Morphological and molecular diversity and phylogenetic relationships among anuran trypanosomes from the Amazonia, Atlantic Forest and Pantanal biomes in Brazil

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(*Received 6 March 2007; revised 24 April 2007; accepted 25 April 2007; first published online 19 June 2007*)

SUMMARY

We examined for the presence of trypanosomes in blood samples from 259 anurans (47 species from 8 families), the majority of which were from the Brazilian Amazonia, Atlantic Forest and Pantanal biomes. Trypanosomes were detected by a combination of microhaematocrit and haemoculture methods in 45% of the anurans, and 87 cultures were obtained: 44 from Hylidae, 22 from Leptodactylidae, 15 from Bufonidae, 5 from Leiuperidae and 1 from an unidentified anuran. High morphological diversity (11 morphotypes) was observed among blood trypanosomes from anurans of different species and of the same species as well as among trypanosomes from the same individual. Conversely, morphologically similar trypanosomes were found in anurans from distinct species and biomes. ITS and SSU rDNA polymorphisms revealed high diversity among the 82 isolates examined.† Twenty-nine genotypes could be distinguished, the majority distributed in 11 groups. Phylogenetic relationships based on rDNA sequences indicated that isolates from more phylogenetically related anurans are more closely related. Comparison of anuran trypanosomes from Brazil and other countries revealed several new species among the isolates examined in this study. Phylogenetic relationships suggest that host restriction, host switching and overall ecogeographical structure may have played a role in the evolution of the anuran trypanosomes.

Key words: *Trypanosoma*, Amphibia, Anura, Amazonia, genetic polymorphism, phylogeny, evolution, ribosomal sequences, morphology.

INTRODUCTION

Amphibians belonging to the orders Anura (frogs and toads) and Caudata (salamanders) have long been known to be infected with trypanosomes. Trypanosomes in Anura were discovered in 1842 in Europe in the blood of the frog *Rana esculenta* and initially classified as *Amoeba rotatoria*. A year later, this species was denominated *Trypanosoma rotatorium* by Gruby (1843), who thus created the genus *Trypanosoma*. Anuran trypanosomes have been recorded in all continents, as reviewed by Bardsley and Harmsen (1973). After their review, new descriptions of trypanosomes in anurans in Canada and the USA (Werner and Walewski, 1976; Levine and Nye, 1977; Woo and Bogart, 1984; Barta and Desser, 1984), China and Japan (Miyata, 1978; Werner, 1993), Europe (Barta *et al*. 1989; Zickus, 2002) and Costa Rica in Central America (Desser, 2001) were published. No surveys were carried out in South America, where trypanosomes were only incidentally mentioned in anurans in Venezuela, Colombia, Argentina, Peru and Brazil (Bardsley and Harmsen, 1973).

Low parasitaemias make microscopical detection of trypanosomes difficult in the blood of anurans. Nevertheless, anuran trypanosomes have traditionally been classified according to the morphology of a small number of blood trypanosomes, their host and geographical origin and, sporadically, the results of cross-infection experiments. Unfortunately, this approach is not supported by the extreme polymorphism of blood trypanosomes within the same anuran species from the same region or by the marked pleomorphism of life-cycle developmental forms of these parasites. Moreover, trypanosomes in anurans from distant geographical regions and distinct host species can be morphologically indistinguishable (Bardsley and Harmsen, 1973; Werner and Walewski, 1976; Reilly and Woo, 1982; Woo and Bogart, 1984; Martin and Desser 1991*a*; Desser, 2001; Martin *et al*. 2002). Data from natural and laboratory cross-infections suggested that some toad trypanosomes evolved through host switching from frogs to toads. However, these data also revealed a certain degree of host restriction among

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† Nucleotide sequence data reported in this paper are available in the GenBank database under the Accession numbers listed in Table 2.

Parasitology (2007), **134**, 1623–1638.
doi:10.1017/S0031182007003058 Printed in the United Kingdom

anuran trypanosomes, the potential for host-switching being inversely proportional to the evolutionary distance between their hosts (Reilly and Woo, 1982; Martin and Desser, 1991*b*; Martin *et al*. 1992*a*, 2002).

Therefore, morphological, behavioural and host-parasite features render unreliable the identification of anuran trypanosomes based solely on traditional taxonomic parameters. However, before this was recognized, Diamond (1965) referred to 26 species of anuran trypanosomes in a compilation of the literature worldwide. In the largest review of amphibian trypanosomes, Bardsley and Harmsen (1973) examined 68 species of anuran trypanosomes and considered most species non-valid. They proposed guidelines for the taxonomy of these organisms that included morphology, geographical origin, vertebrate and invertebrate host species, life-cycles and biochemical features. Miyata (1978) did not take into account these guidelines and recognized 34 valid species including 6 new ones based mainly on morphology. All these reviews focused on morphology and host origin, and no consensus currently exists as to which species are valid.

Recent phylogenetic studies have revealed that anuran trypanosomes cluster together with fish trypanosomes in the 'Aquatic' clade, which comprises species that infect water vertebrates (fishes, turtles and platypus) and are thought to be transmitted by leeches, a fact favouring host switching (Stevens *et al*. 2001; Jakes *et al*. 2001; Hamilton *et al*. 2004; Gibson *et al*. 2005; Simpson *et al*. 2006). Anurans may live all or part of their lives in an aquatic environment where they may be also preyed upon by leeches (Martin and Desser, 1991*a*; Siddall and Desser, 1992). In addition, anurans are also prey to terrestrial arthropods such as sand flies and mosquitoes (Anderson and Ayala, 1968; Ayala, 1970; Desser *et al*. 1973, 1975) and to terrestrial leeches (Hamilton *et al*. 2005). The interplay of habitats, hosts and vectors makes anuran trypanosomes a unique model for evolutionary studies of trypanosomatids (Simpson *et al*. 2006).

Although anuran trypanosomes occur worldwide and have long been cultured, not many cultures are available, and most studies have included a limited set of trypanosome species, namely, *T. chattoni*, *T. fallisi*, *T. rotatorium* and *T. ranarum* from North America, *T. neveulemairei* from Europe and *T. mega* from Africa. Biochemical and molecular data for anuran trypanosomes are limited and include analysis of zymodemes (Martin *et al*. 1992*a*, *b*), riboprinting (Clark *et al*. 1995), karyotyping (Lun and Desser, 1995) and RAPD patterns (Lun and Desser, 1996). Phylogenetic studies based on SSU rDNA sequences revealed that all the above species, with the exception of *T. chattoni*, which is far from the other anuran trypanosomes, were very closely related (Martin *et al*. 2002). Molecular studies showed that the traditional taxonomy was insufficient to properly address the genetic diversity and phylogenetic relationships between anuran trypanosomes (Martin and Desser, 1990, 1991*b*; Clark *et al*. 1995; Lun and Desser, 1996; Desser, 2001; Martin *et al*. 2002). Since most trypanosome species from Anura were described on the basis of their morphology and host origin, their host-parasite and phylogenetic relationships remain far from understood, and a reliable taxonomy of these organisms is still badly needed. Dealing properly with these questions requires the collection, culturing and comparative analysis of a large number of trypanosomes from anuran of distinct species and geographical origins. In light of this, the aims of the present study were (a) to estimate the occurrence of trypanosomes in the blood of anurans from different Brazilian biomes; (b) to evaluate the morphological diversity of blood and culture forms; (c) to assess the molecular diversity of these trypanosomes by analysing the polymorphisms of their ITS ribosomal sequences and (d) to infer phylogenetic relationships between anuran trypanosomes from Brazil and other countries by analysis of ITS1 and SSU ribosomal sequences.

MATERIALS AND METHODS

Capture and identification of anurans

In this paper we report on the presence of trypanosomes in anurans captured during different seasons in the period 2000 to 2005 in the following Brazilian biomes separated by large geographical distances (the regions in Brazil and the states and cities within the biomes in which the anurans were collected are given in parentheses): Amazonia (AM) (northern region, Rondonia state, Monte Negro); Atlantic Forest (AF) (southeast region, São Paulo, São Paulo, Guarulhos and Biritiba Mirim); Pantanal (PA), wetland (central region, Mato Grosso do Sul, Miranda); and Guaporé (GU), a transition region between the Cerrado and Amazonia (western Brazil, Mato Grosso, Pontes de Lacerda). Four anuran specimens from Cerrado (CE), tropical savanna (southeast region, São Paulo, Rio Claro) captured by Dr Carlos Jared (Butantan Institute, São Paulo, Brazil) were also examined (Fig. 1, Table 1). Anuran captures were performed according to IBAMA (The Brazilian Institute for the Environment and Renewable Natural Resources) recommendations with the collaboration of Dr Miguel T. Rodrigues (Department of Zoology, University of São Paulo, Brazil), who also identified and deposited the captured specimens in the Museum of Zoology of the University of São Paulo. The taxonomy of anurans was recently revised according to Frost (2006). The present report is only concerned with the occurrence of trypanosomes in anurans and their

https://doi.org/10.1017/S0031182007003058 Published online by Cambridge University Press

