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Diversity and Phylogeny of Insect Trypanosomatids Based on Small Subunit rRNA Genes: Polyphyly of *Leptomonas* and *Blastocrithidia*

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ABSTRACT. With the aim of further investigating phylogenetic relationships in insect trypanosomatids, we have determined the sequences of small subunit rRNA genes from ten isolates, which were originally classified as *Leptomonas, Blastocrithidia,* and *Wallaceina* based on their morphology in the hosts. The inferred maximum likelihood, parsimony, and distance trees indicate that the *Leptomonas* and *Blastocrithidia* are polyphyletic, and confirm the polyphyly of *Herpetomonas* and *Crithidia. Blastocrithidia triatoma* and *Leptomonas collosoma* were among the earliest branching lineages among the insect trypanosomatids, while most other isolates were found within a closely related terminal clade, which also included *Crithidia fasciculata.* This analysis has clearly demonstrated that the morphological classification system of insect trypanosomatids does not always reflect their genetic affinities warranting its revision in the future.

Key Words. Ribosomal RNA, taxonomy, Trypanosomatidae, *Wallaceina.*

TRYPANOSOMATIDS (family Trypanosomatidae Doflein 1901, order Kinetoplastida Honigberg 1963, suborder Trypanosomatina Kent 1880) are defined as a group of kinetoplastid protozoa with a single flagellum and a relatively small kinetoplast containing densely packed DNA (Vickerman 1976). The family includes four genera of digenetic organisms that parasitize vertebrate (*Trypanosoma, Leishmania,* and *Endotrypanum*) or plant (*Phytomonas*) hosts, with insects or leeches serving as vectors, and the monogenetic genera (*Leptomonas, Crithidia, Blastocrithidia, Herpetomonas, Rhynchoidomonas,* and *Wallaceina*) that are found largely in hemipteran and dipteran insect hosts with a limited distribution in seven other orders of insects, as well as in ciliates.

So far, only light microscopy has been used to establish the trypanosomatid taxonomy. Eight genera were described before 1910, one genus in 1959 and the last one in 1990 (see Podlipaev 1990). The assignment of isolates or species of trypanosomatids to a certain genus is determined by the morphological characters and the host range (Hoare and Wallace 1966; Molyneux and Ashford 1983). The relative positions of the nucleus and the kinetoplast and the shape of cells are the important morphological features. However, it has become increasingly clear that this system does not always reflect the genetic affinities of trypanosomatids on one hand, and their real diversity on the other. The discrepancies between the groups established by biochemical, molecular or phylogenetic analyses and the traditionally defined taxa have been observed in a number of works (Camargo et al. 1982; Dollet 1994; Hollar, Lukeš, and Maslov 1998; Kolesnikov, Maslov, and Podlipaev 1990; McGhee and Cosgrove 1980; Podlipaev, Malysheva, and Kolesnikov 1991; Podlipaev and Lobanov 1996; Vickerman 1994; Wallace et al. 1983; Wright et al. 1999) demonstrating the necessity to establish new genera and revise the existing ones.

Recently the small subunit (SSU) rRNA gene-based phylogenies have been used to establish the major natural groups within the family (reviewed in Philippe 1998; Vickerman 1994). Several monophyletic clades have emerged with only a few of them agreeing with the current taxonomy. The family Trypanosomatidae itself is monophyletic in contrast to the other kinetoplastid families Bodonidae and Cryptobiidae (Doležel et al. 2000). *Trypanosoma,* which appeared paraphyletic in earlier works due to unequal rate effects (Fernandes, Nelson, and Beverley 1993; Maslov et al. 1996), was shown to be monophyletic when the fast evolving lineages were subdivided by the inclusion of additional taxa of salivarian trypanosomes (Haag, O'hUigin, and Overath 1998; Lukeš et al. 1997; Stevens et al. 1999; Wright et al. 1999). Another monophyletic group was obtained for *Phytomonas* (Hollar and Maslov 1997; Marche et al. 1995), although this result did not mean that all promastigote isolates from plants would necessarily belong to this group. A strong monophyletic assembly was found for the symbiont-bearing trypanosomatids, which included species from *Blastocrithidia, Crithidia,* and *Herpetomonas* (Du, Maslov, and Chang 1994; Hollar, Lukeš, and Maslov 1998). The endosymbiont-free species of *Herpetomonas* formed a separate monophyletic group, and a single endosymbiont-free crithidia (*Crithidia fasciculata*) grouped elsewhere in the tree together with the only analyzed species of *Leptomonas* sp. and several *Leishmania* and *Endotrypanum* species. Thus, at least two genera, *Crithidia* and *Herpetomonas,* were found to be polyphyletic prior to this study. No conclusions could be drawn for *Blastocrithidia* or *Leptomonas,* with a single isolate analyzed for each genus. The dearth of data on isolates from insects represents the major gap in our knowledge of the trypanosomatid phylogeny.

