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Monophyly of Endosymbiont Containing Trypanosomatids: Phylogeny versus Taxonomy

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ABSTRACT. To obtain additional information on the phylogenetic relationships within the family Trypanosomatidae (order Kinetoplastida), we have sequenced the small subunit ribosomal RNA genes from the endosymbiont containing species *Herpetomonas roitmani* TCC080, *Herpetomonas* sp. TCC263, *Crithidia oncopelti* ATCC 12982 and a partial large subunit rRNA gene from *H. roitmani*. The small subunit sequences in the two isolates of *Herpetomonas* are very similar but not identical, and so are their restriction digest profiles of kinetoplast DNA. The size of minicircles in both isolates is 4.2 kilobases. The inferred ribosomal RNA phylogenetic trees shows the genera *Herpetomonas* and *Crithidia* as polyphyletic. Endosymbiont-bearing herpetomonads cluster with the endosymbiont-bearing crithidias and a blastocrithidia to form a monophyletic clade, whereas the endosymbiont-free members of these genera are found elsewhere in the tree. These data support the hypothesis of a monophyletic origin of endosymbiosis in trypanosomatid evolution and also suggest that a taxonomic revision is needed in order to better describe the natural affinities in this family.

Supplementary key words. *Crithidia oncopelti*, endosymbiont, *Herpetomonas roitmani*, kinetoplast DNA, Kinetoplastida, minicircles, phylogeny, taxonomy, Trypanosomatidae.

TAXONOMY of the family Trypanosomatidae is based on the morphology and host range [12, 31]. For example, the genus *Herpetomonas* is defined as a taxon which consists of monogenetic parasites of insects with promastigote and opisthomastigote body shape, while the genus *Trypanosoma* represents digenetic parasites of vertebrates and invertebrates with trypomastigote, epimastigote, promastigote and amastigote morphologies [31]. Existing taxonomic criteria have been repeatedly criticized for the dearth of informative characters and it should not be surprising that some taxa represent artificial rather than natural groups. Continuous efforts are being made to improve these criteria (reviewed in [6, 32]).

Molecular phylogenetic approach can be used to test taxonomic relationships originally inferred from morphological data. A taxon is considered to be natural when it appears on phylogenetic trees as a monophyletic clade. So far, phylogeny derived from rRNA sequence analysis has been in agreement with morphological taxonomy in case of four members of the genus *Herpetomonas* [16], eleven species of trypanosomes [17] and seven isolates of *Phytomonas* [13]. The genus *Leishmania* is possibly monophyletic as well, provided its apparently misclassified members, such as *L. hertigi* and *L. herreri*, are excluded [4, 22].

On the contrary, the genus *Crithidia* is found to be polyphyletic with molecular data. The bacterial endosymbiont-containing members *C. oncopelti*, *C. deanei* and *C. desouzai* form a well-defined clade together with *Blastocrithidia culicis* which is also endosymbiont-containing [7, 8, 17, 20], whereas the endosymbiont-free species *Crithidia fasciculata* grouped with *Leptomonas, Leishmania* and *Endotrypanum* [7, 8, 10, 16, 17, 19, 20]. It has been concluded that the acquisition of bacterial endosymbionts was a single event which was followed by coevolution of endosymbionts and their trypanosomatid hosts [8].

Like the genus *Crithidia*, the genus *Herpetomonas* contains an endosymbiont-bearing species, *H. roitmani* [27], along with endosymbiont-free species such as *H. muscarum, H. mariadeanei, H. samuelpessoai* and others. The phylogenetic affinities of *H. roitmani* have remained unknown until now. A recent survey of several *Herpetomonas* isolates, including *H. roitmani*, has indicated that there is a substantial heterogeneity within this group with regard to the presence of the polymorphic restriction sites and reactivity with the ribosomal DNA and kinetoplast DNA probes [30]. Remarkably, the subgroup which included *H. roitmani* was found to be more similar to *Crithidia oncopelti* than to other *Herpetomonas* subgroups which contained only endosymbiont-free species [30]. In the present work we have investigated phylogenetic relationships within this group of endosymbiont-containing herpetomonads and crithidias. We have confirmed that the endosymbiont-containing trypanosomatids form a monophyletic group, and, therefore, there is a discrepancy between the current taxonomic status of these organisms and their phylogenetic affinities.