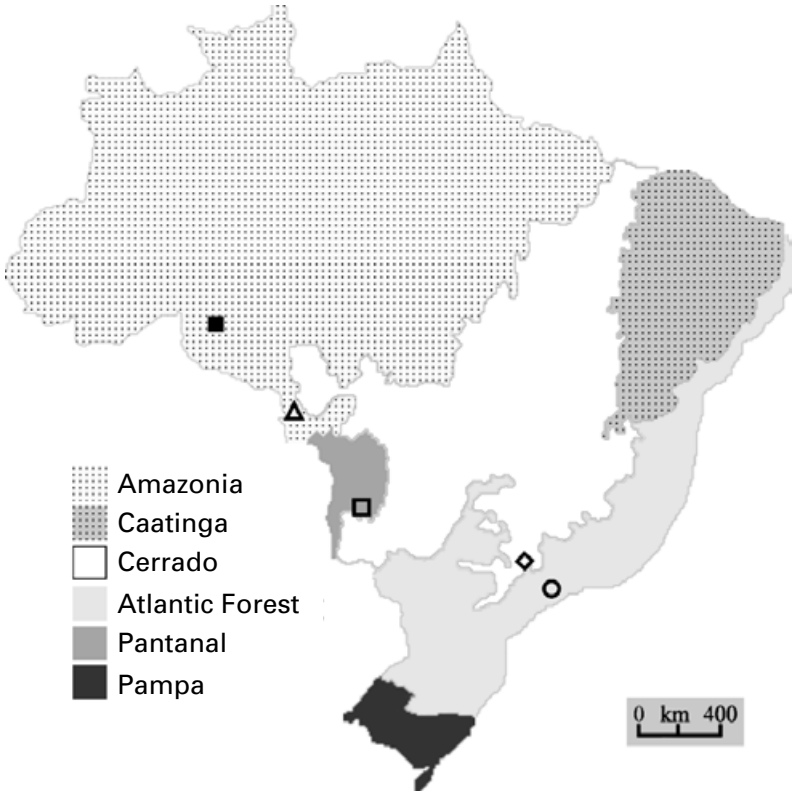

Fig. 1. Geographical origin of Brazilian isolates of anuran trypanosomes. The cities and states within the biomes in which the anurans were collected are: Monte Negro (■), Rondonia in Amazonia; Miranda (□), Mato Grosso do Sul in Pantanal; Mato Grosso; Pontes de Lacerda (△) in Guaporé; São Paulo, Guarulhos and Biritiba Mirim (○) in São Paulo in Atlantic Forest; and Rio Claro (◇), São Paulo in Cerrado.

characterization and was neither planned nor intended to reflect the composition of anuran fauna in any of the regions studied.

Blood survey, isolation in culture and morphology of anuran trypanosomes

The anurans captured were bled by heart puncture using sodium citrate as anticoagulant, and the blood samples examined for the presence of trypanosomes using the microhaematocrit (MH) and haemoculture (HC) methods. In animals captured in Guaporé, part of the anurans from Atlantic Forest, and several hylids from other biomes, the blood was not examined by MH because of the very small blood sample obtained and/or the lack of infield facilities. Haemocultures were performed by inoculating 0·2–0·5 ml of blood in Vacutainer tubes containing a biphasic medium consisting of 15% rabbit red blood cells mixed with 4% Blood Agar Base (DIFCO) overlaid with liquid LIT medium supplemented with 10% FBS. Cultures were maintained in this medium with incubation at 25 °C and expanded for DNA preparation and cryopreservation in liquid N_2. Some positive HCs could not be propagated despite attempts with different media and culture conditions. The culture codes and the anuran hosts and geographical origins are given in Table 2. For morphological analysis, glass-slide smears of the blood from anurans and of the cultures were fixed with methanol and stained with Giemsa.

PCR amplification of ITS1 and SSU rDNA, restriction analysis, sequencing and data analysis

Genomic DNA was extracted from cultured trypanosomes by the classical phenol-chloroform method. The oligonucleotides employed for PCR amplifications of whole ITS rDNA (ITS1/5.8S/ITS2), ITS1 rDNA and the V7–V8 regions of SSU rDNA have been described before (Maia da Silva *et al*. 2004; Rodrigues *et al*. 2006). The PCR-amplified products of SSU and whole ITS genes were cloned, and at least 3 clones from each gene and isolate were sequenced. Length polymorphism of whole ITS rDNA and ITS1 rDNA and restriction site polymorphisms of ITS rDNA digested with *Hin*f I or *Rsa* I enzymes were analysed on 2% agarose gels.

Sequences were aligned using ClustalX and the alignment obtained was refined manually. There are no ITS rDNA sequences from anuran trypanosomes deposited in GenBank. ITS rDNA sequences of other trypanosome species were not included in the analysis due to unreliable alignments. Phylogenetic inferences were assessed by the Parsimony (P) and Bayesian (B) methods. Analysis was conducted in PAUP* v4b10 via 100 random-addition sequence replicates followed by a branch swap (RAS-TBR).

https://doi.org/10.1017/S0031182007003058 Published online by Cambridge University Press

Table 1. Host and biomes of Brazilian anurans examined in this study

(Trypanosome infection determined by MH and HE methods, isolates obtained in culture, and morphotypes associated to trypanosomes observed in blood of anurans.)

Host species of origin			Geographical origin of anurans[a] No. of individuals: examined\positive[b]						No. of isolates in culture[c]	Morpho-type[d]
Family	Genus	Species	AF	AM	PA	GU	CE	Total		
Bufonidae	*Chaunus*	*ornatus*	2\1					2\1	0	
		granulosus		1\0				1\0	0	
		ictericus	20\2					20\2	2	11
		marinus		9\3				9\3	2	1, 3, 5, 6
		schneideri	1\1		20\8	1\0		22\9	6	11
	Rhaebo	*guttatus*		5\0		1\1		6\1	1	
	Rhinella	*margaritifera*		12\5		2\0		14\5	4	2, 3
Centrolenidae	*Hyalino-batrachium*	*eurygnathum*	2\0					2\0	0	
Hylidae	*Aplastodiscus*	*leucopygius*	10\3					10\3	2	
	Bokermannohyla	*circumdata*	6\5					6\5	3	
		hylax	1\0					1\0	0	
	Dendropsophus	*berthalutzae*	3\0					3\0	0	
		leucophyllatus		1\0				1\0	0	
		microps	4\0					4\0	0	
		nanus			1\0			1\0	0	
	Hypsiboas	*albomarginatus*	2\2					2\2	1	
		bischoffi	13\6					13\6	6	
		boans		3\3		3\3		6\6	3	9, 10
		faber	2\2					2\2	2	7, 9
		geographicus		2\0		1\1		3\1	1	
		prasinus	3\2					3\2	2	
		punctatus			1\1			1\1	1	
		raniceps			4\2	5\5		9\7	5	8, 9, 10
	Itapotihyla	*langsdorffii*	1\1					1\1	1	
	Scinax	*acuminatus*			3\2			3\2	2	
		fuscovarius				2\0		2\0	0	
		hayii	2\2					2\2	2	9
		nebulosus				1\0		1\0	0	
		perpusillus	1\0					1\0	0	
		ruber		4\1				4\1	1	9
	Osteocephalus	*taurinus*				1\1		1\1	1	
		sp		1\1				1\1	1	9
	Phyllomedusa	*hypochondrialis*				1\0		1\0	0	
		sp		3\2		1\1		4\3	2	9
		tomopterna		2\1				2\1	0	
	Sphaenorhynchus	sp	1\0					1\0	0	
	Trachycephalus	*venulosus*			16\12	1\1		17\13	8	8, 10
Brachycephalidae	*Eleutherodactylus*	*binotatus*	2\0					2\0	0	
		fenestratus		2\1				2\1	0	
		guentheri	2\0					2\0	0	
		zeuctotylus		5\1				5\1	0	
Hylodidae	*Hylodes*	*phyllodes*	3\0					3\0	0	
Leptodactylidae	*Leptodactylus*	*chaquensis*			23\22	2\1		25\23	19	2, 3, 4, 8, 9, 10
		knudseni		1\0				1\0	0	
		labyrinthicus					4\2	4\2	2	3, 8, 10
		pentadactylus		1\1				1\1	1	3
		stenodema		1\0				1\0	0	
Leiuperidae	*Engystomops*	*petersi*		10\6				10\6	5	3
	Physalaemus	*moreirae*	1\0					1\0	0	
		sp	1\0					1\0	0	
Cycloramphidae	*Proceratophrys*	*boiei*	6\0					6\0	0	
Microhylidae	*Ctenophryne*	*geayi*		3\1				3\1	0	
ND	ND	ND	1\0	9\1				10\1	1	
Total	21	48	90\27	75\27	68\47	22\14	4/2	259\117	87	

[a] Geographical origin of anurans: AF, Atlantic Forest; AM, Amazonia; PA, Pantanal; GU, Guaporé; CE, Cerrado.
[b] Number of anuran specimens examined for the presence of trypanosome by a combination of MH and HE methods.
[c] Isolates obtained from the hemocultures of anuran blood samples that propagated in culture and were cryopreserved.
[d] Morphotype: distinct trypanosome forms observed in Giemsa-stained smears of anuran blood samples (Fig. 2).

https://doi.org/10.1017/S0031182007003058 Published online by Cambridge University Press

Gaps were treated as fifth state and branches whose minimum length was zero were collapsed. Bootstrap analysis (100 replicates) was done using the same parameters described for the searches including only informative characters. Distance matrices were generated using uncorrected *p*-distance. Bayesian analysis was done using MrBayes v3.1.2 (Ronquist and Huelsenbeck, 2003). Tree searches employed GTR plus gamma and proportion of invariable sites. The first 25% of the trees from 100 000 generations were discarded as burn in.