Currently there are more than three hundred descriptions of insect trypanosomatids in the literature (Podlipaev 1990). Many of them may be synonyms or descriptions of organisms of dubious origin. At the same time, only a limited number of isolates have been investigated in culture. Fifteen years ago, 10–15 isolates had been studied in various laboratories (Wallace et al. 1983). Today this number still does not exceed 20–30 isolates obtained from a limited range of hosts and locations. This situation is inadequate for characterization of the diversity of insect trypanosomatids, nor does it allow full investigation of the phylogenetic relationships within the family. In this work, as a step toward solving these problems, we present the SSU rRNA phylogenetic analysis of ten isolates that have been previously assigned to the genera *Leptomonas, Blastocrithidia,* and *Wallaceina.* In order make our study more comprehensive, we combined molecular methodology with examining cell morphotypes in both the hosts and in culture.

MATERIALS AND METHODS

Cultures. Origins of the isolates used in the study are listed in Table 1. Methods of isolation and cultivation have been de-

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Table **1.** Origin of trypanosomatid strains.

Name	Reference	Insect host	Place of isolation	Comments
Blastocrithidia gerricola	Podlipaev 1985	*Gerris lacustris* Hemiptera: Gerridae	North-West Russia	Isolated by S. Podlipaev in 1981
Blastocrithidia triatoma	Cerisola et al. 1971	*Triatoma infestans* Hemiptera: Reduviidae	Argentina	Provided by G. Shaub
Leptomonas collosoma	Wallace et al. 1960	*Gerris dissortis* Hemiptera: Gerridae	USA	ATCC30261
Leptomonas peterhoffi	Podlipaev 1985	*Nabicula flavomarginata* Hemiptera: Nabidae	North-West Russia	Isolated by S. Podlipaev in 1982
Leptomonas seymouri	Wallace 1977	*Dysdercus suturellus* Hemiptera: Pyrrhocoridae	USA	ATCC30220
Leptomonas sp. Cfm	this work	*Nabicula flavomarginata* Hemiptera: Nabidae	North-West Russia	Isolated by A. Frolov in 1983
Leptomonas sp. F6	Kolesnikov, Maslov, and Podlipaev 1990	*Nabicula flavomarginata* Hemiptera: Nabidae	North Russia	Isolated by S. Podlipaev in 1986
Leptomonas sp. Nfm	this work	*Nabicula flavomarginata* Hemiptera: Nabidae	North-West Russia	Isolated by A. Frolov in 1991
Wallaceina inconstans[a] ZK	Kolesnikov, Maslov, and Podlipaev 1990; Podlipaev, Frolov, and Kolesnikov, 1990	*Calocoris sexguttatus* Hemiptera: Miridae	North-West Russia	Isolated by A. Frolov in 1986
Wallaceina brevicula Nbr	Kolesnikov, Maslov, and Podlipaev 1990; Podlipaev, Frolov, and Kolesnikov 1990	*Nabis brevis* Hemiptera: Nabidae	North-West Russia	Isolated by A. Frolov in 1986

[a] We use the generic name *Wallaceina* Podlipaev, Frolov et Kolesnikov, 1999 as a replacement name for *Proteomonas* Podlipaev, Frolov et Kolesnikov, 1990 because the name *Proteomonas* is preoccupied by a cryptomonad flagellate (*Proteomonas* Hill et Wetherbee 1986). The replacement name is given after the late Professor F. G. Wallace (Bulat, Mokrousov, and Podlipaev 1999).

scribed previously (Podlipaev 1985; Podlipaev, Malysheva, and Kolesnikov 1991). The cultures are stored in the Zoological Institute, St. Petersburg, Russia, and in the Max-Planck Institute for Molecular Physiology, Dortmund, Germany.

