Trypanosomatid biodiversity in Costa Rica: genotyping of parasites from Heteroptera using the spliced leader RNA gene

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SUMMARY

The biodiversity of insect trypanosomes is largely unknown, resulting in significant gaps in the understanding of pathogen evolution. A culture-independent preliminary survey of trypanosomatid fauna was conducted for the parasites of Heteroptera (Hemiptera) from several localities in Costa Rica. Trypanosomatid infections were detected by light microscopy of smeared gut contents. Out of 257 insects representing 6 families, infections were found in 62 cases; cultures were obtained for 29 new isolates. Gut material from infected hosts was preserved in the field using an SDS–EDTA buffer solution for subsequent DNA extraction in the laboratory. PCR amplification of the trypanosomatid-specific spliced leader (SL) RNA gene repeats was successful for 60 field samples. Eighteen distinct SL RNA typing units were identified in a set of 28 samples analysed in detail. Cluster analysis indicated that these typing units were unique and thus could represent new species and, in some cases, new genera. This study reveals only a minor fraction of the trypanosomatid biodiversity, which is anticipated to be high.

Key words: Trypanosomatidae, biodiversity, mini-exon, SL RNA, genotyping.

INTRODUCTION

Trypanosomatid protozoa include several monoxenous (1 host) genera, including *Blastocrithidia*, *Crithidia*, *Herpetomonas*, *Leptomonas*, *Rhynchoidomonas* and *Wallaceina*, that are often referred to as 'insect trypanosomatids' on the basis of their host. In contrast to the insect-transmitted dixenous (2 hosts) parasites of vertebrates from the genera *Trypanosoma* and *Leishmania*, very little is known about the diversity, phylogeny, host specificity and geographical distribution of insect trypanosomatids. Thus our knowledge of the entire family remains fragmentary. The lack of sufficient attention to the monoxenous parasites is only partially responsible for this situation. Insect trypanosomatids are usually difficult to distinguish from each other using light microscopy. An ideal solution to this problem would be the recovery of each new isolate in axenic culture, serving both as a species voucher and a source of material for comprehensive biochemical and molecular analyses (Sbravate *et al.* 1989; Jankevicius *et al.* 1993; Batistoti *et al.* 2001; Catarino *et al.* 2001). However, cultivation is not possible for all of the known trypanosomatids. Consequently, although there are more than 400 published reports describing insect trypanosomatids (Wallace, 1966; Podlipaev, 1990), the identity of most of these organisms remains uncertain.

Insect trypanosomatids are broadly distributed. Two insect orders, Hemiptera and Diptera, account for nearly 35 and 55%, respectively, of the known hosts (Podlipaev, 1990). A recent 18S rRNA phylogenetic study (Merzlyak *et al.* 2001) identified several previously undescribed lineages of insect trypanosomatids (Hollar, Lukeš & Maslov, 1998), and led to the suggestion that the group is extremely diverse (Stevens, 2001).

Application of PCR is a viable alternative to cultivation in microbial biodiversity surveys, as a minimal amount of genetic material is sufficient for genotyping, such as might be contained in a field sample. The main advantages of a culture-independent approach are that it allows for identification of organisms that are difficult or impossible

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to cultivate and avoids culture-induced homogenization of mixed infections. Culture-independent surveys are rapidly expanding the investigated range of eukaryotic biodiversity (Baldauf, 2003) including the discovery of potentially deep branching free-living kinetoplastids (Lopez-Garcia *et al*. 2003).

The multicopy spliced leader (SL) RNA (also referred to as mini-exon donor RNA) gene array represents one of the best studied and most useful markers for genotyping of trypanosomatids (Murthy, Dibbern & Campbell, 1992); its highly conserved exon sequence allows for specific and efficient amplification from a broad selection of species, a moderately variable intron is useful for selective grouping of related species, while a hypervariable intergenic region provides the highest level of discrimination among isolates. In addition, SL RNA is not present in the insect or vertebrate hosts of trypanosomatids. SL RNA genotyping has been used to investigate relationships within the genera *Crithidia* (Fernandes *et al*. 1997), *Herpetomonas*, *Leptomonas* and *Blastocrithidia* (Podlipaev *et al*. 2004), *Phytomonas* (Sturm, Fernandes & Campbell, 1995; Dollet, Sturm & Campbell, 2001), *Leishmania* and *Endotrypanum* (Fernandes, Degrave & Campbell, 1993; Fernandes *et al*. 1994), and *Trypanosoma* (Souto *et al*. 1996; Fernandes *et al*. 1998; Grisard, Campbell & Romanha, 1999; Grisard, Sturm & Campbell, 2003). Development of group-specific amplification protocols and hybridization probes has further increased the versatility of the SL RNA gene marker (Ramos *et al*. 1996; Teixeira *et al*. 1996; Fernandes *et al*. 1997; Harris *et al*. 1998; Serrano *et al*. 1999; Fernandes *et al*. 2001; Godoi *et al*. 2002; Marfurt *et al*. 2003), such that it is a logical choice for conducting culture-independent surveys of trypanosomatids.

Our long-term goal is to describe the full range of diversity among insect trypanosomatids. In this work we apply a SL RNA gene repeat-based PCR strategy for a survey of trypanosomatids from Heteroptera (Hemiptera) in Costa Rica, the country known for its great insect diversity and a relative proximity of multiple ecosystems.