MATERIALS AND METHODS

Strain origin, cultivation conditions, DNA extraction and sequencing. The strain *Herpetomonas roitmani* TCC080 was isolated from a syrphid fly *Ornidia obesa* [27], and the endosymbiont-containing strain *Herpetomonas* sp. TCC231 was isolated from a calliphorid fly *Chrysomia putoria* [30]. Clonal lines of these strains were provided by Erney P. Camargo (University of Sao Paulo). *Crithidia oncopelti* strain ATCC12982 and *Blastocrithidia culicis* strain ATCC30268 were obtained from American Type Culture Collection. Cells were cultivated at 26° C in brain heart infusion medium with an optional addition of 10 μg/ml hemin. Isolation of the total cell DNA, PCR amplification and sequencing of the small subunit (SSU) and partial large subunit (LSU) ribosomal RNA genes were performed as described previously [20]. Kinetoplast DNA (kDNA) networks were isolated be centrifugation through the CsCl cushion [28]. Hydrodynamic shearing of kDNA was performed by two passages of the DNA sample (5 μg) in 200 μl of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) through a # 27 gauge (1/2" long) needle. Restriction endonuclease digests were performed according to the manufacturers' suggestions (Life Technologies).

Phylogenetic analysis. Sequences determined in this work are available from the GenBank™ under the following accession numbers: AF038023 (SSU of *H. roitmani* TCC080), AF038024 (SSU of *Herpetomonas* sp. TCC263), AF038025 (SSU of *C. oncopelti* ATCC12982) and AF038026 (partial LSU of *H. roitmani*). The sequences were aligned manually using the sequence editing program SeqEdit [24]. The alignment is available on request. Maximum likelihood analysis was performed with the fastDNAml program, version 1.0.8 [25]. One hundred bootstrapped trees were generated with the corresponding option of fastDNAml, and the consensus tree was derived with the CONSENS program of PHYLIP [9]. Maximum likelihood with constraints, maximum parsimony and distance analyses

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were performed using the tester version of PAUP 4d57 provided by D. Swofford.

RESULTS

Phylogenetic analysis of the rRNA genes. The alignment of the SSU rRNA genes contained 1,771 alignable positions for twenty eight taxa including twenty four trypanosomatids and four bodonids/cryptobiids. In order to ease the computations we did not include in our analysis some available sequences of *Phytomonas* and *Leishmania* since the SSU sequences within each group are rather similar to each other. In addition, in order to reduce the potential unequal rate effect on the phylogenetic inference, we omitted the fast evolving sequences of *T. vivax*, *T. simiae* and *T. congolense*. We have demonstrated previously [17] that all Salivarian trypanosomes form a highly supported monophyletic clade. In the current analysis this clade is represented only by *T. brucei*. The tree was rooted using the sequence of *Bodo caudatus* [10].

The SSU sequences of the *Herpetomonas* strains TCC080 and TCC263 determined in this work are nearly identical to each other. They are also identical to the SSU sequence obtained previously from another strain of *H. roitmani* (unpubl. data of D. A. M. and L. Simpson). These sequences are also very close to the previously published SSU sequence shared by three reportedly similar species, *C. oncopelti*, *C. deanei* and *C. desouzai* [7]. Surprisingly, the SSU sequence of *C. oncopelti* strain ATCC12982 determined in this work is only 91% identical to the sequence of the three *Crithidia* species. In support of this observation, it has also been noticed that the SSU riboprint from *C. oncopelti* strain ATCC12982 does not exactly correspond to the sequence of *C. oncopelti-C. deanei-C. desouzai* [3]. The reason for this discrepancy is not clear, but authenticity of the ATCC12982 strain of *C. oncopelti* is confirmed by analysis of its kDNA (see below).

In contrast to the situation with *C. oncopelti*, it has been noted that the published sequence of *C. oncopelti-C. deanei-C. desouzai* does correspond to the SSU riboprint of *C. deanei* strain ATCC30255 [3]. Therefore, in this paper we regard the published sequence of the three crithidias [7] as an authentic sequence of *C. deanei*.