Trypanosomatids were cultivated at 26 °C in Brain Heart Infusion medium (Difco) with 10 μg/ml hemin. For *Blastocrithidia triatoma* we used TC100 medium (Gibco BRL) supplemented with 10% heat-inactivated fetal bovine serum.

Cell morphology was investigated by light microscopy of the fixed and Giemsa-stained samples and the images drawn using the camera lucida.

DNA preparation, amplification and sequencing. Isolation of total cell DNA and amplification of the SSU rRNA genes were performed as described previously (Maslov et al. 1996). The amplified PCR fragments were directly sequenced using a set of the conserved sequence primers described earlier (Maslov et al. 1996). The sequencing was performed by SeqLab GmbH (Göttingen, Germany). Nucleotide sequences were deposited to GenBank™ under following accession numbers: *Blastocrithidia gerricola*—AF153036; *Blastocrithidia triatoma*—AF153037; *Leptomonas collosoma*—AF153038; *Leptomonas peterhoffi*—AF153039; *Leptomonas seymouri*—AF153040; *Leptomonas* sp. Cfm—AF153041, *Leptomonas* sp. F6—AF153042; *Leptomonas* sp. Nfm—AF153043; *Wallaceina inconstans*—AF153044; *Wallaceina brevicula*—AF153045.

Phylogenetic analysis. The following sequences were retrieved from GenBank™ and included in the analysis: *Crithidia fasciculata* (X03450), *Leptomonas* sp. (X53914), *Leishmania tarentolae* (M84225), *Leishmania donovani* (X07773), *Endotrypanum monterogeii* (X53911), *Herpetomonas mariadeanei* (U01016), *Herpetomonas pessoai* (U01013) [*Herpetomonas pessoai* (Galvao, Oliveira, Carvalho et Veiga, 1970) is often referred to as *Herpetomonas samuelpessoai* Roitman, Brener, Roitman et Kitajima, 1976, which is incorrect by the rules of zoological nomenclature (Levine 1978; Podlipaev 1990)], *Herpetomonas megaseliae* (U01014), *Herpetomonas muscarum* (L18872), *Phytomonas serpens* (AF016323), *Phytomonas* sp. EM1 (AF016322), *Phytomonas* sp. E.hi.Se (L35077), *Phytomonas* sp. Hart1 (L35077), *Blastocrithidia culicis* (U05679), *Crithidia oncopelti* (AF038025), *Herpetomonas* cf. *roitmani* (AF267738), *Herpetomonas roitmani* (AF038023), *Herpetomonas* sp. TCC263 (AF038024), *Trypanosoma boissoni* (U39580), *Trypanosoma triglae* (U39584), *Trypanosoma caraassii* (L14841), *Trypanosoma rotatorium* (U39583), *Trypanosoma avium* (U39578), *Trypanosoma cruzi* (M31432), *Trypanosoma scelopori* (U67182), *Bodo saltans* (AF208887).

The sequences were aligned manually based on the previous alignments (Hollar, Lukeš, and Maslov 1998; Lukeš et al. 1997) and using a multiple alignment editor program SeqEdit, version 3.1 (Olsen 1990). Hypervariable regions that could not be aligned unambiguously were omitted. The alignments are available from D.A.M on request or can be retrieved from the following URL: http://www.lifesci.ucla.edu/RNA/trypanosome/alignments.html.

Maximum likelihood (Felsenstein 1981), several distance, including paralinear distance (Lake 1994), and parsimony trees were inferred using PAUP* 4.0 beta version (Swofford 1998). Heuristic search was used with maximum likelihood and branch-and-bound search was used with parsimony and distance. Bootstrap analyses included 100 replicates for likelihood and 500 replicates for other methods. The best and suboptimal likelihood and parsimony trees were compared using the Kishino-Hasegawa test (Kishino and Hasegawa 1989.). The null-hypothesis was that the trees were not significantly different. It was rejected and the difference between the two trees considered statistically significant at $P \leq 0.05$. Additional distance analyses were performed using neighbor-joining (Saitou and Nei 1987) in the TREECON program (Van de Peer and De Wachter 1997).