MATERIALS AND METHODS

Sample handling: field stage

Heteropteran bugs were collected at 5 sites in Costa Rica: 2 locations in the Carara National Park, near the northern boundary of the Guanacaste National Park, near the La Selva Biological Station, and at the western boundary of the Braulio Carrillo National Park (El Ceibo). Insects were collected by sweep-netting, attraction to black and mercury vapour light, or by hand picking. Live specimens were kept individually in small plastic vials before dissection, which usually took place the same day. Insects were dissected using disposable fine-point wooden toothpicks. After opening the abdomen, the contents were suspended in a few drops (0·2–0·3 ml) of 1 × SSC buffer (150 mM NaCl, 15 mM sodium citrate) on a microscope slide. The slides were examined using phase-contrast light microscopy at 400 × magnification with a cover-slip. Upon detection of a trypanosomatid infection, the material from the slide was divided between two 1·5 ml Eppendorf tubes: one tube containing 1 ml of 1% SDS, 0·1 M EDTA for DNA preservation, and another tube with 1 ml of brain heart infusion (BHI) medium, supplemented with 10 μg/ml haemin, 100 μg/ml ampicillin, 100 μg/ml tetracycline and 50 μg/ml chloramphenicol to initiate a primary culture. The remaining material on the slide was fixed with ethanol and air-dried for subsequent staining with Giemsa. The post-dissection insects ('xenotypes') were deposited in the UCR Research Entomology Museum as voucher specimens.

Sample handling: laboratory stage

Extraction of DNA from the material preserved in the SDS–EDTA buffer was performed after digestion with pronase (200 μg/ml) at 65 °C for 30 min. The lysate was extracted with Tris-buffered phenol (pH 8), followed by extraction with phenol–chloroform. Nucleic acid from the aqueous phase was precipitated with isopropanol after addition of glycogen (5 μg/ml) as a carrier. The pellets were washed with ethanol and resuspended in 100 μl of 10 mM Tris–HCl, pH 8·0, 0·1 mM EDTA.

PCR amplification, cloning and sequencing

The SL RNA gene repeats were amplified from sample DNAs using the overlapping oligonucleotides M167, 5′-gggaagcTTCTGTACT(A/T)TATTGGTA, and M168, 5′-gggaattCAATA(A/T)AGTACAGAAACTG, wherein the lower case letters indicate nucleotides added to the genomic sequence to facilitate PCR. These oligonucleotides were based on the exon-specific primers suggested originally (Murthy *et al*. 1992). The DNA contained in 1 μl of each sample was used for PCR with *Taq* polymerase with the following cycling profile: initial denaturation at 95 °C for 5 min, followed by 3 low-stringency cycles (95 °C for 30 s, 42 °C for 1 min, 65 °C for 2 min 30 s) and 32 high-stringency cycles (95 °C for 30 s, 50 °C for 1 min, 72 °C for 2 min 30 s). Agarose gel electrophoresis was performed by standard procedures (Sambrook, Fritsch & Maniatis, 1989). The *Phytomonas*-specific hybridization oligonucleotide probe ('probe 42') was 5′-TTGGACTCGGGGCCTTCGG. The DNA blots were hybridized in 6 × SSC buffer containing 1 × Denhardt's solution, 0·1% SDS at 42 °C; the post-hybridization washes were performed in 2 × SSC, 0·1% SDS at 55 °C. For

