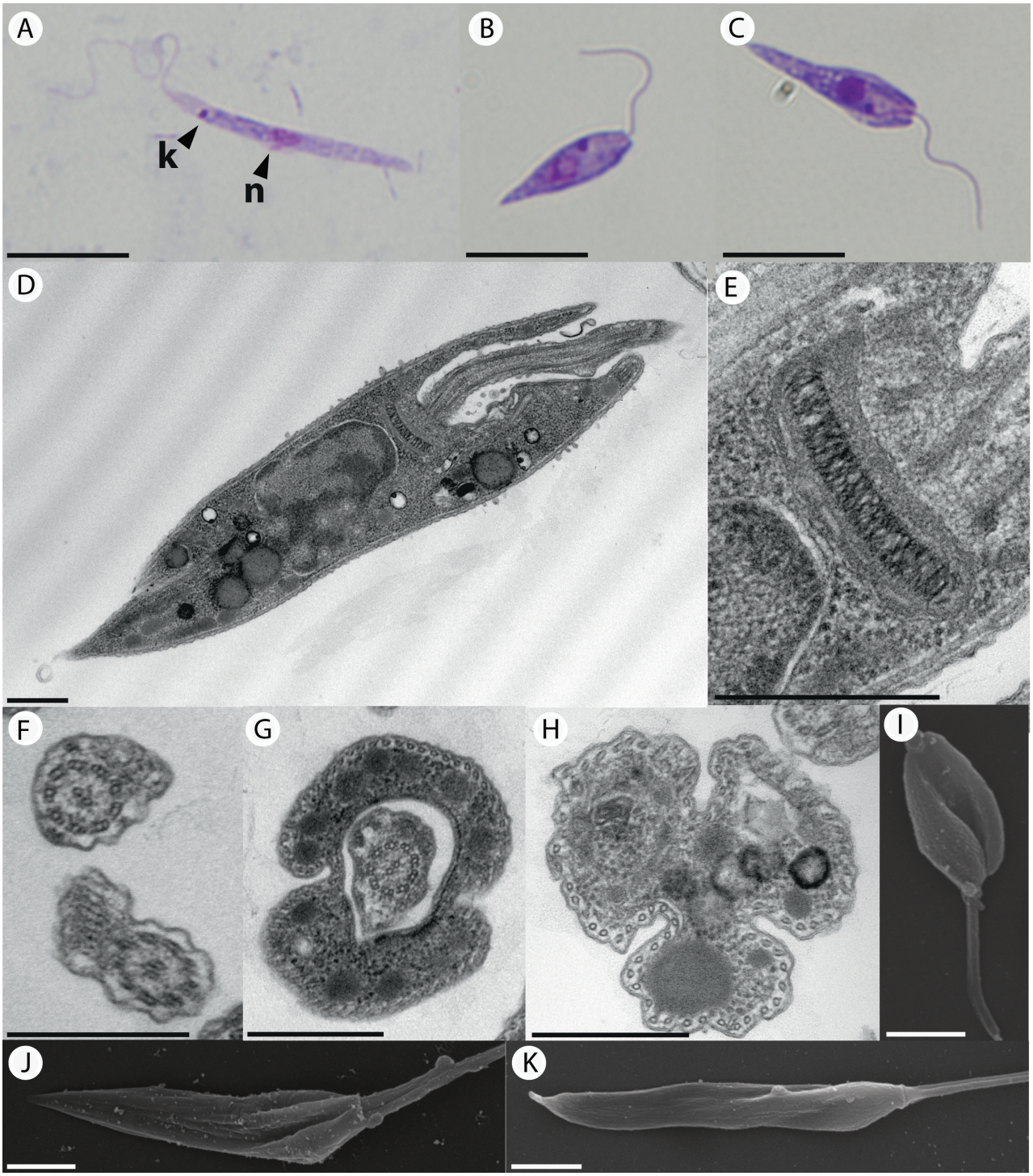

Figure 6. Morphology and ultrastructure of *Leptomonas scantii* n. sp. Giemsa-stained promastigotes (**A-C**) of various size and shape; nucleus (n) and kinetoplast (k) are shown in **A** with arrowheads. Transmission electron microscopy (**D-H**) images showing the longitudinally sectioned cell with a deep flagellar pocket (**D**), the kinetoplast DNA disk area (**E**), cross-section of the free flagellum with a prominent paraflagellar rod (**F**), cross-section of a flagellum inside the flagellar pocket (**G**) and mid-section of a cell with deep furrows (**H**), a typical feature of this species. Scanning electron microscopy images (**I-K**) of a short (**I**) and longer (**J** and **K**) promastigotes illustrate the existence of deep surface furrows and a slight longitudinal twist of the cells. Scale bar corresponds to 10 μm (**A** – **C**), 2 μm (**I** – **K**) and 250 nm (**D** – **H**).