To infer phylogenetic relationships between anuran trypanosomes from Brazil and those from other countries, ~750 bp of SSU rDNA corresponding to the V7–V8 variable region plus the conserved flanking region were identified in this study and aligned with sequences from the following anuran trypanosomes from GenBank (Accession number): *T. rotatorium* (B2-II) (AJ009161); *T. neveulemairei* (AF119809); *T. mega* (AJ223567); *T. fallisi* (AF119806); *T. ranarum* (AF119810); and *T. chattoni* (AF119807). *T. therezieni* (AJ223571) from *Chamaeleo brevicornis*, which clustered with anuran trypanosomes, was also included. The following sequences of trypanosomes from fish were used as outgroup for anuran trypanosomes (Hamilton *et al*. 2004): *T. boissoni* (U39580); *T.* sp. CLAR (AJ620555); *T. granulosum* (AJ620552) and *T. triglae* (U39584). In addition, *T.* sp. K&A from aquatic leech (AJ009167); *T. binneyi* from platypus (AJ620565) and *T. chelodinae* from aquatic turtle (AF297086), which clustered with fish trypanosomes, were also included in the alignment. Alignments used in this study are available from the authors upon request.

RESULTS

Occurrence of trypanosomes in anurans

Detection of trypanosomes by MH yielded 48 positive individuals out of 124 examined (39%) whereas HC yielded 111 positive individuals out of 259 (43%) (Table 1). Positive haemocultures were obtained from both MH-positive and MH-negative individuals. The overall prevalence of blood trypanosomes in anurans assessed by the combination of the two methods was 45% (117 of 259). Of the 117 positive animals, 100 belong to 48 nominal species, 7 had only been identified at the generic level at the time this study was carried out, and 10 remained unidentified (Table 1). The prevalence (%), the number of positive individuals (+), the total number of individuals examined (N) and the total number of anuran species recovered (S) in the different biomes were, respectively, 69% (+47, N 68, S 7) in the Pantanal; 64% (+14, N 22, S 12) in Guaporé; 36% (+27, N 75, S 17) in Amazonia; 30% (+27, N 90, S 21) in the Atlantic Forest; and 50% (+2, N 4, S 1) in Cerrado (Table 1). The percentage of different anuran species infected by trypanosomes was highest in the PA (86%), followed by Amazonia (65%), Guaporé (58%) and the Atlantic Forest (52%). The occurrence of trypanosomes in the anuran families examined was 28% (+21, N 74, S 6) in Bufonidae; 57% (+60, N 106, S 26) in Hylidae; 81% (+26, N 32, S 5) in Leptodactylidae; 18% (+2, N 11, S 4) in Brachycephalidae; 50% (+6, N 12, S 2) in Leiuperidae; and 33% (+1, N 3, S1) in Microhylidae (Table 1).

Different trypanosome infection indices were found among anuran of distinct species independent of their family, namely, 92% for *Leptodactylus chaquensis* (Leptodactylidae); 77% for *Trachycephalus venulosus* (Hylidae); 60% for *Engystomops petersi* (Leiuperidae); 46% for *Hypsiboas bischoffii* (Hylidae); 41% for *Chaunus schneideri* (Bufonidae); 36% for *Rhinella margaritifera* (Bufonidae); 30% for *Aplastodiscus leucopygius* (Hylidae), and 10% for *Chaunus ictericus* (Bufonidae). Only species for which more than 10 individuals were examined are mentioned.

Morphology of blood trypanosomes

Microhaematocrit analysis revealed that most anurans had low parasitaemias, with few trypanosomes found in Giemsa-stained blood smears or even in smears of buffy-coat layers from MH capillaries. Microscopy revealed trypanosomes that varied greatly in size and shape not only between distinct anuran species but also between individuals of the same species and even within the same individual. On the other hand, very similar trypanosomes were found infecting anurans of distinct species from the same or different families (Fig. 2).

Comparison of the main morphological features of Giemsa-stained blood trypanosomes, including shape, size, kinetoplast position and features of the nucleus and undulating membrane, revealed at least 11 major morphotypes (M1 to M11) separable in 2 groups (I and II), as shown by the selected photomicrographs in Fig. 2. In addition to the major 11 morphotypes, unusual forms represented by a very small number of individuals and not associated with a particular species were also found in blood samples (data not shown). Whenever possible, morphotypes were associated with previously described species of anuran trypanosomes, but despite careful analysis of drawings and photomicrographs in the literature, association of the morphotypes described here with reported species was very often a particularly difficult and highly subjective task (Diamond, 1965; Bardsley and Harmsen, 1973; Miyata, 1978; Reilly and Woo, 1982; Woo and Bogart, 1984; Barta and Desser, 1984; Werner, 1993; Desser, 2001).

A greater number of group I trypanosomes than group II trypanosomes was detected in blood samples, indicating a higher parasitaemia for this type of trypanosome in the anurans examined.

Table 2. Host and geographical origin of anuran trypanosome isolates obtained in this study

(Genotyping by polymorphisms of ITS rDNA and morphotypes of blood trypanosomes from their respective anuran of origin.)

Host species		Isolate TryCC[a]	Geographical origin[b]	Length of ITS rDNA[c]		Genotype[d]	Morpho-type[e]	GenBank Accession numbers	
Family	Species			wITS	ITS1			ITS1rDNA	SSUrDNA
Buf.	*C. marinus*	364	AM	760 & 700	(180) 190 & 250	A1		EF457247-50	EF457295
	R. margaritifera	367	AM	760 & 700		A1			
	R. margaritifera	399	AM	760 & 700		A1	3		
Lep.	*L. pentadactylus*	398	AM	760 & 700		A1	3		
	N.D.	365	AM	760 & 700		A1			
Lei.	*E. petersi*	401	AM	760 & 700		A1	3		
	E. petersi	402	AM	760 & 700		A1			
	E. petersi	405	AM	760 & 700		A1	3		
	E. petersi	408	AM	760 & 700		A1	3		
Buf.	*C. marinus*	339	AM	780 & 730	(210) 210 & 280	A2	1, 3, 5, 6	EF457244-46	EF457294
Hyl.	*B. circumdata*	933	AF	990	290	B			
	H. bischoffi	932	AF	990		B			
	H. bischoffi	944*	AF	990		B			
Buf.	*C. ictericus*	858	AF	1020	(287)	C1	11	EF457266-69	EF457290
	C. ictericus	868	AF	1020		C1	11		
	C. schneideri	311	PA	1020		C2			
	C. schneideri	322	PA	1020	(293)	C2		EF457263-65	EF457289
	C. schneideri	325	PA	1020		C2			
	C. schneideri	441	PA	1020		C2	11		
	C. schneideri	598	AF	1020		C2			
Hyl.	*S. hayii*	645*	AF	1020		C3	9		
	B. circumdata	287*	AF	1020		C4			
	H. faber	641	AF	1020		C4	7		
	H. faber	950*	AF	1020		C4			
	A. leucopygius	288	AF	1020		C4			
	I. langsdorffii	644	AF	1020	320	C4			
Lep.	*L. chaquensis*	324	PA	1020		C5			
	L. chaquensis	439	PA	1020		C5	10		
	L. chaquensis	440	PA	1020		C5	8		
	L. chaquensis	443	PA	1020		C5			
	L. chaquensis	445	PA	1020		C5			
	L. chaquensis	446	PA	1020		C5	8		
	L. chaquensis	447	PA	1020		C5	4, 8, 10		
	L. chaquensis	966	PA	1020		C5			
Hyl.	*H. bischoffi*	282*	AF	1070		D1			
	H. prasinus	290	AF	1070		D1			
Buf.	*R. margaritifera*	346	AM	1070	(262) 280	D2		EF457255-58	EF457292
	R. margaritifera	362	AM	1080	(266) 280	D3		EF457259-62	EF457293
Lep.	*L. chaquensis*	317	PA	1090		E			
	L. chaquensis	327	PA	1090		E			
	L. chaquensis	436*	PA	1090		E	8, 10		
	L. chaquensis	444	PA	1090	(305) 330	E	10	EF457270-73	EF457288