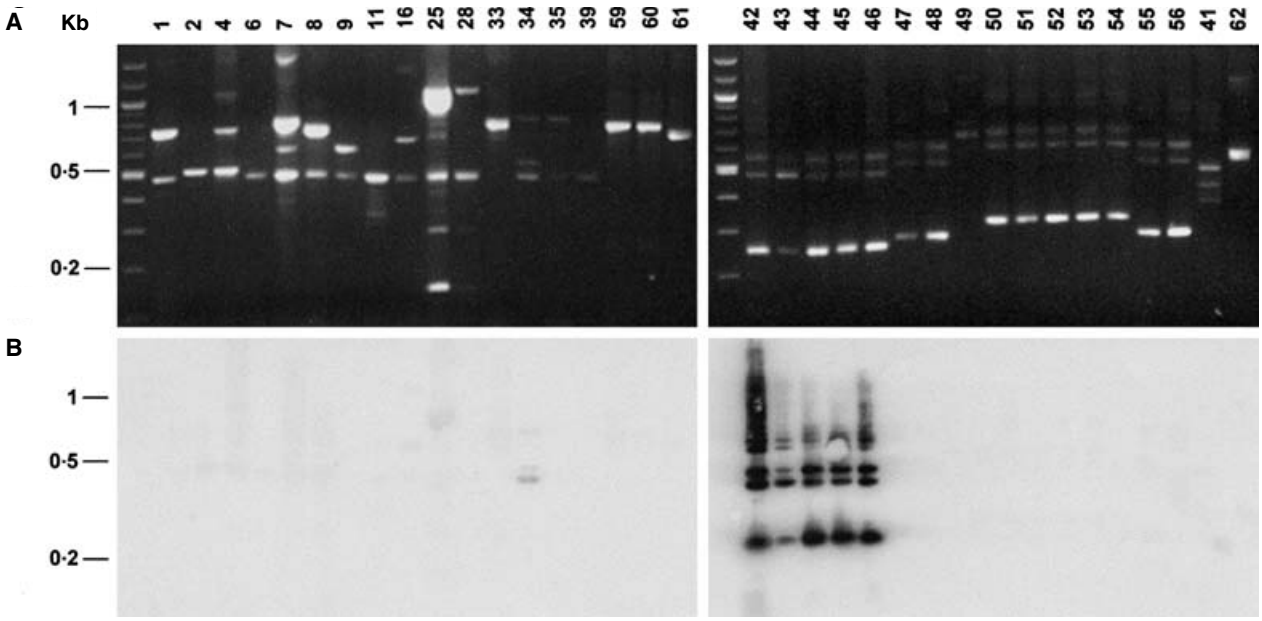

Fig. 1. Analysis of the trypanosomatid-specific SL RNA gene repeat from field samples. (A) Ethidium bromide staining of a 2% agarose gel electrophoresis of PCR products from a representative set of samples. (B) DNA blot hybridization of a replicate gel with the *Phytomonas* group-specific oligonucleotide 'probe 42'.

cloning, amplified DNA products of interest were size-selected and eluted from electrophoretic gels using the QIAquick gel extraction kit (QIAGEN), cloned in the TOPO® TA vector (Invitrogen) and sequenced by a commercial facility (Laragen). Sequences were analyzed with Vector NTI Suite 6.0 (InforMax).

Cluster analysis

Partial SL RNA gene repeats were aligned using ClustalX, version 1.81 (Thompson *et al*. 1997). The region corresponding to the amplification primers (positions 21 to 42 of the SL RNA) and most of the intergenic region except for positions −100 to −1 were excluded from the analysis. Clustering was performed using the neighbour-joining method with the Kimura 2-parameter distance model in PAUP* 4.0, beta version (Swofford, 1998). For preliminary analyses the trees were also constructed by ClustalX using default parameters and viewed using TreeViewX, version 0.4. The following SL RNA gene repeat sequences were retrieved from the GenBank™: *Phytomonas* sp. Mar1 (AF250986), *Phytomonas* sp. Hart1 (X87136), *Phytomonas serpens* (X87137), *Phytomonas* sp. EM1 (X87138), *Herpetomonas pessoai* (X62331), *Leptomonas seymouri* (X07488), *Leptomonas collosoma* (S76723), *Trypanosoma cruzi* (K02632), *T. cruzi* CL Brener (U57984), *T. cruzi* Y (K02634), *T. cruzi* Silvio (X62674), *T. cruzi* Tulahuen (X00632), *T. cruzi* M6241 (AF050522), *T. cruzi* M5631 (AF050521), *T. cruzi* MT4167 (AF050523), *T. cruzi* Cutia (AY367129), *T. cruzi* 92122102r (AY367124), *T. cruzi* SC43 (AY367127), *T. cruzi* MN (AY367128), *T. cruzi* CanIII (AY367123), *Trypanosoma rangeli* (AJ012419), *Trypanosoma lewisi* (AJ250740), *Trypanosoma desterrensis* (AF124146), *Crithidia oncopelti* (U96172), *Crithidia fasciculata* (U96170), *Crithidia luciliae* (U96171), *Crithidia deanei* (U96168), *Crithidia acanthocephali* (U96167), *Endotrypanum schaudinni* (L05398), *Leishmania mexicana* (AY155508), *Leishmania amazonensis* (L05395), *Leishmania colombiensis* (AY155502), *Leishmania brasiliensis* (X69442), *Leishmania panamensis* (AY155509), *Leishmania guyanensis* (X69452), *Leishmania shawi* (X69455). The alignment and other supplementing material are available on-line (http://www.kinetoplast.ucr.edu/CostaRica03/Genotyping.htm).

RESULTS AND DISCUSSION

SL RNA gene repeats can be amplified from field DNA samples

DNA contained in insect gut material from infection-positive samples was preserved efficiently in a solution of high EDTA concentration and a strong ionic detergent. After several weeks at the ambient temperature of the tropical environment, the samples preserved in the EDTA–SDS buffer contained enough intact trypanosomatid DNA for amplification of SL RNA gene repeats in 60 out of 62 preparations. Since trypanosomatid cells represented a minor fraction in the original crude samples that also contained insect cells, fungi, and various protist and microbial components, this result testifies to the effectiveness of our sample preservation and PCR amplification strategies.

A representative gel of the amplification products is shown in Fig. 1A. The sizes of the bands varied

Table 1. Summary of the trypanosomatid SL RNA repeat sequences and isolates analysed in this study