In the maximum likelihood tree (Fig. 1), several shortest parsimony trees and the distance tree (not shown) *H. roitmani* TCC080 and *Herpetomonas* sp. TCC263 are found within the clade of endosymbiont-containing trypanosomatids as a sister group of *C. deanei*. In the majority rule consensus parsimony tree (not shown) they form a trichotomy. *C. oncopelti* is more closely related to *B. culicis* than to other crithidias with maximum likelihood and the other methods. Bootstrap support for each of these affiliations is 100% with all methods, and support for the entire clade of endosymbiont-containing trypanosomatids is 100% with likelihood (Fig. 1), 100% with parsimony (not shown) and 99% with distance (not shown).

Two additional clades—the clade of *Phytomonas* spp. and the clade of slowly-evolving *Crithidia fasciculata, Leptomonas* sp. and *Leishmania donovani*—are also supported at the 99–100% level. Endosymbiont-free members of the genus *Herpetomonas* are found in a separate monophyletic clade which has low support level in the likelihood (78%), parsimony (75%) and distance (79%) analyses. These four clades: 'Endosymbiontic', 'Herpetomonas', 'Phytomonas' and 'Slowly-Evolving'—form a monophyletic group ('Non-trypanosomes') highly supported with the likelihood (90%) and parsimony (96%) analyses, but not the distance (77%) analysis. The clade of trypanosomes appears to be monophyletic, as it did previously [17].

Arrangement of the four non-trypanosome clades with the clades of 'Herpetomonas' and 'Phytomonas' being most closely

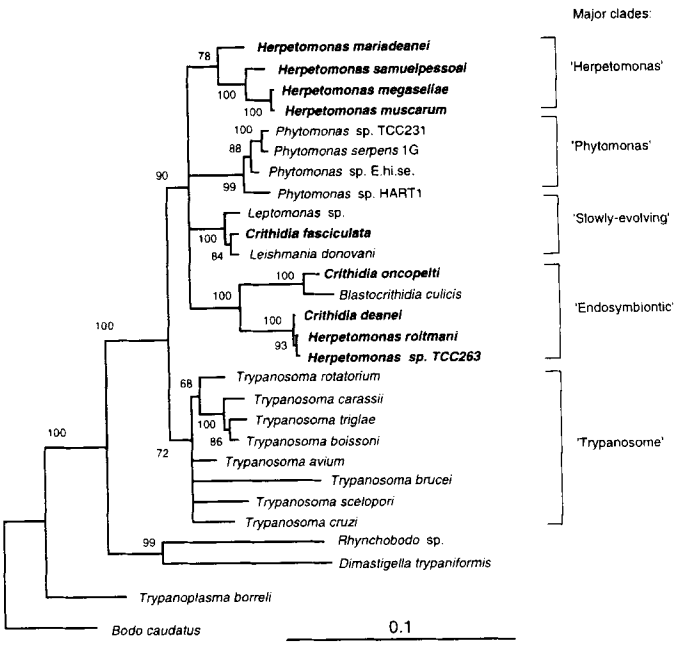

Fig. 1. Majority consensus maximum likelihood SSU rRNA tree of Trypanosomatidae. Search options with fastDNAml program included empirical base frequencies, randomized sequence addition order and global rearrangements. Ln-likelihood value of the maximum likelihood tree is −9092.81296. Scale bar corresponding to 0.1 substitutions per site is shown under tree. Bootstrap analysis was performed with 100 pseudoreplicates. Although the monophyly of the 'Phytomonas' and 'Herpetomonas' clades is supported at the level of 71%, the branching order of the four non-trypanosome clades is shown as unresolved (see also Table 1). Other bootstrap values are as indicated. Members of the polyphyletic genera *Herpetomonas* and *Crithidia* are shown in boldface.

related is similar on the maximum likelihood tree, distance tree and eight out of twenty two shortest parsimony trees (Table 1). The remaining shortest parsimony trees display three alternative types of topology. When these topologies are enforced in the likelihood or distance analysis, the trees produced are not significantly longer than the non-constrained minimal trees (Table 1), indicating that the exact branching order is only marginally resolved from the SSU data. To underline this uncertainty the topology of the non-trypanosome clade is presented as a polychotomy in Fig. 1.