Hyl.	*H. boans*	616	GU	1100		F			
	Osteocephalus sp	357	AM	1100	270	F	9		
Lep	*L. labyrinthicus*	920	CE	1120	350	U1	3		
Lei.	*E. petersi*	407	AM	1130		G1	3		
Hyl.	*S. hayii*	660	AF	1130	370	G2			
	S. ruber	406	AM	1150	310	U2	9		
Lep.	*L. labyrinthicus*	928	CE	1160		H1	8, 10		
Hyl.	*O. taurinus*	614	GU	1160	370	H2			
	T. venulosus	448*	PA	1160		H3	8		
	T. venulosus	457	PA	1160		H3			
	H. boans	617	GU	1200		I1	9, 10		
	H. bischoffi	291*	AF	1200	440	I2			
	H. albomarginatus	647	AF	1260		J1			
	A. leucopygius	646	AF	1260	(402)	J1		EF457278-80	EF457285
	H. bischoffi	939	AF	1260		J1			
	H. boans	615*	GU	1260		J2			
	H. geographicus	612	GU	1260		J2			
	H. punctatus	304	PA	1260		J2			
	H. raniceps	618	GU	1260		J2			
	H. raniceps	613	GU	1260		J2			
	H. raniceps	619	GU	1260		J2			
	H. raniceps	622*	GU	1260		J2			
	T. venulosus	305	PA	1260	(401)	J2		EF457281-84	EF457286
	T. venulosus	620*	GU	1260		J2			
	T. venulosus	313	PA	1260		J2			
	Phyllomedusa sp	400	AM	1260		J2	9		
	Phyllomedusa sp	358	AM	1260	440	J2			
	S. acuminatus	442	PA	1260		J2			
	S. acuminatus	321	PA	1260		J2			
	T. venulosus	334	PA	1260		J3			
	T. venulosus	465	PA	1260		J3			
	H. raniceps	467	PA	1260		J4	8, 9, 10		
	T. venulosus	315	PA	1260	(385)	J4		EF457274-77	EF457287
	H. bischoffi	653	AF	1380	600	U3			
	H. prasinus	934*	AF	1420 & 1070	520 & 330	U4			
Lep.	*L. chaquensis*	306	PA	1460		K			
	L. chaquensis	316	PA	1460	(570) 580	K	2, 4, 8	EF457251-54	EF457291
	L. chaquensis	326	PA	1460		K	3, 8, 10		
	L. chaquensis	449	PA	1460		K	8		
	L. chaquensis	492	PA	1460		K	8		

[a] Cultures of anuran trypanosomes are cryopreserved in the Trypanosomatid Culture Collection of the Department of Parasitology, University of São Paulo, São Paulo, Brazil. TryCC correspond to number codes of isolates cryopreserved in this collection.

[b] Geographical origin (biomes) of anurans from which cultures were isolated: AM, Amazonia; AF, Atlantic Forest; PA, Pantanal; GU, Guaporé; CE, Cerrado.

[c] Length in base pairs (bp) of PCR-amplified whole ITS (wITS) or ITS1 rDNA sequences determined by separation in agarose gel (approximated length) or by sequencing (in parentheses).

[d] Groups (A–K) and genotypes (indicated by letters and numbers) defined by length and sequencing polymorphism of ITS and SSU rDNA sequences; U, unique genotypes; * Mixed cultures.

[e] Morphotypes (M) of trypanosomes found in blood smears of anurans from which cultures were obtained. Anuran families: Buf, Bufonidae; Lep, Leptodactylidae; Lei, Leiuperidae; Hyl, Hylidae.

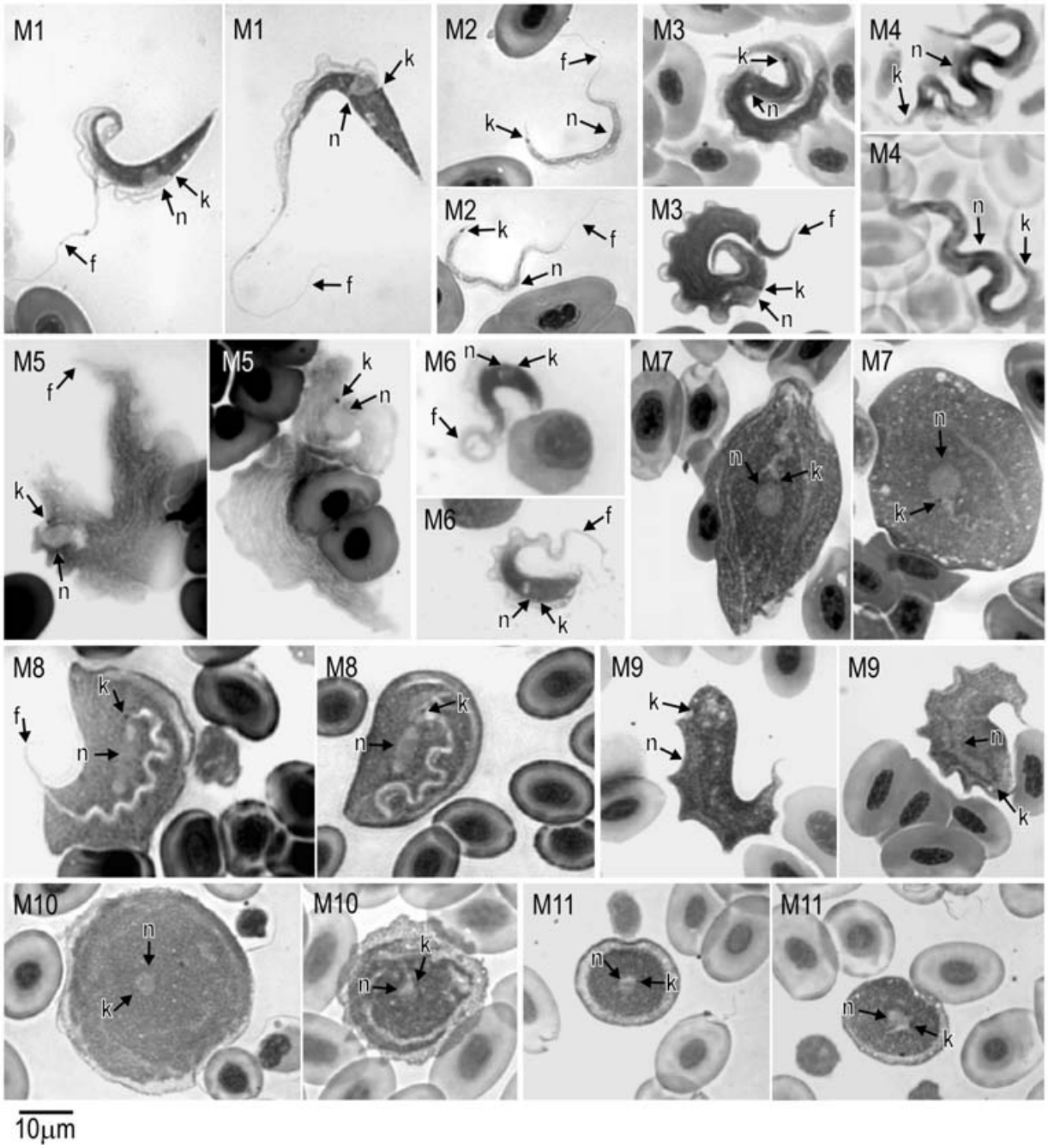

Fig. 2. Photomicrographs selected to illustrate the morphological diversity of trypanosomes found in blood smears (Giemsa stained) of Brazilian anurans, which are distributed in 2 major groups (I and II) comprising 11 morphotypes (M1–M11): Group I, elongated trypomastigotes with pointed ends observed in Bufonidae (M1, M2, M3, M5 and M6), Leiuperidae (M3) and Leptodactylidae (M2, M3, M4), and Group II, leaf-shaped, rounded or elliptical trypanosomes found mostly in Hylidae (M7, M8, M9, M10) and Leptodactylidae (M8, M9, M10). Morphotypes associated with previously described species of anuran trypanosomes are: M1 (*T. bocagei*-like); M3 (*T. leptodactily*-like); M5 (*T. fallisi*-like); M7 (*T. loricatum*-like); M8 (*T. rotatorium*-like), M10 (*T. chattoni*-like); and M11 (*T. tsunezomiytai*-like). k, Kinetoplast; f, flagellum; n, nucleus.

Group I morphotypes were observed in Bufonidae (M1, M2, M3, M5 and M6), Leiuperidae (M3) and Leptodactylidae (M2, M3, M4) whereas group II morphotypes were found mostly in Hylidae (M7, M8, M9, M10) and Leptodactylidae (M8, M9, M10). Interestingly, morphotypes found in Bufonidae were not found in Hylidae and group II morphotypes were reported in North American ranids and bufonids (Bardsley and Harmsen, 1973).