DNA sample	Collection locale	Heteropteran host species (family)	SL sequence name	Repeat size, bp	5S gene	Typing unit	Culture available
1	Carara-1	*Largus* sp. 1 (Largidae)	1B	719	+	***9***	−
2	Carara-1	*Largus* sp. 1 (Largidae)	2	475	+	***12***	−
4	Carara-1	*Largus* sp. 1 (Largidae)	4A	478	+	***12***	−
			4B	718	+	***9***	−
6	Carara-1	*Largus* sp. 2 (Largidae)	6B	719	+	***9***	−
			7B1	784	+	***13***	−
7	Carara-1	*Largus* sp. 2 (Largidae)	7B2	770	+	***13***	−
8	Carara-1	*Largus* sp. 2 (Largidae)	8B	708	+	***10***	−
9	Carara-1	*Largus* sp. 2 (Largidae)	9A	476	+	***12***	−
			9B	590	+	***14***	−
			9C	715	+	***10***	−
11	Carara-1	*Butinus* sp. (Alydidae)	11A	307	−	***6***	−
16	El Ceibo	*Dolichomiris linearis* (Miridae)	16B	647	+	***17***	−
25	El Ceibo	*Dysdercus* sp. 1 (Pyrrhocoridae)	25E	1063	−	***1***	+
28	El Ceibo	*Dysdercus* sp. 1 (Pyrrhocoridae)	28B	1062	−	***1***	+
33	El Ceibo	*Acanthocephala bicoripes* (Coreidae)	33	756	+	***16***	−
34	El Ceibo	N.D. (Rhopalidae)	34B	514	−	***2***	+
35	El Ceibo	N.D. (Rhopalidae)	35B	514	−	***2***	+
39	El Ceibo	*Collaria oleosa* (Miridae)	39	797	+	***15***	−
41	La Selva	*Lyrnessus geniculatus* (Alydidae)	41B	382	−	***7***	−
42	La Selva	*Schraderia hughsae* (Pentatomidae)	42A	225	−	***5***	−
			42B, dimer	450	−	***5***	−
44	La Selva	*Schraderia hughsae* (Pentatomidae)	44B, dimer	450	−	***5***	−
47	Carara-1	*Prepops* sp. 1 (Miridae)	47B, dimer	489	−	***4***	+
48	Carara-1	*Prepops* sp. 1 (Miridae)	48B, dimer	489	−	***4***	+
49	Carara-1	*Prepops* sp. 1 (Miridae)	49A	624	+	***18***	−
50	Carara-2	*Pachypoda* sp. 1 (Miridae)	50A	286	−	***3***	+
			50B, dimer	571	−	***3***	−
52	Carara-2	*Pachypoda* sp. 1 (Miridae)	52B, dimer	571	−	***3***	−
55	Carara-1	*Prepops* sp. 1 (Miridae)	55B, dimer	488	−	***4***	+
59	Guanacaste	*Dysdercus* sp. 2 (Pyrrhocoridae)	59	775	+	***15***	+
60	Guanacaste	*Dysdercus* sp. 2 (Pyrrhocoridae)	60	784	+	***15***	+
61	La Selva	*Heza* sp. 1 (Reduviidae)	61	695	+	***11***	−
62	La Selva	*Zicca rubricator mayorgae* (Coreidae)	62	505	−	***8***	−

from 0·25 to 1·0 kb. In many cases more than one band was observed which, in some, but not all, cases could be attributed to amplification of tandem repeat 'dimers' as observed previously (Fernandes *et al*. 1993, 1994; Sturm *et al*. 1995; Dollet *et al*. 2001). This was particularly evident for smaller repeats such as those in samples 42–56. When multiple products were amplified from a single sample, the bands were designated A, B, C, and so on, by increasing size. Products representing different size classes were eluted from the gel, cloned and sequenced. The DNA samples that were used for this analysis are listed in Table 1.

Thirty-four sequenced DNA fragments were SL RNA gene-specific: they contained recognizable SL RNA features including the conserved exon and intron followed by a T-tract and a variable intergenic region. In several cases, the intergenic region of the SL RNA gene repeat contained the 5S rRNA gene, which is linked with SL RNA genes in some trypanosomatids and bodonids (Santana *et al*. 2001). Several cloned and sequenced bands (42B, 44B, 47B, 48B, 50B, 52B, 55B) were dimers, some of which (47B, 48B, 50B, and 52B) differed internally by a single nucleotide in the intergenic region, as seen previously (Dollet *et al*. 2001).

Diagnostic features of the SL RNA exon allow preliminary grouping of new sequences

Exon positions 1 to 20, the region immediately upstream of the amplification primers, contain specific nucleotides that may be diagnostic for certain species, groups of species or genera (Sturm *et al*. 1995, 2001). To further explore the potential of exon variability for genotyping and identification, we grouped the new sequences and those from several known species according to the exon patterns (Table 2). The previously described species formed several groups corresponding to their phylogenetic affinities within the family (Croan, Morrison & Ellis, 1997; Hollar & Maslov, 1997; Noyes *et al*. 1997; Haag, O'Huigin & Overath, 1998; Hollar, Luke & Maslov, 1998; Stevens *et al*. 1999; Merzlyak *et al*. 2001). Thus, the species of *Leishmania*, *Endotrypanum*, some species of *Crithidia*, and *L. seymouri* were all found within the same group. All known species of *Phytomonas* formed a distinct group, as did

Table 2. Species groups defined by the SL RNA exon (positions 1–20 of the *Leishmania* sequence)

Previously described species	Exon sequence	Putative new species (typing units)
Leishmania and *Endotrypanum* spp., *C. fasciculata*, *C. luciliae*, *C. acanthocephali*, *L. seymouri*	AACTAACGCTATATAAGTAT	***1***, ***2***, ***3***, ***4***
Phytomonas spp.	..........TCT..ATA	***5***
	.........A.ATC..AGTA	***6***, ***7***
	.........A.ATA..AGTA	***8***
	.........GT.T.TT..TA	***9***, ***10***, ***11***, ***14***
	.........AT.T.TT..TA	***12***, ***13***, ***15***, ***16***, ***17***
	.........T..CT....A	***18***
C. oncopelti	..............TT..TA	
C. deanei	......A..AT....TAG..	
H. pessoai	............TATT..TA	
L. collosoma	......AA.A..T.TT.A.G	
T. cruzi, *T. desterrensis*, *T. lewisi*, *T. rangeli*	............TATT.ATA	

the South American trypanosomes. Trypanosomatids containing endosymbionts, such as *H. pessoai*, *C. oncopelti*, and *C. deanei*, although monophyletic, are characterized by rapid sequence evolution (Hollar, Luke & Maslov, 1998) and thus belong to separate exon groups.