The genera *Herpetomonas* and *Crithidia* appear polyphyletic in the phylogenetic trees obtained. Due to uncertain topology of the nontrypanosome clade, the corresponding constrained monophyletic trees were also investigated (Table 1). These trees were found to be significantly longer than the unconstrained polyphyletic trees. This indicates that regardless of the uncertainty mentioned above, the monophyly of *Herpetomonas* and *Crithidia* is not supported.

These conclusions were corroborated by a phylogenetic analysis of the LSU data set (not shown).

Restriction enzyme analysis of kinetoplast DNA. Since SSU rRNA gene sequences from the *Herpetomonas* isolates TCC080 and TCC263 are almost identical, as is their morphology [30], we have investigated whether these trypanosomatids still can be distinguished by a restriction digest analysis of their kinetoplast DNA.

Kinetoplast DNA networks of *H. roitmani* TCC080, *Herpetomonas* sp. TCC263, *B. culicis* ATCC30268 and *C. oncopelti* ATCC12982 were digested with restriction endonucleases and analyzed by electrophoresis in agarose gels. In order to release

Table 1. Parameters of the phylogenetic trees with alternative topologies.

Topology of the non-trypanosome clade[a]	Number of steps in parsimony[b]	Minimum evolution score in distance analysis[c]	Ln-likelihood value in maximum likelihood[d]
(E,(S,(H,P)))	1163, 8 trees	0.59868, 1 tree	−9083.05, 1 tree
(S,(E,(H,P)))	1163, 2 trees	0.60142, 1 tree	−9084.69, 1 tree
(H,(S,(P,E)))	1163, 6 trees	0.60184, 1 tree	−9086.63, 1 tree
(S,(H,(P,E)))	1163, 6 trees	0.60203, 1 tree	−9089.89, 1 tree
Monophyly of *Herpetomonas*	1231, 1 tree	0.64034, 1 tree	−9386.66, 1 tree
Monophyly of *Crithidia*	1295, 12 trees	0.67506, 1 tree	−9721.35, 1 tree

[a] S—'Slowly-evolving' clade (*C. fasciculata, L. donovani, Leptomonas* sp.), H—'Herpetomonas' (endosymbiont-free species: *H. samuelpessoai, H. mariadeanei, H. muscarum* and *H. megaseliae*). P—'Phytomonas' (*Phytomonas* spp.), E—'Endosymbiontic' (*B. culicis, C. oncopelti, C. deanei, H. roitmani, Herpetomonas* sp. TCC263).

[b] Parsimony analysis was performed by heuristic search.

[c] Distance analysis was performed by heuristic search using Kimura 2-parameter distances, with the starting tree obtained by neighbor-joining followed by the tree rearrangements.

[d] These values were obtained with PAUP 4d57.

unit-length minicircles, the networks were hydrodynamically sheared. The results are shown in Fig. 2. With kDNA of *Herpetomonas* isolates, restriction enzymes *Kpn*I, *Xho*I, *Eco*RI and *Hind*III produce a single major band of 4.2 kb and several minor bands. The cleavage patterns from the two isolates are very similar but not identical, and this is confirmed by cleavage with more frequently cutting enzymes *Msp*I, *Hae*III, *Sau*3AI and *Acc*I (not shown). Hydrodynamic shearing produces a major band of 4.2 kb for both the isolate TCC263 (Fig. 2, indicated by the arrow) and the isolate TCC080 (not shown). The results demonstrate that in both *Herpetomonas* isolates there is a major minicircle size class of 4.2 kb. Bands larger than 4.2 kb probably correspond to fragments of maxicircles and/or minicircle oligomers. Smaller size bands may represent either minor classes and/or fragments of the 4.2 kb class. In addition, nonstoichiometry of the products released by *Kpn*I (Fig. 2), *Msp*I, *Hae*III, *Sau*3AI and *Acc*I (not shown), all of which cleave the entire network, suggests that in both isolates, similar to other studied trypanosomatids, minicircles are heterogeneous in sequence.