Academy of Sciences, Biology Centre, Institute of Parasitology, České Budějovice, Czech Republic.

Etymology: The species name refers to the taxon of most frequent hosts occurring in Europe.

Leptomonas scantii n. sp. Votýpka, Lukeš and Maslov

Generic assignment: Based on predominance of promastigote-shape cells and presumed monoxenous life cycle the new species is assigned to the genus *Leptomonas* Kent 1880.

Species diagnosis: The new species, mainly defined by the SL RNA, SSU rRNA and gGAPDH gene sequences, belongs phylogenetically to a group of typical leptomonads, including *L. seymouri*, as well as *L. pyrrhocoris*, but occupying a distinct position within this clade. The GenBank™ accession numbers of the sequences are JN036654 (SSU rRNA), JN036652 (gGAPDH) and JN009104 (SL RNA gene repeat). Cells are typically promastigotes of variable shapes and sizes, with a few deep furrows on the surface.

Type host: Intestine (abdominal part of midgut) of *Scantius* (= *Lodosiana*) *aegyptius* Linnaeus, 1758 (Heteroptera: Pyrrhocoridae).

Type locality: In the vicinity of Sète (43° 28′ 37″ N, 03° 46′ 03″ W), southern France.

Additional hosts and localities: Intestine (abdominal part of midgut) of *S. aegyptius* in France, Cyprus and Tunisia and *P. apterus* (Insecta, Heteroptera) in Cyprus.

Type material: The designated hapantotype is the axenic culture of isolate F221 deposited in the collection of the Department of Parasitology, Faculty of Science, Charles University, Prague, Czech Republic, and in the collection of the Czech Academy of Sciences, Biology Centre, Institute of Parasitology, České Budějovice, Czech Republic.

Etymology: The species name was given after the host in which that species was discovered.

Discussion

In this work we have demonstrated the existence of a cosmopolitan monoxenous trypanosomatid species, *L. pyrrhocoris* that was found in Central and South America, various regions of Europe and Mediterranean, as well as in some locations in Africa and Asia. The coherence of this species has been demonstrated by several molecular markers (SL RNA, SSU rRNA and gGAPDH genes), as well as by RAPD. This flagellate has been found exclusively in members of the Pyrrhocoridae family (Insecta, Heteroptera) including the genera *Pyrrhocoris*, *Scantius* and *Dysdercus*. Apparently, the broad distribution of the hosts, in particular the genus *Dysdercus*, played a crucial role in the worldwide dissemination of the parasite. Another major factor behind the successful expansion of *L. pyrrhocoris* was a relaxed host-specificity of these parasites, which were able to cross the species and genus boundaries, yet remained restricted to a particular host family (Table 1). It is likely that additional investigations would expand the list of *L. pyrrhocoris* hosts: so far, only a few pyrrhocorid species tested have not been found to harbor infections and each of such cases can be explained by a relatively small sampling size (data not shown).

The strains of *L. pyrrhocoris* have been found to display the intraspecies variability pattern in SL RNA gene repeats that correlated with the geographic origin of these organisms. The isolates from different parts of the Old World grouped according to their origin and collectively were separated from the Neotropical isolates. The data also suggest that the observed vicariance pattern is primarily defined by geographic separation instead of host-driven divergence: thus, the isolate KYPR10 from *P. apterus* in Cyprus grouped together with its compatriot (isolate KYPR06) from *S. aegyptius* and not with the European isolates from *P. apterus* that clustered separately. The European clade also includes isolates from two *P. marginatus* populations. Additionally, the Costa Rican isolates from several species of *Dysdercus* are mingled within the Neotropical clade regardless of their host identity. These findings indicate that a distinct pool of parasites in each geographic region is shared by all suitable hosts inhabiting this region. This observation argues against the notion that the regional variability of *L. pyrrhocoris* lineages was driven by the diversity of their hosts and that leaves the geographic separation as the major factor that created the differences among the pools of parasites.

Yet, the variability observed within the group of Neotropical isolates, most of which originated from a relatively small biogeographic region (Costa Rica), was noticeably larger compared to the European and Mediterranean lineages collectively spanning much greater distances. The seemingly random mingling of individual repeats from various isolates (Fig. 4) suggests that the flagellates are freely exchanged between the co-inhabiting species. Even a population of parasites within a single infected specimen can be highly diverse, as exemplified by the intestinal samples 14BT, 25EC or 28EC with repeats scattered across the Neotropical clade. Barring the unusually high divergence rate in the Neotropics compared to the Old World, this might indicate an accumulation of diversity over a longer period of time in the Neotropics compared to the other parts of the world. This scenario implies the origin of *L. pyrrhocoris* in the Neotropics and the subsequent spread to other locations, in all likelihood facilitated by migrations of hosts or transmission of parasites to the adjacent populations of the same or a different *Dysdercus* species.