The new sequences fell into several groups, some of which matched groups composed of known species, indicating potential relationships among them. The other sequences formed 5 unique groups, suggesting that they represent distinct undescribed lineages in the family.

Cluster analysis of a portion of the SL RNA gene repeat

Our next goal was to explore the full potential of SL RNA gene repeats for genotyping, including identification of new species or higher taxonomic ranks. We wanted to challenge the exon-based groups presented above at the level of the complete genes. Due to the extreme length and sequence variability of intergenic regions, SL RNA gene repeats from the entire set of isolates could not be fully aligned except from closely-related isolates. Cluster analysis performed with complete repeats could be used to identify the clades of the most similar sequences but the rest of the tree would be biased due to extensive misaligned regions (data not shown).

In order to obtain maximally-informative and minimally-biased SL RNA gene repeat-based clustering, we constructed an alignment from position −100 relative to the exon to the T-tract. The region contains the upstream promoter and the SL RNA gene transcribed region (Saito, Elgort & Campbell, 1994; Campbell, Sturm & Yu, 2000). The result of cluster analysis performed by neighbour joining is shown in Fig. 2. The unrooted tree included all available sequences of monoxenous insect trypanosomatids and several dixenous representatives including Neotropical leishmanias, trypanosomes and *Phytomonas*.

The bootstrapping of our data set indicated that most of the subterminal clades were weakly supported and the deep branches unresolved (data not shown), while the small terminal clades were highly supported (data not shown). Of particular importance was the question of which of the larger clades faithfully reflected genetic relatedness among the isolates. In this regard, it was remarkable that the dixenous organisms known to form distinct clades in SSU trees also formed the corresponding groups in our tree, although with variable support. For example, the isolates of *T. cruzi* formed a monophyletic clade (Tibayrenc, 1998; Brisse, Verhoff & Tibayrenc, 2001) that was monophyletic with other South American trypanosomes in accordance with the monophyly of the genus *Trypanosoma* (Lukeš *et al*. 1997; Haag *et al*. 1998; Stevens *et al*. 1999). The *Leishmania* spp. formed a clade with *Endotrypanum* (Croan *et al*. 1997; Noyes *et al*. 1997).

Relationships among the reference monoxenous trypanosomatids were resolved only for *C. fasciculata*, *C. luciliae* and *C. acanthocephali*, albeit with low bootstrap support. For the rest, the high levels of sequence divergence apparently exceeded the resolving power of the SL RNA marker. A high level of divergence was expected among *C. fasciculata*, *C. oncopelti*, *C. deanei*, *H. pessoai*, *L. collosoma*, for which the phylogeny is known (Hollar *et al*. 1998; Merzlyak *et al*. 2001).

SL RNA gene repeats are indicative of new typing units or potential new species

We considered the results from the exon comparison, the cluster analysis, and the sequence in the entire SL RNA gene repeat to determine the relationships among the new and described trypanosomatids. We have chosen arbitrarily the threshold level of 90%