Group I comprises elongated trypomastigotes with pointed ends, which were separated into 6 morphotypes (M1–6), of which only 3 were associated with a previously described species: M1 (*T. bocagei*-like that is similar to *T. bufophlebotomi*), large and

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wide forms with central nucleus and kinetoplast, conspicuous undulant membrane and long free flagellum; M2, the thinnest trypomastigotes, with the kinetoplast at the end of a posterior extremity, long free flagellum and slight undulant membrane; M3 (*T. leptodactily*-like), with S-like or roll-shaped bodies with conspicuous, many-folded undulating membranes and the kinetoplast at the posterior extremity; M4, slender trypanosomes with serpentine bodies and small undulating membranes; M5 (*T. fallisi*-like), the largest and widest trypomastigotes with many longitudinal striations and large nucleus at the posterior extremity; and M6, the smallest forms, with a well-developed undulating membrane and rounded posterior end (Fig. 2).

Group II is formed by leaf-shaped, rounded or elliptical trypanosomes distributed in 5 morphotypes: M7 (*T. loricatum*-like), flagellates with elliptical, broad and costate bodies, spherical nucleus, and many-folded undulating membranes without a free flagellum; M8 (*T. rotatorium*-like), forms with wide and large bodies, fusiform nucleus, conspicuous undulating membranes and a short free flagellum; M9 (probably *T. rotatorium*-like), small dark-staining forms with a rounded anterior end and a small free flagellum; M10 (*T. chattoni*-like), trypanosomes with largest and irregular rounded bodies with clear borders, a central small and spherical nucleus with the kinetoplast appended to it, without undulating membranes; and M11 (*T. tsunezomiytai*-like), forms similar to *T. chattoni* but with small spherical bodies, clear and regular borders.

The same anuran species had a maximum of 3 morphotypes (Fig. 2; Table 1). Exceptions were *Leptodactylus chaquensis* and *Chaunus marinus*, which had 6 and 4 morphotypes, respectively, although no more than 4 morphotypes were observed in the same animal. Some morphotypes were detected in only 1 anuran species: for example, M1, M5 and M6 in *Chaunus marinus*, M7 in *Hypsiboas faber* and M4 in *Leptodactylus chaquensis* (Fig. 2; Table 1).

Isolation in culture of anuran trypanosomes and morphology

Trypanosomes could be observed after approximately 10 days in most haemocultures. In recent cultures, the flagellates multiplied as small round bodies known as spheromastigotes, which often clustered into rosettes (Fig. 3I, M, N). After a few days, the flagellates began to elongate while the rosettes disintegrate, releasing free epimastigotes (Fig. 3I, M). Multiplication occurs by either binary and/or multiple divisions. Like the blood forms, the cultured forms of anuran trypanosomes showed high polymorphisms both among and within cultures. Although the cultures were not cloned, it was found by means of molecular analysis that most pleomorphic cultures probably consisted of only 1 isolate, except for a few mixed samples disclosed by ITS rDNA analysis (Fig. 4). Some cultures shared morphological features while others exhibited unique and peculiar morphologies (Fig. 3). Epimastigotes varied in body shape and size, in the length and width of the body, and in the size and position of the nucleus and kinetoplast, in the development of the undulating membrane and in the length of the free flagellum. While the kinetoplast in all the different blood morphotypes is always very small (Fig. 2), in the cultured epimastigotes it varies considerably in size and position (Fig. 3A, B). A few cultures had very large, slender and pointed epimastigotes, with the kinetoplast positioned far from the nucleus (Fig. 3A). Most cultures had smaller epimastigotes with slender (Fig. 3B, E, K, L), wide (Fig. 3C, D, F) or short (Fig. 3G, H, J) predominant forms. A small number of trypomastigote forms, all of which were distinct from blood trypomastigotes, could be observed in stationary-phase cultures; these included large and wide forms with a very well-developed undulant membrane (Fig. 3O, P) and very small trypomastigote forms (Fig. 3Q, R).

Genetic diversity among anuran trypanosomes evaluated by ITS rDNA length and restriction polymorphisms

Analysis of length polymorphism of amplified whole ITS rDNA or ITS1 rDNA disclosed high diversity among the 82 trypanosomes examined, which were isolated from 25 anuran species, but did not allow phylogenetic relationships to be inferred. Amplified fragments ranged from ~700 to 1460 bp for wITS (whole ITS) and from ~180 to 600 bp for ITS1 (Fig. 4, Table 2). To evaluate the polymorphism within the groups, isolates were analysed by restriction patterns of amplified wITS. Length and restriction site polymorphisms of the ITS rDNA revealed high genetic diversity among the 82 isolates examined. Together, length, restriction and sequence polymorphisms permitted most isolates to be distributed into 11 major groups (A–K). Some isolates showed 2 amplified fragments, suggesting mixed infections (Fig. 4, Table 2). Polymorphisms of ITS rDNA, together with sequence polymorphisms of SSU rDNA sequences, from 11 selected isolates presenting different genotypes representative of 6 groups disclosed high genetic variability, with at least 29 distinguishable genotypes. Most isolates, representing 25 genotypes distributed in 11 major groups, each group comprising isolates that shared genotypes and morphological features in cultures. Four isolates showed unique and ungrouped genotypes (U1 to U4) (Fig. 4, Table 2). Some considerations regarding the distribution of genotypes among the anuran families deserve to be mentioned. Firstly, there appears to be a consistent association between trypanosome genotype and anuran family, with trypanosome genotypes

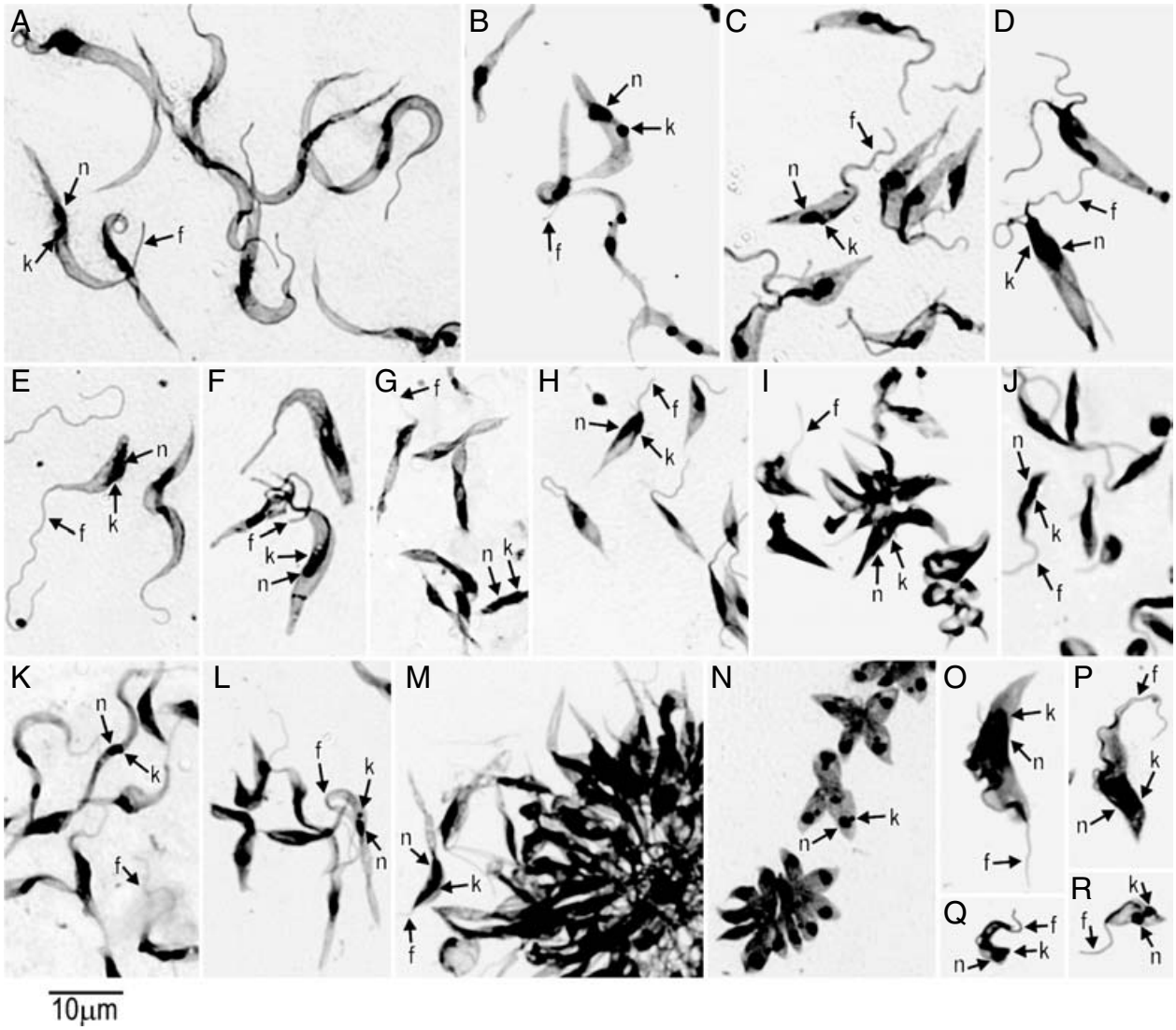

Fig. 3. Photomicrographs illustrative of the morphological diversity of culture forms (Giemsa stained) of isolates from Brazilian anurans. Logarithmic-phase culture epimastigote forms varying in body shape and size, length of free flagellum and in size and position of kinetoplast, with the following predominant body forms: large and slender (A); of medium size and slender (B, E, K, L); of medium size and wide (C, D, F); and small (G, H, J). Stationary-phase cultures showing large and wide (O, P) and very small (Q, R) trypomastigote forms. The following cultures of anuran isolates are represented: A, 322; B, 858; C, 367; D, 346; E, 339; F, 321; G, 316; H, 407; I, 444; J, 315; K, 291; L, 357; M, 316; N, 287; O, 448; P, 620; Q and R, 321. k, Kinetoplast; f, flagellum; n, nucleus.