In *C. oncopelti* we have observed four major size classes (Fig. 2, arrows) corresponding to the classes of 1.3, 1.6, 1.9 and 2.2 kb which were found previously in *C. oncopelti* Pasteur Institute strain S14 [18, 26]. This finding confirms the authenticity of the ATCC strain. In *B. culicis* two size classes (1.35 kb and 1.55 kb, released by *Eco*RI and *Kpn*I, respectively) are

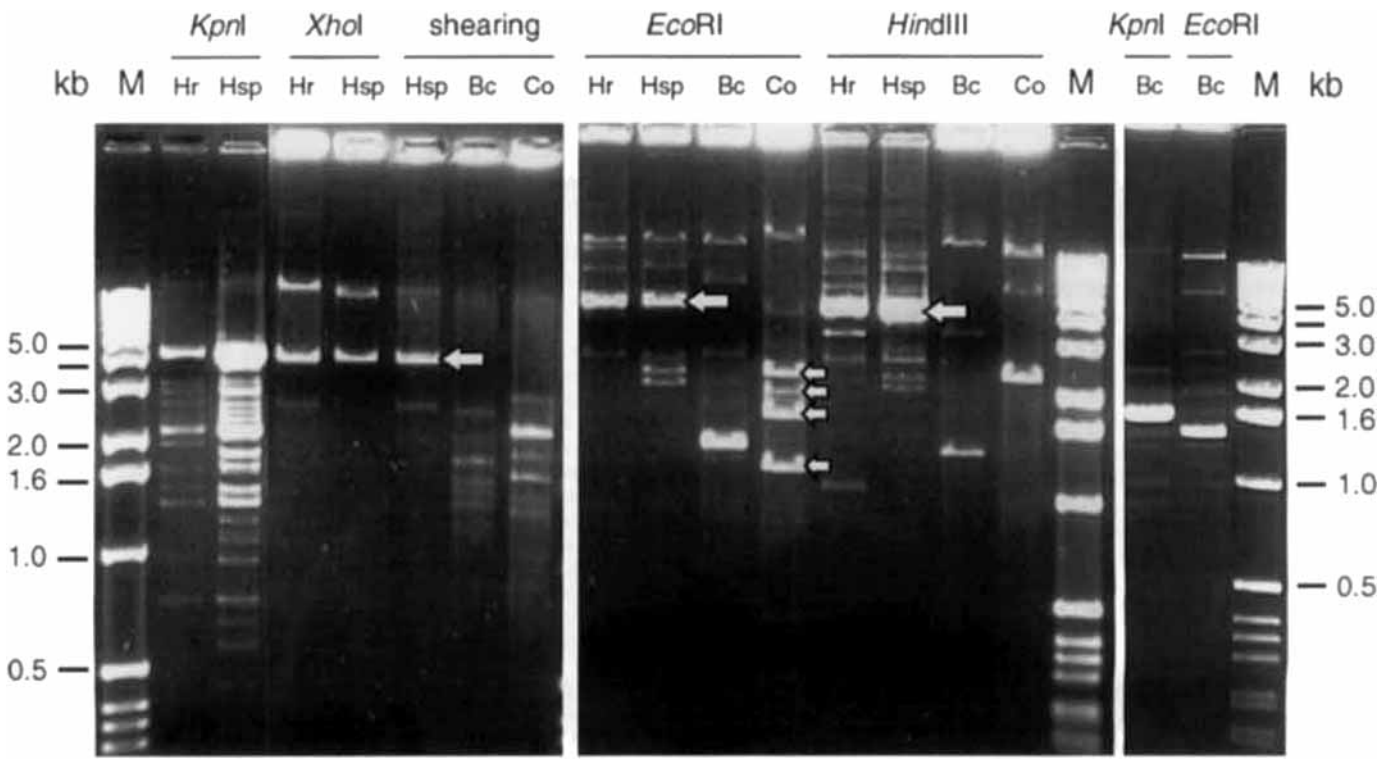

Fig. 2. Restriction digest profiles of the kDNA from *H. roitmani* TCC080 (Hr), *Herpetomonas* sp. TCC263 (Hsp), *B. culicis* ATCC30268 (Bc) and *C. oncopelti* ATCC12982 (Co). The cleavage products were resolved in 1% agarose gel. The lanes shown were derived from three different gels. Original lane order was changed for the clarity of presentation. Marker lanes (M) represent a 1 kb size ladder (Life Technologies). Large arrows point at the bands of the putative full-length 4.2 kb minicircles in *H. roitmani* and *Herpetomonas* sp. Small arrows indicate the major minicircle size classes in *C. oncopelti*.

found consistent with the results reported for this species previously [2]. Multiple bands which are seen in the sheared samples for both species are due to the size heterogeneity and different topology of the released minicircles.