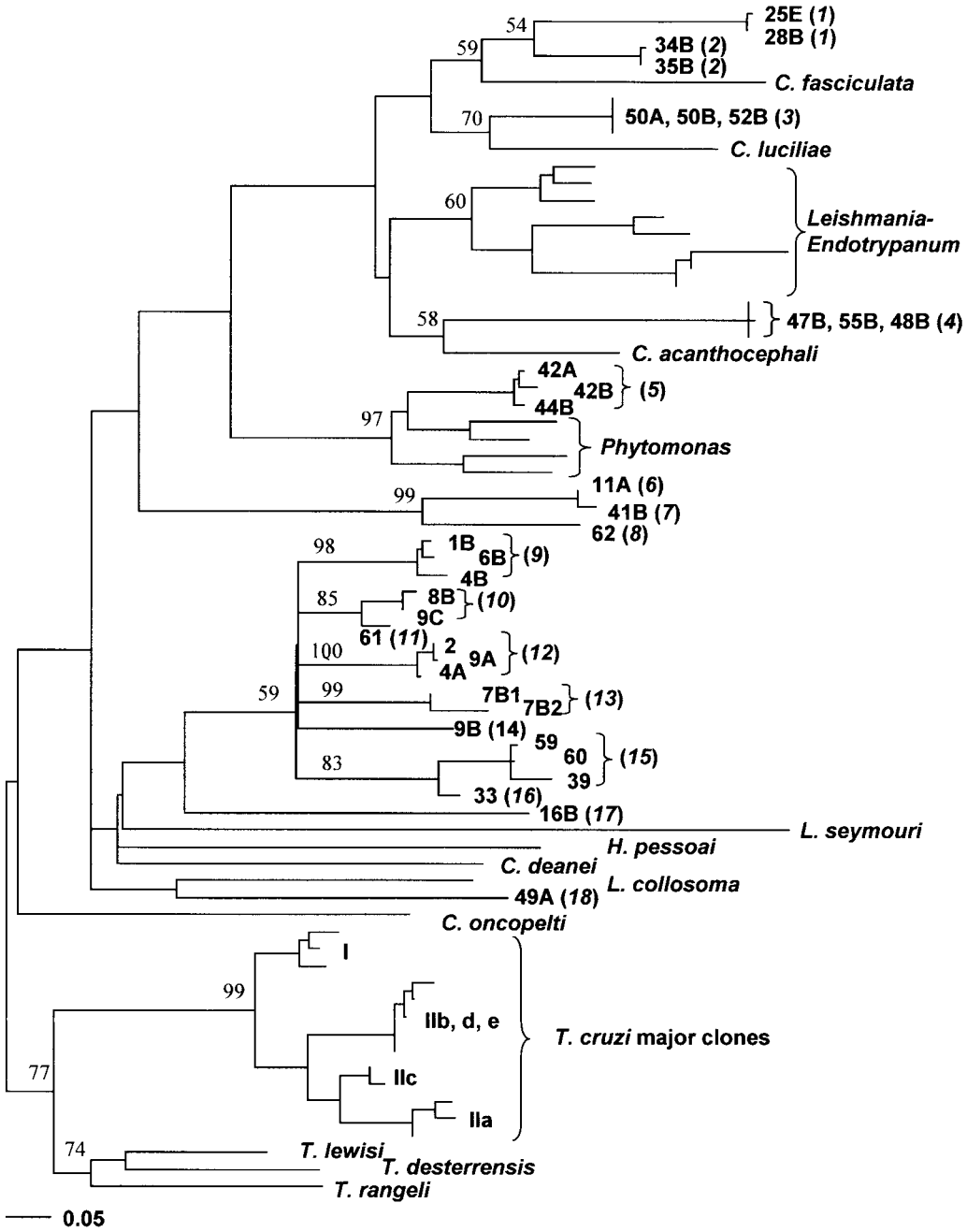

Fig. 2. Cluster analysis of the SL RNA gene with new and previously described trypanosomatids. Neighbour-joining tree inferred from the aligned SL RNA gene sequence encompassing positions −100 to the 3′ end of the intron. Kimura 2-parameter distances were used. Bootstrapping was performed using 500 replicates and the values are shown at the selected clades that were preserved in the 50% majority-rule consensus tree. Most of the terminal clades were also preserved, although the bootstrap values are not shown. Numbers given at the tips of the branches correspond to the new trypanosomatid sequences followed by designations of the corresponding typing units (in parentheses).

identity of the whole SL repeat as a limit of intraspecies variation. Thus, if the identity of two sequences exceeds 90%, they would belong to the same species or, due to the uncertainty of the species concept in asexual parasites (Kunz, 2002), to the same discrete typing unit (Tibayrenc, 1998). We realize that this definition, as a trypanosomatid species definition itself, is debatable, as the level of phylogenetic divergence warranting 'splitting' versus 'lumping' is still a matter of subjective judgment in molecular parasitology. This situation is well illustrated by the *Leishmania* – *T. cruzi* taxonomic inconsistency (Tibayrenc, 1998). The identity levels observed for SL RNA gene repeats from closely related *Leishmania* species ($\geqslant$92% for the *Viannia* species and $\sim$94% between *L. m. mexicana* and *L. m. amazonensis*) were much higher than among the major *T. cruzi* natural clones, wherein it dropped below 60% in the intergenic region (Souto *et al.* 1996; Brisse *et al.* 2001). Following our level of

90%, the single *T. cruzi* species should be broken up into several typing units. On the other hand, sequence identity level, while sufficient to define typing units, is not the only criterion for defining species, which must include consideration of the biological properties of the organisms in question.

The cluster tree showed that none of the new sequences fell into close association with the named species. Rather, the new sequences formed several distinct clusters and thus may represent new taxa. Some of the new sequences were identical among themselves, or nearly so. Given the common origin of these sequences, it is likely that they were derived from members of the same species. For example, two nearly identical sequences, 25E and 28B (typing unit ***1***), corresponded to trypanosomatids found in the same population of hosts (Table 1), as do the sequences within the clusters 34A/35B, 50A/50B/52B, and 47B/48B/55B (units ***2***, ***3*** and ***4***, respectively).

Another potential new species (typing unit ***5***) was formed by SL RNA gene repeats 42B and 44B which were 96·4% identical and shared the same origin (Table 1).

A group of 3 related sequences was represented by the cluster 11A/41B/62. The corresponding isolates came from 3 different host species collected at 3 different locales. The identity between the whole repeats 11A and 41B was 79·1%, and the longer sequence 62 was only 38–44% identical to the two other sequences, thereby relegating these trypanosomatids to independent typing units ***6***, ***7*** and ***8***, respectively.

A large group of sequences (from sequence 1B to sequence 33 moving down the tree) is found in 1 weakly (59%) supported clade. The corresponding samples were obtained from 6 host species collected at 4 different locales (Table 1). The sequences in this cluster formed several terminal clades with 83–100% support levels. Eight typing units (***9*** to ***16***) were identified by applying the 90% threshold rule with sequences 61 and 33 separate from the rest of their clades.

Two additional typing units (***17*** and ***18***) were formed by sequences 16B and 49A.

Cluster analysis allows assignment of new species to the known genera or designation of potential new genera

A remarkable feature of the SL RNA gene tree is that new species were distributed consistently with their associations identified by exon positions 1 to 20 (Table 2) indicating that there was no bias in nucleotide substitution patterns of the exon compared to the rest of the alignable SL RNA gene sequence. Therefore changes in the exon sequence are indicative of phylogeny.