C1 and C2 found in Bufonidae and genotypes B, C4, D1, F, H3 and J1–J4 found in Hylidae. There also appears to be a consistent association between some anuran species and certain genotypes such as *Leptodactylus chaquensis* and genotypes C5, E and K; *Chaunus ictericus* and genotype C1; *Chaunus scheneideri* and genotype C2; and *Trachycephalus venulosus* and genotypes J3 and H3. This may suggest the existence of a certain degree of host-restriction between anuran species and their trypanosomes. However, isolates of group A occurred in Bufonidae, Leptodactylidae and Leuperidae and some anurans harboured more than 1 distinct genotype, as is the case of *Leiptodactylus chaquensis* (3 genotypes), *Hypsiboas bischoffii* (5) and *Trachycephalus venulosus* (4) (Fig. 4). Thus, these data indicate that if host restriction does indeed exist, it is not an absolute axiom for anuran trypanosomes, as some genotypes are shared by more than 1 species of different genera and sporadically by species from distinct families of anurans.

The distribution of the trypanosome genotypes in the different biomes deserves some considerations. Some genotypes, in addition to having a host-family association, were found in only 1 biome. Thus, all trypanosomes of genotype A (from bufonids, leptodaclylids and leiuperids) are from Western Amazonia; trypanosomes of genotypes B, C4, D1 and J1 (hylids) and C1 (bufonids) are all from the Atlantic Forest; trypanosomes of genotypes C5, E and K (leptodaclylids) and H3 and J3–4 (hylids) are from the Pantanal. Exceptions were some genotypes

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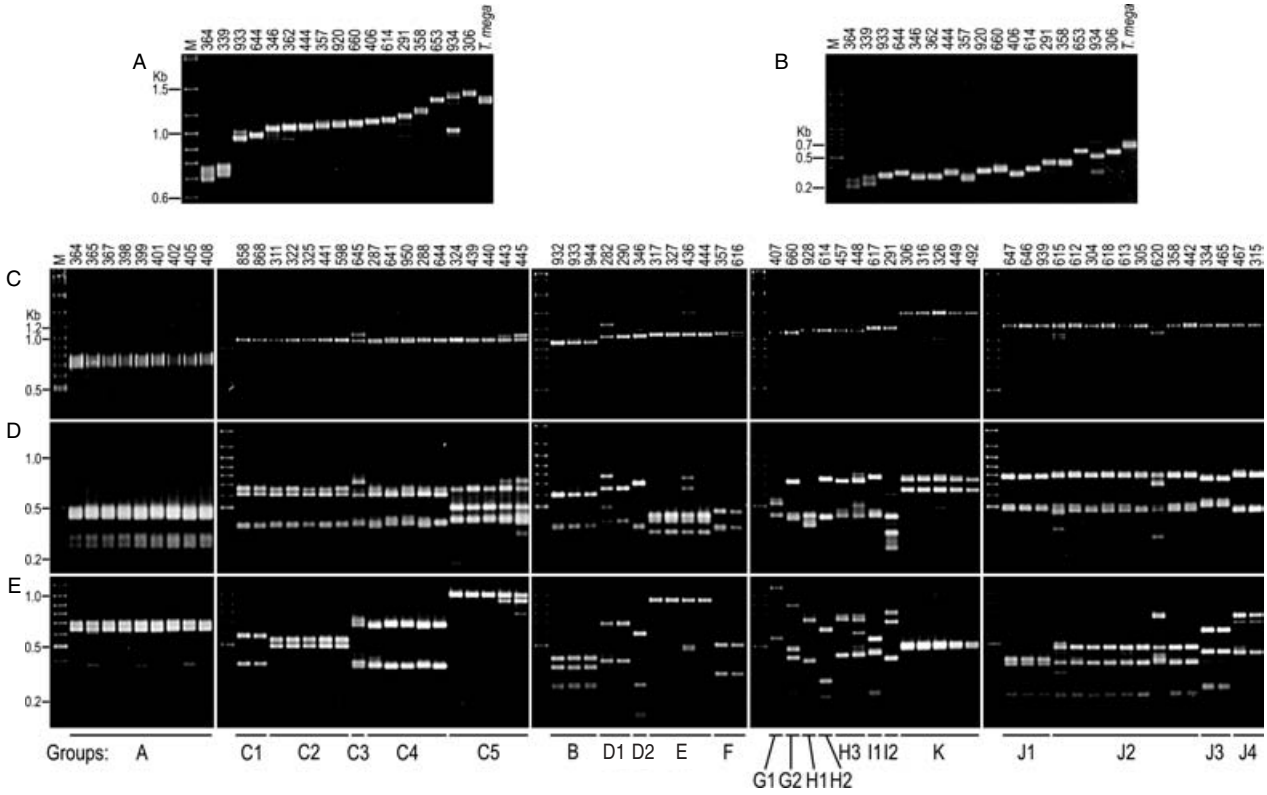

Fig. 4. Analysis of length and restriction site polymorphism of trypanosome isolates from Brazilian anurans. PCR-amplified wITS rDNA (A) and ITS1 rDNA (B) sequences from 17 selected isolates plus *T. mega* to illustrate the high degree of polymorphism among the anuran trypanosomes. Restriction fragment length polymorphism (RFLP) of PCR-amplified wITS rDNA (C) digested with the restriction enzymes *Hinf* I (D) or *Rsa* I (E). Agarose gels (2%) stained with ethidium bromide. M, molecular marker (1 kb).

of hylid trypanosomes: J2, from Amazonia, Guaporé and the Pantanal, and F, from Amazonia and Guaporé. It must be remembered, however, that Amazonia and the Atlantic Forest are biomes separated by a very large geographical distance and that there is a high level of endemism among anuran species living in these biomes. In contrast, the Pantanal is located between these biomes and shelters anurans from Amazonia and the Atlantic Forest, and Guaporé is a small transition area between Amazonia and the Pantanal in which anuran fauna consists of species from both these biomes (Table 2).

Phylogenetic relationships among Brazilian anuran trypanosomes using ITS1 rDNA sequences

To infer the phylogenetic relationships among anuran trypanosomes ascribed to different genotypes, we selected 11 genotypes from 8 groups to compare their ITS1 and SSU rDNA sequences (Table 2). The high heterogeneity of ITS1 sequences detected is in accordance with the ITS1 length polymorphism and with the polymorphisms on restriction profiles of wITS rDNA (Fig. 4, Table 2). Analysis of ITS1 aligned sequences disclosed large blocks of deletions and insertions in addition to regions with numerous substitutions and few blocks of conserved sequences (data not shown). Altogether, ITS rDNA sequences from the 11 isolates shared only 47% ITS1 sequence similarity, with divergences ranging from ~80% to 4·2% among the isolates. Besides significant sequence divergence among the isolates, considerable divergence was also observed among the 3 or 4 cloned sequences of ITS1 rDNA from the same isolate, with divergences ranging from 0·0% (isolate 316, 346, 362) up to 9·2% (isolate 339). However, sequences from the same isolate clustered together despite divergences, indicating that they belong to the same isolates and are not sequences from mixed cultures. Dendrograms of ITS1 sequences segregated the isolates into the following 3 major clusters: An01 (average sequence divergence of ~28·3%); An02 (~40·0%); and An03 (~37%). Very similar branching patterns resulted using the Parsimony (Fig. 5) and the Bayesian (data not shown) methods.