RESULTS

Cell morphology. Initial classification of the new isolates (Table 1) used the morphotypes observed in insects. Availabil-

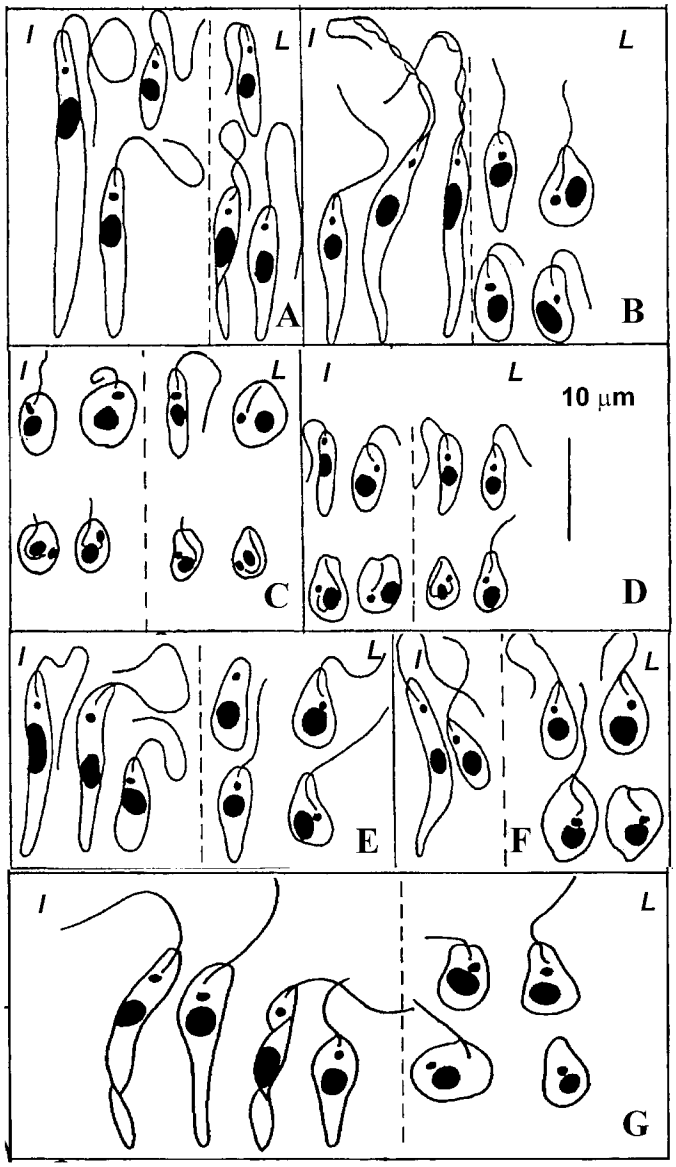

Fig. **1**. Camera lucida drawings of the insect trypanosomatids in the original insect vector (I) and in laboratory culture (L). A, *Leptomonas* sp. Nfm; B, *Blastocrithidia gerricola;* C, *Wallaceina inconstans*; D, *Wallaceina brevicula*; E, *Leptomonas* sp. Cfm; F, *Leptomonas peterhoffi*; G, *Leptomonas* sp. F6. Additional explanations are given in the text. Bar = 10 μm.

ity of the corresponding cultures now allows us to investigate if there have been morphological changes upon cultivation.

Typical promastigotes were observed for *Leptomonas* sp. Nfm, both in insects and in culture, supporting its genus assignment (Fig. 1A). Both isolates of *Wallaceina* maintained the set of morphotypes typical for this genus (Podlipaev, Frolov, and Kolesnikov 1990): promastigotes and endomastigotes, the morphotype characterized by the flagellum not extending beyond the deep flagellar pocket (Frolov 1994; Janovy, Lee, and Brumbaugh 1974), were found in culture, as well as in insect. There were epimastigotes in the culture of *B. triatoma* and mostly promastigotes, with the rare occurrence of choanomastigote-like types, in *Leptomonas collosoma* and *Leptomonas seymouri* (not shown), in agreement with their taxonomic names and original descriptions (Cerisola et al. 1971; Wallace 1977; Wallace et al. 1960).

A change of predominant morphotypes was observed in culture for four isolates (Fig.1). *Leptomonas* sp. Cfm and *Leptomonas* sp. F6 displayed a promastigote phenotype in the hemipteran host, while the cell morphology in culture was more choanomastigote-like. *Leptomonas peterhoffi* showed a promastigote morphology in the bug intestine, but was represented by a mixture of choanomastigote- and endomastigote-like cells in culture. The morphological changes were more drastic in *Blastocrithidia gerricola*. The original identification of this parasite from the insect gut was based on the observed predominant epimastigotes with some rare promastigotes. However, cell morphology in culture varied between pro- and choanomastigote, and no epimastigotes were observed.

