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Phylogenetic analysis of Trypanosomatina (Protozoa: Kinetoplastida) based on minicircle conserved regions

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Abstract. Phylogenetic relationships within the suborder Trypanosomatina were inferred from the kinetoplast DNA minicircle conserved region sequences. Trees built using distance-matrix (Neighbor-Joining) and maximum parsimony methods showed that the minicircle conserved regions (CRs) provide a sensitive and specific molecular marker suitable for phylogenetic analyses of subspecies and strains of trypanosomatid flagellates, as testified by the subdivision of the genus *Leishmania* into the subgenera *Leishmania*, *Viannia* and *Sauroleishmania*. However, since *Phytomonas* and monogenetic parasites of insects represent the earliest diverging groups, the CRs do not seem to be useful for inference of relationships among major lineages of the order Kinetoplastida.

Abbreviations: CR – conserved region, SSU and LSU – small and large subunit ribosomal RNA, kDNA – kinetoplast DNA.

Since the beginning of this century scientists have tried to elucidate the origin and evolution of parasitism of kinetoplastid protozoa. Two major alternative hypotheses have been proposed, suggesting that either the monogenetic (invertebrate host only) or the digenetic (vertebrate and invertebrate hosts) life cycle appeared first in the evolution of this ancient group (for review see Maslov et al. 1996).

Several molecular markers have been used for the reconstruction of evolutionary relationships within the order Kinetoplastida, focusing mainly on the important and widely studied suborder Trypanosomatina. The small (SSU) and large (LSU) subunits of nuclear ribosomal RNA genes (Maslov et al. 1996, Lukeš et al. 1997, Wright et al. 1999), mitochondrial 9S and 12S ribosomal RNAs (Lake et al. 1988, Lukeš et al. 1994), mitochondrial proteins (Maslov et al. 1994, Blom et al. 1998), and various conserved nuclear-encoded proteins (Alvarez et al. 1996, Croan and Ellis, 1996, Philippe 1998) have been used for this purpose. In general, phylogenetic analyses performed so far favoured the scenario in which the ancestral trypanosomatid was digenetic, while the monogenetic life cycle seems to be a derived feature. Presented herein is a phylogenetic analysis based on the conserved regions (CRs) of kinetoplast DNA (kDNA) minicircles which, as we suggest, provide further source of information for the studies of evolution of these fascinating flagellates.

MATERIALS AND METHODS

The cloning, in our laboratories, of minicircles of *Trypanosoma carassii*, *Trypanosoma scelopori*, *Leishmania (Sauroleishmania) guliki* and *Leishmania (S.) gymnodactyli* was described elsewhere (Fu and Kolesnikov 1994, Kolesnikov et al. 1995, Yurchenko et al. 1998). The CR of *Trypanosoma triglae* was amplified using primers CSB3out – TTAAGCTTAGGGGTTGGTGTAAT and CSB3in– TTGGAT CCTATCGAAGCACCAC under conditions described previously (Yurchenko et al. 1998). The minicircles of *Wallaceina* (formerly *Proteomonas*) *brevicula* and *Wallaceina inconstans* (Kolesnikov et al. 1990) were released from the kDNA network by *Bam*HI or *Hind*III digestion, gel purified, cloned into the pUC19 cloning vector digested with *Bam*HI or *Hind*III, respectively, and then sequenced using "ABI PRISM™ Cycle Sequencing Kit" (Perkin Elmer). With the exception of *Trypanosoma triglae* and *T. scelopori* (Jirků et al. 1995, Yurchenko et al. 1998), all CRs were derived from the full-length minicircles. The remaining sequences were retrieved from the "Kinetoplast Minicircle Sequences Database" (Brewster et al. 1998).

The phylogenetic relationships were analysed within a subset of twenty nine species belonging to the suborder Trypanosomatina, out of which ten were *Trypanosoma* species, twelve *Leishmania* species and seven representatives of the genera *Crithidia, Herpetomonas, Phytomonas* and *Wallaceina* (Table 1). The CR derived from the minicircle-like molecules of the cryptobiid flagellate *Trypanoplasma borreli* (Bodonina) was used as an outgroup.