DISCUSSION

By demonstrating that the endosymbiont-containing trypanosomatids *H. roitmani*, *Herpetomonas* sp. and *C. oncopelti* together with the other endosymbiont-containing species, *B. culicis* and *C. deanei*, form a highly supported monophyletic clade, we have provided additional evidence for the monophyletic origin of bacterial endosymbiosis in the family Trypanosomatidae [7, 8]. With inclusion of these species, the possibility of coevolution of the hosts and their endosymbionts [8] can now be more thoroughly investigated.

The relationships within the 'Endosymbiontic' clade are well resolved by the phylogenetic analysis of rDNA and confirmed by restriction digest analysis of kDNA. *H. roitmani* TCC080 and *Herpetomonas* sp. TCC263 are very closely related. The level of differences in their kDNA restriction profiles is comparable to the differences between different strains and isolates of *Trypanosoma cruzi* [21] or *Leishmania tarentolae* [11]. Both sequences are also nearly identical to the SSU sequence of *C. deanei* [7]. In addition, the minicircle size in *C. deanei* is 4 kb (Camargo, E. P., pers. commun.) and this is close to the size (4.2 kb) observed in the herpetomonads. These three isolates substantially differ from two other members of the same clade, *C. oncopelti* and *B. culicis*, an unique feature of which is a size heterogeneity of minicircles.

It is unclear whether there are any minicircle size classes in *H. roitmani* besides the 4.2 kb class. This size in unusually large, but similarly large minicircles were observed in *Trypanosoma boissoni* (3.7 and 4.1 kb) and *Trypanosoma triglae* (5.2 kb) [14]. The size of minicircles in endosymbiont-free herpetomonads is significantly smaller: 0.9 kb in *H. muscarum* (data of S. Hajduk, J. H. J. Hoeijmakers, P. Borst and K. Vickerman cited in [29]), 1.1 kb in *H. megaseliae* [23], and 1.3–1.35 kb in *H. samuelpessoai* (= *Leptomonas pessoai*) [1, 15].

Our phylogenetic analysis has clearly demonstrated that the genus *Herpetomonas* is an artificial taxon in which the two endosymbiont-containing isolates form a clade together with other endosymbiont-containing species, while the endosymbiont-free members of the genus form a separate clade. The genus *Crithidia* is also polyphyletic [7, 8, 17, 20] and, thus, represents another artificial taxon. A recent analysis of the nineteen isolates of *Crithidia* by riboprinting [3] corroborates this conclusion.

Interestingly, not only molecular but also some morphological data indicate that the taxonomic assignment of endosymbiont-containing herpetomonads and crithidias needs a revision. While classical choanomastigotes possess a kinetoplast in the antenuclear position [12], the choanomastigotes observed in a culture of *C. deanei* contain a kinetoplast in the postnuclear position [5]. Such forms, called 'opisthomorphs', were also observed instead of classical opisthomastigotes in the *H. roitmani* group and *C. oncopelti* [30].

As natural classification of trypanosomatids is a goal of molecular and biochemical taxonomic studies, a revision of the nomenclature has now become well justified. The new genus name *Angomonas* or the old name *Strigomonas* were suggested for the taxon of endosymbiont-containing crithidias [3, 5]. Regardless of the future choice, the close relatedness of these organisms with the isolates of *H. roitmani* suggests that both groups be united in the same taxon. Additional phylogenetic analysis is still required in order to delineate the boundaries of this taxon and, in particular, investigate its relationships with other endosymbiont-containing organisms. It would also be of interest to investigate whether this taxon includes any endosymbiont-free organisms.

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