Typing units ***1***, ***2***, ***3*** and ***4*** were associated with three species of *Crithidia* on the tree, as they were based on exon sequence. Since bootstrap support with the SL RNA data set was generally low, additional analyses are required to verify this relationship. None of the sequences contained a 5S rRNA gene in the intergenic region, similar to the *Crithidia* species.

The sequences 42 and 44 (typing unit ***5***) were monophyletic with *Phytomonas* spp., thus confirming their preliminary assignment to this group. According to the SSU rRNA phylogenetic analyses (Marche *et al*. 1995; Hollar & Maslov, 1997), several phloem, latex and fruit isolates classified as *Phytomonas* species form a monophyletic group. The criterion of monophyly is thus a useful definition for the genus *Phytomonas*, which is sometimes considered arbitrary as its original criteria (morphology and host specificity) are also met by other trypanosomatid taxa (Jankevicius *et al*. 1993; Catarino *et al*. 2001), and because plant parasites themselves are a diverse group (Muller *et al*. 1994, 1995; Dollet *et al*. 2000). Nevertheless, the sequences of SL RNA gene arrays from several fruit, phloem and latex isolates (Nunes *et al*. 1995; Sturm *et al*. 1995; Dollet *et al*. 2001) clearly distinguish these organisms from other trypanosomatids. The alignment of exon-intron and the upstream regions of the isolates 42 and 44 with several *Phytomonas* species (Fig. 3) demonstrated that the new isolates are distinguishable from the other phytomonads by their intron and the upstream intergenic regions. These differences are reflected by a separate branch of the new isolates within the *Phytomonas* clade in the tree. There were no 5S rRNA genes in SL RNA gene repeats from new isolates, similar to the species of *Phytomonas*.

Sequences 11A, 41B and 62 (typing units ***6***, ***7***, ***8***) formed a well-supported clade that branches off deeply, and is not associated with any known trypanosomatids. They also formed 2 separate but obviously similar groups based on exon sequence. This cluster is a likely candidate for a new genus if the deep branching position is verified using additional markers. There were no 5S rRNA genes in the intergenic regions of these sequences.

The existence of a large clade formed by typing units ***9–16*** is also consistent with the corresponding sequences forming 2 similar exon groups. The sequence 16B weakly associated with this clade in the tree is a member of one of these groups. These sequences contained 5S genes in intergenic regions. It is likely that the entire clade represents a separate genus.

Sequence 49A is another candidate for a new genus as it forms a long branch on the tree with a distinctive exon. This sequence contains a 5S rRNA gene in the intergenic region.

These results provide credibility to the exon-based typing. The position of *L. seymouri*, separate from other members of its exon group, is due to its extraordinary exon length and high sequence divergence;