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Table 1. The descriptions of species and isolates used in this work. Positions of CRs are indicated as they occur in the GenBank entries. In the case of *Leishmania (S.) gymnodactyli* it is assumed that minicircle is a physical circle.

	Name	Description	Isolate	GenBank accession #	Conserved region, nt
	Genus *Leishmania* Ross, 1903, subgenus *Leishmania* Ross, 1903				
1	*Leishmania (L.) donovani*	Laveran et Mesnil, 1903	AJS-PEKIN	Z35275	40–169
2	*Leishmania* (*L.*) *infantum*	Nicolle, 1908	AJS-D2PST	Z35292	109–238
3	*Leishmania* (*L.*) *chagasi*	DaCunha et Chagas, 1937	AJS-PPECO	Z35276	40–169
4	*Leishmania (L.) mexicana (amazonensis)*	Lainson et Shaw, 1972	IFLA/BR/ 67/PH8	M21325	42–170
5	*Leishmania* (*L.*) *mexicana*	Biagi, 1953		Z11550	719–848
6	*Leishmania (L.) amazonensis*	Lainson et Shaw, 1972		Z11554	43–173
	Genus *Leishmania* Ross, 1903, subgenus *Viannia* Lainson et Shaw, 1987				
7	*Leishmania* (*V.*) *guyanensis*	Floch, 1954		M87316	40–170
8	*Leishmania* (*V.*) *peruviana*	Herrer, 1951		M87317	40–170
9	*Leishmania* (*V.*) *braziliensis*	Vianna, 1911		M87315	39–169
	Genus *Leishmania* Ross, 1903, subgenus *Sauroleishmania* Saf'janova, 1982				
10	*Leishmania* (*S.*) *guliki*	Saf'janova, 1982	3214	Z32857	454–582
11	*Leishmania* (*S.*) *tarentolae*	Wenyon, 1921		M28567	207–335
12	*Leishmania* (*S.*) *gymnodactyli*	Khodukin et Sofiev, 1940	GR20	Z32855	834–95
	Genus *Endotrypanum* Mesnil et Brimont, 1908				
13	*Endotrypanum* sp.	Mesnil et Brimont, 1908	341	Unpubl.[1]	1–130
14	*Endotrypanum* sp.	Mesnil et Brimont, 1908	58	Unpubl.[1]	1–130
	Genus *Herpetomonas* Kent, 1880				
15	*Herpetomonas samuelpessoai*	Galvao, Oliveira, Carvalho et Veiga, 1970	Her 70	AF064385	1–122
	Genus *Trypanosoma* Gruby, 1843				
16	*Trypanosoma carassii*	Mitrophanov, 1883	El (*Esox lucius*)	AF169953	1–131
17	*Trypanosoma carassii*	Mitrophanov, 1883	Ab-Tb (*Abramis brama*)	AF169954	1–131
18	*Trypanosoma carassii*	Mitrophanov, 1883	Cac-Ra (*Carassius carassius*)	AF169955	1–131
19	*Trypanosoma triglae*	Neumann, 1909		AF085735	1–131
20	*Trypanosoma scelopori*	Ayala, 1970		AF044840	160–279
21	*Trypanosoma brucei*	Luhe, 1906	EATRO 164	L11652	10–117
22	*Trypanosoma equiperdum*	Doflein, 1901		M14763	311–423
23	*Trypanosoma congolense*	Broden, 1904		M19750	51–182
24	*Trypanosoma rangeli*	Tejera, 1920	H9	L28038	1700–60
25	*Trypanosoma cruzi*	Chagas, 1909	strain CL	M19190	32–164
	Genus *Crithidia* Leger, 1902				
26	*Crithidia fasciculata*	Leger, 1902		U12625	48–175
	Genus *Phytomonas* Lafont, 1909				
27	*Phytomonas* sp.	Lafont, 1909	30T	M74195	108–236
	Genus *Wallaceina*, Podlipaev, Frolov et Kolesnikov, 1999				
28	*Wallaceina brevicula*	Frolov, Kolesnikov et Podlipaev, 1999	NBR	Z32854	268–397
29	*Wallaceina inconstans*	Podlipaev, Frolov et Kolesnikov, 1999	ZhK	AF124056	594–723
	Genus *Trypanoplasma* Laveran et Mesnil, 1901				
30	*Trypanoplasma borreli*	Laveran et Mesnil, 1901	Pg - JH	U14184	346–477