Cluster An01 comprises 4 flagellates: 3 are tightly clustered isolates of hylids from the Atlantic Forest (isolate 646) and Pantanal (305 and 315), while 1 is an isolate of a leptodactylid from the Pantanal (444). Cluster An02 grouped 5 isolates: 2 from bufonids (isolates 322 and 858) from the Pantanal and Atlantic Forest, respectively, 2 tightly clustered bufonid isolates (346 and 362) from Amazonia, in addition to more distant isolate 316 of a leptodactylid from the Pantanal. Cluster An03 consisted of 2 bufonid

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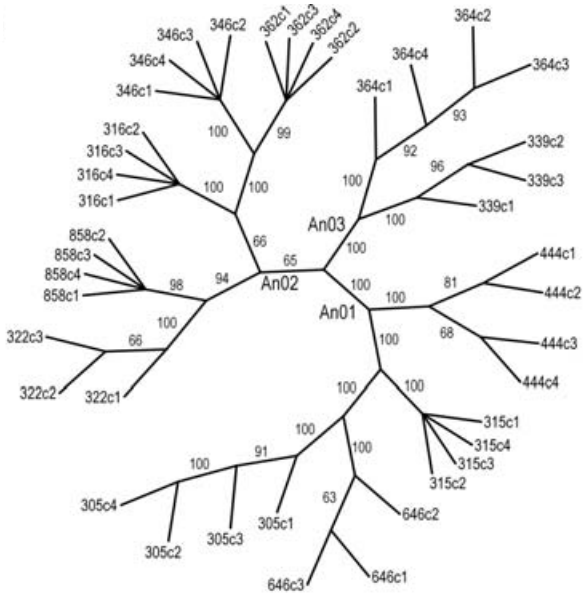
A



B

	1	2	3
1 An01	28.27 (10.6-41.5)		
2 An02	57.17 (39.3-71.8)	40.00 (4.2-62.4)	
3 An03	68.74 (62.0-71.5)	62.62 (58.1-80.0)	36.98 (-)

Fig. 5. (A) Unrooted dendrogram based on Parsimony analysis of 41 cloned ITS1 sequences from 11 isolates (3 to 4 clones from each isolate) of Brazilian anuran trypanosomes. The numbers at nodes correspond to percentage of bootstrap values derived from 100 replicates. (B) Matrix of average sequence divergence among anuran trypanosomes clustered in An01, An02 and An03 clades. Range given in parentheses. Length of 4 most parsimonious trees: 941 steps. Length of strict consensus: 942 steps; CI = 0·87; RI = 0·97.

isolates (364 and 339) from Amazonia (Fig. 5B). Although leptodactylid isolates were clustered in clades An01 and An02, their sequences were the most divergent within each clade, suggesting distant relationships of leptodactylid trypanosomes with both hylid and bufonid trypanosomes.

Phylogenetic inferences among anuran trypanosomes from Brazil and other countries using SSU rDNA sequences

To infer the phylogenetic positioning of Brazilian anuran trypanosomes in relation to anuran trypanosomes from other countries, comparative analysis was performed using partial SSU rDNA sequences from the 11 selected isolates and sequences available on GenBank from trypanosomes of exotic anurans. All the analyses corroborated the clustering of all the anuran trypanosomes and indicated the position of fish trypanosomes as a basal group for the anuran clade. The branching pattern of the phylogenetic trees showed very similar topologies in both Parsimony (Fig. 6A) and Bayesian (data not shown) analysis. Analysis based on SSU rDNA sequences confirmed the phylogenetic relationships inferred by ITS1 sequence analysis, with the isolates also segregating in the 3 main clades: An01, An02 and An03. Clades An01 (divergence ranging from 0·4% to 1·4%), and An02 (0·0% to 3·0% divergence), clustered together (99% bootstrap) and shared ~94·8% similarity (Fig. 6B). All the Brazilian isolates separated from most species of exotic trypanosomes (Fig. 6A). *T. chattoni* was the closest to the Brazilian isolates despite high divergence (12·39–14·95%) and was positioned close to clade An03, which consisted of 2 bufonid isolates from Amazonia (2·3% divergence). Anuran trypanosomes from North America (*T. fallisi*, *T. ranarum*, *T. rotatorium*), Europe (*T. neveulemairei*) and Africa (*T. mega*) clustered together (100% bootstrap) in clade An04 despite significant divergence (15·3%). The divergence separating Brazilian isolates from the exotic isolates in clade An04 ranged from 19·2 to 19·9% (Fig. 6B).

Brazilian isolates showed significant sequence polymorphism in the V7–V8 region of the SSU rDNA (5·7% average divergence). Because the V7–V8 SSU rDNA sequences were very conserved compared with the highly polymorphic ITS1 sequences, only 8 of the 11 isolates distinguished by ITS rDNA polymorphism were separated by significant SSU rDNA sequence divergence. Trypanosomes from the same or from very closely related anuran species showed identical or very similar SSU rDNA sequences. This was true for (a) isolates 322 (genotype C2) and 858 (C1) respectively from the bufonids *Chaunus schneideri* and *Chaunus ictericus*, which shared 100% and ~93% SSU and ITS1 sequence similarity, respectively; (b) isolates 346 (D2) and 362 (D3) both from the bufonid *Rhinella margaritifera* (99·9% and ~96% SSU and ITS1 similarity, respectively) and (c) isolates 364 (A1) and 339 (A2) from the bufonid *Chaunus marinus* (97·7% and ~91% SSU and ITS similarity, respectively).

DISCUSSION

Anuran trypanosomes represent the largest known assemblage of trypanosomes among vertebrate orders (Bardsley and Harmsen, 1973). However, their taxonomy was largely built on the proven insufficient criteria of morphology and host origin, with studies including molecular markers being relatively scarce (Clark *et al.* 1995; Martin *et al.* 1992*a*, *b*; Lun and Desser, 1995, 1996). As a result, trypanosomes that probably belong to the same species have been classified in separate ones, leading to a profusion of species. Similarly, distinct species may have been considered as a single species merely because the trypanosomes come from the same host and/or are morphologically indistinguishable. The use of unreliable taxonomic parameters over a long period caused considerable confusion and prevented correct appraisal of the taxonomy, biology, diversity and

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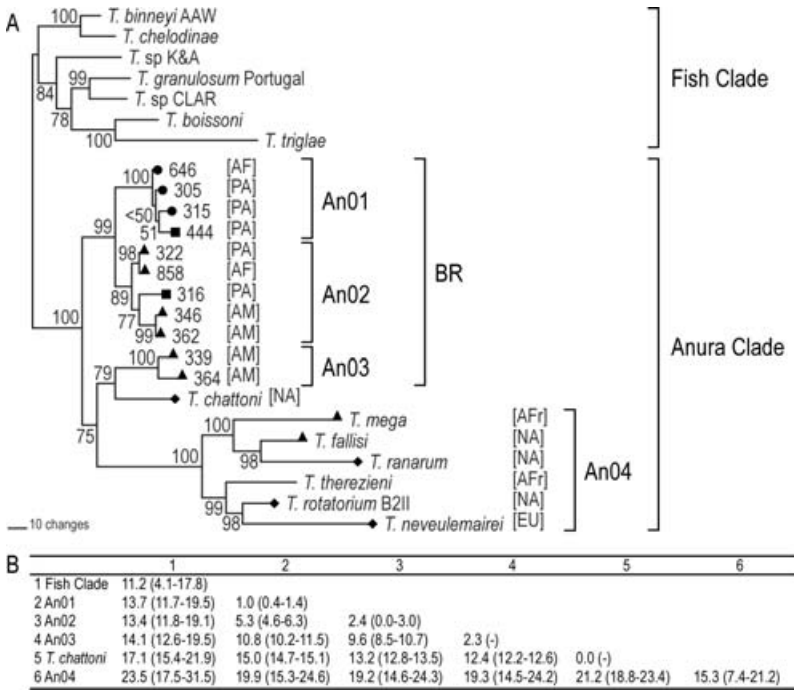

B

	1	2	3	4	5	6
1 Fish Clade	11.2 (4.1-17.8)					
2 An01	13.7 (11.7-19.5)	1.0 (0.4-1.4)				
3 An02	13.4 (11.8-19.1)	5.3 (4.6-6.3)	2.4 (0.0-3.0)			
4 An03	14.1 (12.6-19.5)	10.8 (10.2-11.5)	9.6 (8.5-10.7)	2.3 (-)		
5 *T. chattoni*	17.1 (15.4-21.9)	15.0 (14.7-15.1)	13.2 (12.8-13.5)	12.4 (12.2-12.6)	0.0 (-)	
6 An04	23.5 (17.5-31.5)	19.9 (15.3-24.6)	19.2 (14.6-24.3)	19.3 (14.5-24.2)	21.2 (18.8-23.4)	15.3 (7.4-21.2)

Fig. 6. (A) Phylogenetic tree inferred by Parsimony analysis of partial SSU rDNA sequences (~725 bp of V7–V8 regions) of trypanosomes from Brazil (BR), Africa (AFr), North America (NA) and Europe (EU) from anurans of the families Hylidae (●), Leptodactylidae (■), Bufonidae (▲) and Ranidae (◆). The Brazilian isolates included in this analysis are from Atlantic Forest (AF), Pantanal (PA) and Amazonia (AM). Sequences from fish trypanosomes (Fish clade) were used as outgroup for anuran trypanosomes (Anura clade). The numbers at nodes correspond to percentage of bootstrap values derived from 100 replicates. (B) Matrix of average sequence divergence indexes among anuran trypanosomes clustered An01, An02, An03 and An04 clades. Range given in parentheses. Length of most parsimonious tree: 846 steps; CI = 0·80; RI = 0·83.

host range of anuran trypanosomes. To date, few anuran trypanosomes have been included in phylogenies of trypanosomes in general. The only study that has specifically dealt with the phylogeny of these trypanosomes is that of Martin *et al*. (2002). Their data showed that 5 species of trypanosomes from ranids and bufonids in North America, Europe and Africa clustered together despite the considerable divergence among them. In this phylogeny, *T. chattoni*, isolated from a North American ranid, clearly stood apart from all the trypanosomes analysed, nesting more closely to the trypanosomes of fishes (Martin *et al*. 2002). However, in a phylogeny using a larger collection of fish trypanosomes, *T. chattoni* clustered with anuran trypanosomes, although in a long and separate branch (Gibson *et al*. 2005). Therefore, additional data from a larger number of trypanosomes from a wider cohort of hosts of varied geographical origin are badly needed to allow correct appraisal of the phylogenetic relationships of anuran trypanosomes.