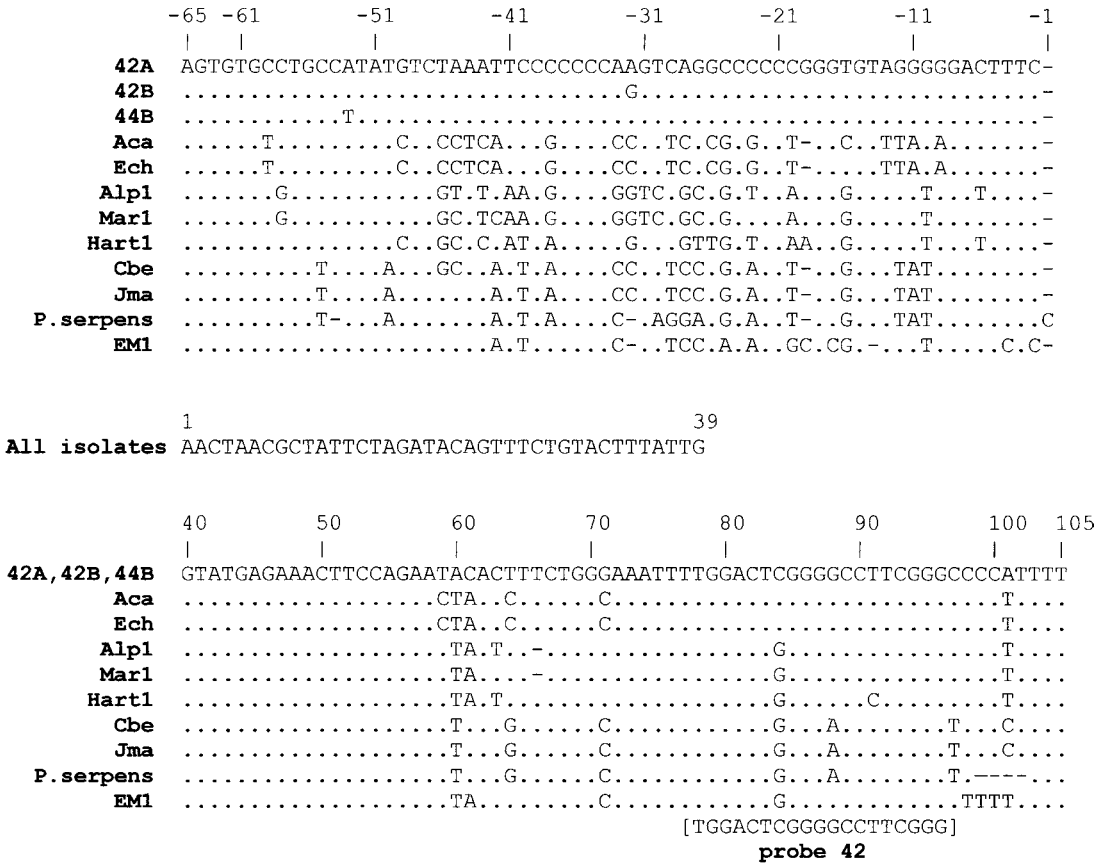

Fig. 3. Sequence analysis of new *Phytomonas*-like isolates from insects. Alignment of SL RNA exon (positions 1 to 39), intron (positions 49 to 105) and upstream region (positions −65 to −1) sequences of the isolates 42 and 44 with several trypanosomatids isolated from plants. The sequence 42A is a monomer, and the sequences 42B and 44B represent second units from the corresponding dimers. The additional *Phytomonas* sequences included in the alignment are isolates *Aca* (L42376), *Ech* (E42382), *Alp1* (AF250967), *Cbe* (L42377) and *Jma* (L42378).

the SSU rRNA phylogeny showed that *L. seymouri* was indeed monophyletic with other members of the *Leishmania* exon group (Merzlyak *et al.* 2001).

Group-specific probes can be used for assigning additional isolates to typing units

We investigated if group-specific hybridization probes could be used to investigate relationships of additional samples with the typing units. In particular, we asked if the DNA samples 43, 45 and 46 (Fig. 1A), which were obtained from the same population of hosts and have the same amplified repeat patterns as the samples 42 and 44, also belonged to the same new putative species of *Phytomonas* (typing unit ***5***). An analogous blot of the gel presented in Fig. 1A was hybridized with labelled oligonucleotide probe matching intron positions +78 to +96 of the 42B and 44B sequences ('probe 42', Fig. 3). Specific and efficient hybridization of this probe to samples 42–46 (Fig. 1B) indicated that the corresponding SL RNA genes were closely related. Given their common origin, they likely belong to the same typing unit.

Analysis of SL RNA gene repeats detects multiple infections

Three sequences obtained from sample 9 (9A, 9B and 9C) fell into different typing units, as did two sequences obtained from sample 4 (4A and 4B). Multiple infections of individual hosts would produce these results; however, the existence of related but non-identical repeat arrays within the same genome could not be eliminated (Maslov *et al.* 1993; Yu *et al.* 2002). While sequence 9B was unique and likely authentic, questions remained regarding the origin of the sequences 9A and 9C which are ~96% identical to their counterparts from the corresponding typing units. Likewise, sequences 7B1 and 7B2 were obtained from the same individual host and are 93·6% identical; they could originate from the same trypanosomatid or from two closely related trypanosomatids infecting the same host. Levels of SL RNA gene repeat sequence variability within an array or between an allelic and non-allelic array, as well as within populations, have not been sufficiently investigated. The clonal mode of reproduction that is predominant in nature can result in a rapid accumulation of differences between closely related

organisms, as seen on a larger scale in *T. cruzi* (Tibayrenc, Kjellberg & Ayala, 1990).

Culture-independent approach allows for a more complete and less biased sampling of diversity

We have attempted to cultivate all trypanosomatids encountered during this survey. Stably propagating cultures were obtained in 29 cases. Out of the 33 failed cultivation attempts, the parasites grew in the primary culture in 24 cases but were lost during subsequent transfers, and no growth of parasites in the primary culture was seen in the remaining 9 cases. It is possible that the use of more complex media (e.g. LIT supplemented with foetal bovine serum) would allow for a better recovery of viable cultures. Analysis of the successful cultures is in progress.

Among the trypanosomatid groups identified by the culture-independent approach, the distribution of cultivable organisms was non-random (Table 1). Thus, most of the recovered cultures correspond to typing units ***1–4*** that have been associated tentatively with 3 described species of *Crithidia*. Most of the known members of this genus are easy to cultivate. On the contrary, we did not recover the many putative *Phytomonas* species, and this failure was consistent with the reported difficulties in cultivating plant parasites (Dollet, 1994). Moreover, only two cultures were recovered for the large group of isolates representing units ***9*** to ***17***. Thus only 5 out of the 18 putative new species could be cultivated. Without negating the importance of cultures, these results demonstrate advantages of the culture-independent approach, without which the new typing units would not have been found.

Concluding remarks

The work presented here describes the potential of a culture-independent survey strategy based on PCR amplification and sequencing of SL RNA gene repeats. Amplification of DNA extracted from the preserved field samples was largely successful. The trypanosomatid diversity was revealed efficiently by sequencing of the amplified repeats followed by cluster analysis. Diversity was found to be high, especially considering the relatively narrow host range and limited geographical scope of this study. These sequences can be used for developing group-specific hybridization probes that can reduce the amount of future sequencing. The ease and specificity of obtaining SL RNA gene sequences from a large number of samples thus justifies the use of this marker for preliminary grouping or clustering of organisms in a high-throughput survey strategy. These preliminary descriptions will be complemented by use of a more informative phylogenetic marker (e.g. SSU rRNA) to investigate relationships among the SL RNA groups. Due to the occurrence of mixed infections in insects, analyses incorporating multiple genetic markers must rely on the successful generation of axenic, clonal cell cultures. The work also highlights the current dearth of information on the natural diversity of insect trypanosomatids and the need for developing criteria for correlating levels of genetic divergence of isolates with their taxonomic status.

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