[1] available from http://www.ebi.ac.uk/parasites/kDNA/Source.html

The alignment was made by eye using the Pileup sequence alignment program (Genetic Computer Group) and is available at http://www.ebi.ac.uk/parasites/kDNA/Source.html/. The phylogenetic analysis was carried out using the TreeCon program (Van de Peer and De Wachter 1994) and Neighbor-Joining algorithm for the tree topology inference, and the consensus tree was built from the 100 replicates with the bootstrap option of the TreeCon. The maximum parsimony tree was built by the Paup 4.0 Version and 300 bootstrap replicates were performed.

RESULTS AND DISCUSSION

The alignment of the CR sequences contained 132 alignable positions for 29 trypanosomatid and one bodonid taxa (Table 1). Results of the phylogenetic reconstruction using the Neighbor-Joining method are shown in Fig. 1.

All representatives of the genus *Trypanosoma* form a monophyletic group (bootstrap 81%) within the suborder Trypanosomatina. The salivarian, stercorarian and fish trypanosomes form monophyletic groups within the genus *Trypanosoma*, although the bootstrap support for this branching order is low. These results are in good correlation with the data inferred from the SSU and LSU rRNA genes (Lukeš et al. 1997, Haag et al. 1998, Wright et al. 1999). An African marine teleost trypanosome *Trypanosoma triglae* forms a strongly supported monophyletic branch with the isolates of *Trypanosoma carassii* obtained from the blood of several European freshwater fish species. Our analysis confirmed previous conclusion that there are at least two different subgroups (subspecies) of freshwater fish trypanosomes parasitising different fish hosts (Kolesnikov et al. 1995). The affiliation with another trypanosome para-site of reptiles – *Trypanosoma scelopori*, which forms a separate branch, may be resolved only after the CRs of other reptilian and amphibian species will be available.

The branching of *Endotrypanum* spp. that renders the genus *Leishmania* paraphyletic is not surprising since similar result was obtained by the analysis of SSU rRNA genes (Noyes et al. 1997). All *Leishmania* species are considered to be closely related and therefore their resolution in phylogenetic trees based on ribosomal RNA genes is generally low (Briones et al. 1992, Noyes et al. 1997, Philippe 1998). One outcome of the minicircle CR-based phylogeny is that the diversity within the genus *Leishmania* is similar to that of the genus *Trypanosoma*. The subgenera *Leishmania, Viannia* and *Sauroleishmania* appear as monophyletic groups within the *Leishmania* branch, supported by high bootstrap values. Our results testify of the