Aiming to assess the diversity of anuran trypanosomes and better understand their phylogeny as well as to evaluate their current taxonomy, we captured anuran from various species of distinct Brazilian biomes, some of which separated by large geographical distances, evaluated their overall prevalence of trypanosome infection, isolated trypanosomes in culture and compared their morphological and molecular characteristics. The prevalence of blood trypanosomes in anurans was high (45%). The families Leptodactylidae, Hylidae, Leiuperidae and Bufonidae had decreasing infection indices ranging from 81% to 28%. Isolates from bufonids came mainly from the Amazonia and Pantanal; trypanosomes from hylids came mostly from the Atlantic Forest, Pantanal and Guaporé; and isolates from leptodactylids came mainly from the Pantanal and Guaporé. Any explanation of the disparities in prevalence among the families and biomes should take into account biome features, anuran habitat, sampling size and collecting biases. It is known that haematophagous insects (flies and mosquitoes) and leeches transmit trypanosomes between anurans (Bardsley and Harmsen, 1973). The life-cycles and habitats (adult and breeding environments) of anurans affect their availability to potential vectors and thus prevalence of trypanosome infection. In this study, anuran species examined in both dry and rainy seasons exhibited highest infection indices in the rainy season, coinciding with the increased number of vector insects and greater time spent by some anuran species in aquatic breeding environments, where they can be exposed to aquatic leeches for longer periods.

The Brazilian anurans showed a large variety of bloodstream trypanosomes showing at least 11

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distinct morphotypes, 8 similar to forms of previously described trypanosome species, but 3 entirely new. Adoption of the morphological and host-origin taxonomic criteria would lead to the classification of some Brazilian trypanosomes as new species. However, such an approach should be strongly discouraged because (a) the same morphotypes could be found in anurans of different species and from distant locations, including different continents; (b) inversely, anurans of the same species and from the same location could harbour trypanosomes of quite distinct morphotypes and (c) cultures of isolates from anuran blood samples showing identical morphotypes could have distinct molecular characteristics or, inversely, blood samples with distinct morphotypes could generate cultures with identical or very similar molecular features. The extensive pleomorphism of epimastigotes also precludes the use of the morphology of culture forms for species discrimination. Thus, data from this study do not support species identification of anuran trypanosomes based on morphology, host species and geographical origin.

To evaluate host, ecological and geographical diversity, we examined ITS and SSU rDNA sequences of Brazilian trypanosomes of anurans from geographically distant locations corresponding to distinct biomes. Genetic diversity among 82 isolates evaluated by polymorphisms of ITS rDNA distinguished 11 major groups (A–K) comprising 29 genotypes. Phylogenetic analysis using ITS and SSU rDNA sequences of Brazilian trypanosomes of anurans and species from North America (*T. chattoni*, *T. fallisi*, *T. ranarum* and *T. rotatorium*), Africa (*T. mega*) and Europe (*T. neveulemairei*) showed that new isolates from this study and the reference species clustered together in a clade exclusive of anuran trypanosomes, providing evidence of the monophyly of anuran trypanosomes in agreement with previous studies (Hamilton *et al.* 2004; Gibson *et al.* 2005; Simpson *et al.* 2006). Phylogenetic relationships of 11 Brazilian isolates inferred using ITS and SSU rDNA sequences positioned most of them in a major assemblage formed by 2 clades, An01 and An02. These clades were separated by a considerable distance from clade An03, which is composed of 2 Brazilian isolates positioned closer to *T. chattoni* than to other Brazilian isolates. Therefore, although still significantly divergent from all anuran trypanosomes, in our analysis *T. chattoni* was positioned within the clade of anuran trypanosomes, corroborating results from Gibson *et al.* (2005) in conflict with its positioning with fish trypanosomes as shown by Martin *et al.* (2002). The phylogeny based on V7–V8 SSU rDNA was totally congruent with data generated by ITS1 rDNA, in conformity with our previous analysis using the same approach for mammalian trypanosomes (Maia da Silva *et al.* 2004; Rodrigues *et al.* 2006; Cortez *et al.* 2006).

Molecular analysis revealed that the same anuran species could be infected by distinct trypanosomes and that the same presumed trypanosome species could infect distinct anuran species. In addition, our analyses suggest that closely related trypanosomes generally come from closely related anuran species. This is supported by the clustering together of trypanosomes from anurans of the same host families and genera but from different biomes and collection locations, as in the case of trypanosomes from hylids (Clade An01) or bufonids (Clades An02 and An03). Comparison of trypanosomes according to their collection locations might also suggest that there is some degree of association between genotype and geographical origin, since isolates from the same anuran family and genus, but from regions far apart, were separated in distinct subgroups/genotypes. Moreover, some genotypes were found to be restricted to certain biomes regardless of their host family, genus or species. This was the case of group A, which comprises 10 isolates from 4 anuran species from 3 families, all of which are from the same location in Rondônia State in Amazonia. Another example of a relationship related to geographical origin is genotype J2, composed of 14 isolates from 6 hylid species living in Pantanal or Guaporé, whereas trypanosomes from hylids of the Atlantic Forest were clustered in separate groups. This type of association is supported by previous studies employing isoenzyme, RAPD and karyotyping patterns to demonstrate that isolates of *T. rotatorium* and *T. ranarum* from ranids of the same geographical region had high similarity whereas isolates from geographical locations that are far apart exhibited pronounced genetic polymorphism (Lun and Desser, 1995, 1996; Desser, 2001). The association between genotype and geography is expected considering the high degree of endemism of anuran taxa. For the same reason, genotypes shared by anurans from Guaporé and Pantanal are also expected. The Brazilian isolates characterized in this study are from anuran species whose geographical distribution is restricted to Brazil or South America. These isolates were found to be separated from all the trypanosomes from other countries by large genetic distances, indicating that the culture collection of anuran trypanosomes obtained in this study contains several new species.

Finally, in spite of its monophyly, the clade formed by anuran trypanosomes is undoubtedly a complex taxon comprising distinct phylogenetic lineages. Several speciation modes may have played a role in the evolution of trypanosomes of this clade, including co-divergence by host switching and co-evolution, and sympatric and allopatric speciation events. The data from this study also suggest a significant degree of host restriction and that vector transmission, which is largely driven by biome and anuran species-specific ecogeographical features

could be important evolutionary factors for anuran trypanosomes. Leech transmission is thought to be a predominant evolutionary factor for anuran trypanosomes and the entire 'Aquatic' clade (Hamilton *et al*. 2004, 2005; Simpson *et al*. 2006). However, haematophagous insects should be very important vectors for terrestrial and arboreal anurans, despite the aquatic breeding environment of these anurans. We are currently investigating possible trypanosome vectors for the anurans investigated in this study. Several analyses of the phylogeography and co-evolutionary history of diverse host-parasite assemblages have been performed. However, few studies have focused on anuran parasites, one of the most diverse and complex groups in its parasite community, and most of the available data relate to the strictly host-specific Monogenea species (platyhelminth flatworms) (Sinnappah *et al*. 2001; Bentz *et al*. 2006). Further studies into host-parasite interactions and comparative analysis of the ecology and phylogeography of anurans and their trypanosomes would greatly contribute to our understanding of the evolution of this large, widespread and heterogeneous group of trypanosomes. The polymorphic DNA markers we have identified in this paper may facilitate such studies.

We are grateful to several collaborators for their tireless help in the fieldwork. We specially would like to thank Arlei Marcili (ICB-USP), Miguel T. Rodrigues (IB-USP), Sandra Favorito (Universidade Bandeirantes, São Paulo) and Carlos Jared (Instituto Butantan, São Paulo) for inestimable help providing blood samples from anurans. Work in Amazonia was done at the laboratory of the ICB5-USP in Monte Negro, Rondonia. We thank Fernando Paiva (UFMS) for providing good conditions for field work in the Pantanal. Many thanks to Professor Erney P. Camargo for helpful discussions, valuable comments and constructive criticisms in reviewing our manuscript. This work was funded by the Brazilian agencies of FAPESP and CNPq. Robson C. Ferreira and Laerte B. Viola are graduate student fellows respectively from FAPESP and CNPq.

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