The details of this scenario, such as number and routes of these migrations, still need to be elucidated. Moreover, additional sampling in Asia and Africa is also required; these new data may validate or change our conclusions. At this stage it is certain only that sufficient time must have elapsed for the divergence to accumulate and the observed biogeographic pattern to emerge. These migrations were, therefore, ancient events, unlike the recently (in 2009) discovered invasion of *S. aegytius* in Southern California.

The global distribution of *L. pyrrhocoris* extends our previous observations on finding the same parasites in widely separated geographic regions (Maslov et al. 2007; Votýpka et al. 2010). Two mechanisms seem to promote such a global distribution. The case of *L. pyrrhocoris* described herein and the previously observed case of a trypanosomatid genotyped as TU***6***/***7***(=Ch**14**) from the heteropteran family Alydidae represent trypanosomatids with a clearly defined (albeit somewhat relaxed) host specificity that have achieved a broad distribution due to ubiquity of their host families. A different strategy to achieve a broad distribution is to further lower the host specificity. Apparently, this case is represented by the *Blastocrithidia* species genotyped as TU***44***(=Ch**1**) found in four heteropteran host families in the Neotropics and China (Maslov et al. 2007; Votýpka et al. 2010).

Thus, it appears that the primary factor defining the distribution of insect trypanosomatids is host specificity. The secondary factor is the distribution of the hosts themselves. As exemplified by *L. pyrrhocoris*, a parasite with a restricted host specificity still can achieve global distribution provided a wide occurrence of suitable hosts. The correlation of genotypes and large scale distribution pattern indicates that homogenization (e.g. by host migrations) is slower compared to the rates of divergence. Moreover, the existence of a region with a relatively high diversity of intraspecies lineages indicates a possible centre of the species origin. The mechanisms (routes) and the timing of the dispersal still represent open questions.

Methods

Field work and isolation of DNA from intestinal samples: Collecting of insects was performed in the following localities. The Bohemian isolates H10–11 and H18 were collected in October 2007 in Prague (50° 4′ 5″ N, 14° 25′ 35″ W), while the isolate H21 in November 2007 in Předboř (49° 45′ 53″ N, 15° 42′ 39″ W). The Moravian isolates (P59–64) were collected in May 2009 in Sedlec u Mikulova (48° 46′ 05″ N, 16° 41′ 52″ E) and the Slovak isolates (SK01–10) in April 2010 in Likavka (49° 05′ 47″ N, 19° 18′ 08″ E). The French isolates were isolated in October 2007: F19–20 in the vicinity of Générargues (44° 04′ 09″ N, 04° 01′ 32″ W), isolates F115 and F165 in Montferrier-sur-Lez (43° 40′ 49″ N, 03° 51′ 55″ W), and isolates F220–222 in the vicinity of Sète (43° 28′ 37″ N, 03° 46′ 03″ W). The Cypriot isolates (KYPR01–16) originated from October 2009 from the vicinity of Ayos Nikolaos (35° 04′ 43″ N, 33° 53′ 25″ W) and the Tunisian isolate (Tun1) was obtained in September 2007 from Kairouan (35° 39′ 15″ N, 10° 06′ 21″ W). The Ghanian isolates were isolated in July 2009: G03–08 and G58–59 in the vicinity of Kokrobite (05° 29′ 42″ N, 0° 22′ 08″ W) and G14 in the vicinity of Ho–Matse (06° 34′ 47.6″ N, 0° 29′ 02″ W). The Costa Rican isolates with suffix 'RV' were collected in September 2009 in the vicinity of Rincon de la Vieja National Park, province Guanacaste (10° 45′ 14″ N, 85° 20′ 59″ W).

The earlier collection sites in Costa Rica (El Ceibo: labeled with suffix EC; Monteverde: MV; Carara/Tárcoles: VL; Osa: BT and CRT) and Ecuador (Napo-1: AL) and the localities in China were described previously (Maslov et al. 2007; Votýpka et al. 2010; Westenberger et al. 2004).

Insects were dissected and analyzed by light microscopy in the field as described earlier (Votýpka et al. 2010). The gut material from infected hosts was preserved in 2% SDS, 100 mM EDTA solution (Westenberger et al. 2004). Upon transfer to the laboratory, DNA from intestinal samples was purified using PureLink™ Genomic DNA kit (Invitrogen, Carlsbad, CA).