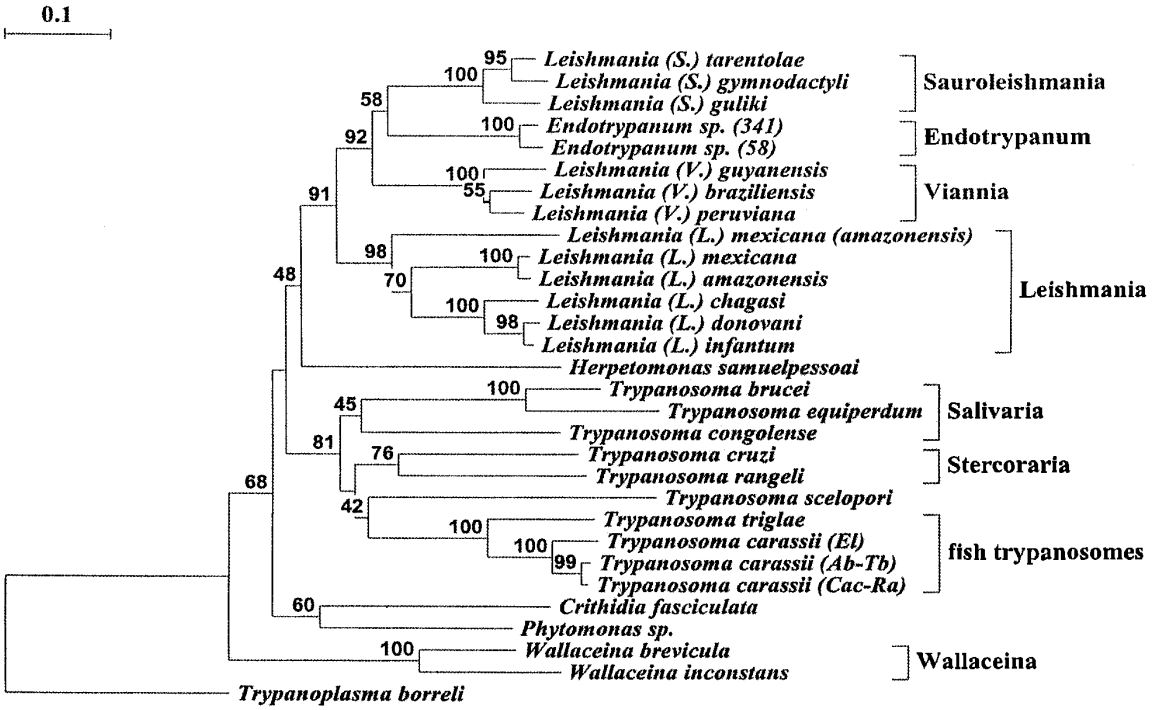

Fig. 1. Neighbor-Joining consensus minicircle CR tree of the suborder Trypanosomatina. A consensus tree was built from 100 pseudoreplicates with the bootstrap option of the TreeCon program.

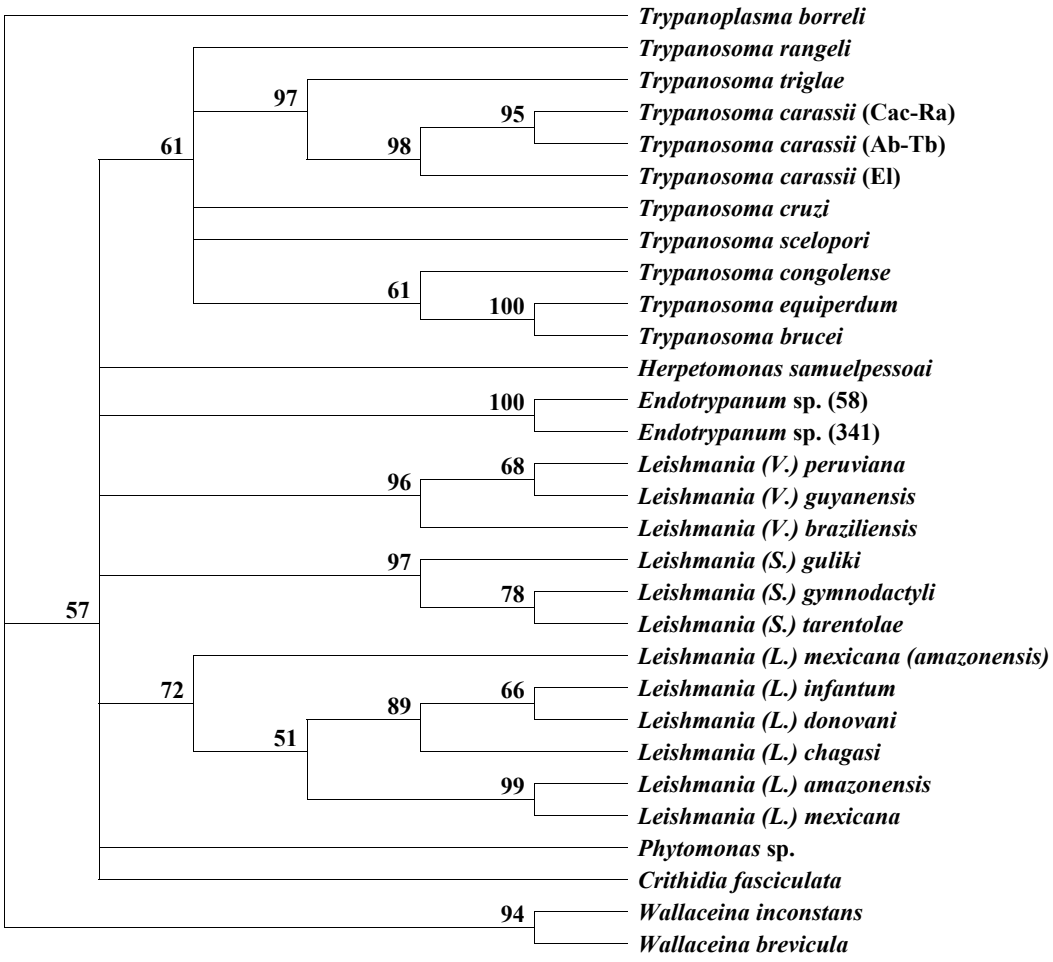

Fig. 2. Maximum parsimony minicircle CR tree (50% majority consensus). The branching order for *Crithidia fasciculata*, *Herpetomonas samuelpessoai* and *Phytomonas* sp. is shown as unresolved, as well as for *Trypanosoma cruzi*, *Trypanosoma rangeli* and *Trypanosoma scelopori* within the *Trypanosoma* group.

informative value of the minicircle CR when used for the phylogenetic analysis of closely related flagellates. Remarkably, our groups are equal to the subgenera of *Leishmania*, the establishment of which was based on morphology and life cycle data (Lainson and Shaw 1987) and further supported by the phylogenetic analysis of the RNA polymerase II subunit gene (Croan and Ellis 1996).

Unexpectedly, the earliest diverging groups within Trypanosomatina are represented by a plant parasite *Phytomonas* sp., and monogenetic parasites of insects – *Wallaceina inconstans*, *Wallaceina brevicula* and *Crithidia fasciculata.* This result contradicts trees constructed on the basis of several conserved nuclear and kinetoplast genes (Lukeš et al. 1997, Haag et al. 1998, Philippe 1998, Wright et al. 1999), and is likely to be an artifact caused by the significant difference between minicircle molecules of insect and plant parasites from minicircles of other trypanosomatids.

To shed more light on this point we analysed the same subset of sequences using the maximum parsimony algorithm. The resulting maximum parsimony tree (Fig. 2) reflects major shortcomings of the use of minicircle CRs for phylogenetic studies, since the CRs are only about 130 bp long, and contain a relatively low number of informative positions. The tree is dominated by a major polytomy within the trypanosomatid clade, to the exclusion of *Wallaceina* species that form a stable early offshoot. However, the overall topology of this tree does not significantly differ from the Neighbor-Joining tree. All major groups discussed above remain unchanged, in particular the existence of subgenera *Leishmania*, *Sauroleishmania* and *Viannia* is supported by high bootstrap values (72, 97, and 96%, respectively). The early branching of the genus *Wallaceina* is confirmed by the parsimonious tree making this insect parasite an interesting object for further studies.

Taken together, our results demonstrate that the minicircle CRs provide a sensitive and specific molecular marker useful on the generic level, and in cases studied so far, information inferred from these regions seems to be sufficient to distinguish between subspecies and strains of trypanosomatid flagellates.

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