PCR amplification and analysis of SL RNA repeats: Primers M167 and M168 (Westenberger et al. 2004) were used to amplify SL RNA gene repeats which usually vary from 0.2 to 1.0 Kb. The amplification products were gel purified, cloned and sequenced as described earlier (Maslov et al. 2007). The GenBank™ accession numbers of SL repeat sequences representing isolates of *L. scantii* determined in this work are: Tun – JF950592; KYPR01 – JF950593; KYPR12 –JF950594; F221 – JN009104. The new SL sequences from the *L. pyrrhocoris* group which are listed in the legend of Figure 4. Accession numbers for isolates of unnamed trypanosomatids are: isolate P63 – accession number JF950596; G07 – JF950596; G14 – JN022575, JN022576; F115 – JF950598; H21 – JF950599, JF950600; SK02 – JF950601; SK03 – JF950602; 124AL – JN036650.

For across-the-family comparisons (Supplementary Fig. S1), only the most conserved section of repeats, beginning at position -100 upstream of the exon and ending at the 3′ end of the intron (approximately 140 nt long), is used. For the intraspecies analysis of TU***1*** parasites (Fig. 4) the entire repeat sequences were used. The sequences were aligned with Clustal-X, ver. 2.0 (Larkin et al. 2007). Neighbor-joining clustering with K2P distances was performed using PAUP* 4.0, beta version (Swofford 1998). The 90% cut-off level applied to the entire sequence was used to delineate individual Typing Units (TU) (Maslov et al. 2007).

Analyses of cells in environmental samples and axenic cultures: Cultures were established in Brain Heart Infusion (BHI) medium (Becton Dickinson, Sparks, MD) supplemented with 10 μg/ml hemin at 27 °C as described previously (Westenberger et al. 2004). Bacterial growth was suppressed with an antibiotic cocktail containing 100 μg/ml ampicillin, 100 μg/ml chloramphenicol, 50 μg/ml tetracycline. Fungal contamination was removed by a U-shaped device described earlier (Podlipaev and Frolov 1987). Light microscopy, and transmission and scanning electron microscopy were performed as described previously (Podlipaev et al. 2004; Svobodová et al. 2007; Yurchenko et al. 2006b; Zídková et al. 2010).

DNA-based analyses of trypanosomatid cultures: Total cell DNA was extracted from cells grown in axenic cultures or from environmental samples (the lysate of infected insect tissue, mostly gut content in the preservation 2% SDS 100 mM EDTA solution) with the DNA tissue isolation kit (Roche) or was isolated by sarcosyl-pronase lysis and phenol-chloroform deproteinization (Maslov et al. 1996). RAPD analyses were performed as described elsewhere (Dvořák et al. 2006) except that new primers OPE5 (5′-TCAGGGAGGT) and OPE12 (5′-TTATCGCCCC) were designed in the course of this work. The products were resolved in 1% agarose gel and stained with SybrSafe (Invitrogen). SSU rRNA and gGAPDH genes were amplified and sequences as described previously (Maslov et al. 1996, 2010; Yurchenko et al. 2006a). GenBank™ accession numbers of the SSU rRNA and gGAPDH sequences determined in this work are given in the caption of Figure 2.

Phylogenetic inference: Sequences of the concatenated gGAPDH and SSU rRNA gene dataset were aligned using Clustal-X, ver. 2.0 (Larkin et al. 2007), with gap opening penalty of 12, gap extension penalty of 5 and the default remaining parameters. The alignment was then edited manually by deleting ambiguously aligned regions and most positions with gaps. The final alignment included 2672 characters. It is available from the authors on request. The dataset was analyzed using maximum likelihood (ML) and Bayesian inference (BI). The ML analysis was carried out using RaxML 7.2.8 (Stamatakis 2006) under the GTR + Γ model of evolution. The best fitting model was estimated using both Akaike and Bayesian Information Criterion (AIC/BIC) as implemented in MrAIC (Nylander 2004). The model parameters were optimized for each gene separately and the topology with the highest likelihood score was selected from 200 independent runs each starting with different randomized maximum parsimony tree. Branching support was estimated using non-parametric bootstrapping from 500 replicates. The BI analysis was performed used Phylobayes 3.2 (Lartillot et al. 2009) with 40 empirical profile mixture (C40) combined with GTR-derived substitution rates (GTR + CAT model). Two chains were run until their maximum observed discrepancy across all bipartitions was lower than 0.1 and effective sample size of all variables was higher than 100. Consensus topology and posterior probabilities were then estimated with first 20% of the generations discarded as a burn-in. The full-length SL RNA repeat dataset was analyzed using the same ML conditions as described above.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.protis.2011.12.004.

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