Phylogenetic analysis. In order to determine a position of the root of the trypanosomatid tree, we have aligned trypanosomatid sequences with the outgroup sequence of *Bodo saltans* (Table 2, taxon set 1). The 35 ingroup taxa contained representatives of the previously defined major groups of trypanosomatids (Hollar, Lukeš, and Maslov 1998) together with the ten new isolates. In order to reduce the unequal rate effects, we did not include the fast-evolving sequences of salivarian trypanosomes (Lukeš et al. 1997). The choice of *B. saltans* as the outgroup was determined both by its close relatedness to trypanosomatids and by the slow rate of divergence of its SSU rRNA sequence (Doležel et al. 2000). The alignment contained 1656 alignable characters representing the most conserved regions of the molecule, and, therefore, was used only to determine a position of the root.

In the maximum likelihood (Fig. 2A), parsimony and distance trees (not shown), consistent with the previous reconstructions (Hollar and Maslov 1997; Hollar, Lukeš, and Maslov 1998; Lukeš et al. 1997), the root was found to be between the monophyletic clades corresponding to trypanosomes (T, Fig. 2) and remaining non-trypanosome (NT, Fig 2). The first diverging lineage among non-trypanosomes was *B. triatoma.* The bootstrap support for the exact position of *B. triatoma* as a sister-group to the other non-trypanosomes was not very high: 49% in likelihood and 40% in parsimony (not shown). The majority consensus tree showed *B. triatoma* within the unresolved clade of non-trypanosomes supported at the 81% level with maximum likelihood (Fig. 2B) and 73% level with parsimony (not shown). The likelihood trees constrained for the earliest divergence of *B. triatoma* in the family (constraint 1) or for the monophyly of *B. triatoma* with trypanosomes (constraint 2) showed the same basal trichotomy composed of *B. triatoma,* trypanosomes, and non-trypanosomes (Fig. 2C). The score of such trees was not significantly different from the best tree (Table 2). Most of the constrained parsimony trees were not significantly different either. Therefore, the alternative branching orders for *B. triatoma* cannot be completely excluded.

During the next step of the analysis we investigated the relationships within the non-trypanosome clade using a second alignment from which *B. saltans* and most trypanosome taxa were omitted and only the relatively slowly evolving *Trypanosoma avium, Trypanosoma rotatorium,* and *Trypanosoma boissoni* were included as outgroups (taxon set 2). The reduction of taxa allowed us to increase the number of alignable characters to 1836 in order to achieve a better phylogenetic resolution in the ingroup. The bootstrapped consensus likelihood tree (Fig. 3) showed that the new isolates were dispersed among different clades. A separate position of *B. triatoma,* excluded from the clade of all remaining non-trypanosomes, was now supported at the 60% level. *Blastocrithidia gerricola,* on the other hand, was found within the late diverging group, which

Table **2.** Parameters of the phylogenetic trees.

Taxon set and the tree	Maximum likelihood		Parsimony (*N* of trees)	
	ln likelihood	*P*-value	*N* of steps	*P*-value
Taxon set 1:				
No constraints	−6885.78202	—	833 (12)	—
Constraint 1 (O,(Bt,(T),(NT)))	−6892.57484	0.1323	837 (12)	0.2483–0.5166
Constraint 2 (O,((Bt,T),(NT)))	−6892.57484	−0.1323	840 (3)	0.0196[a] (1) 0.0522 (2)
Taxon set 2:				
No constraints	−8000.74019	—	1026 (2)	—
Constraint 3 (Divergence of *L. collosoma* prior to all other NT lineages)	−8006.58155	0.2473	1034 (8)	0.1701–0.3098
Constraint 4 (Monophyly of all *Leptomonas* including *L. collosoma*)	−8078.32815	0.0001[a]	1062 (39)	<0.0001[a]
Constraint 5 Monophyly of all *Leptomonas*, except *L. collosoma*)	−8039.86638	0.0135[a]	1045 (8)	0.0023[a] 0.0056[a]
Constraint 6 (Monophyly of *Leptomonas* Nfm, *Leptomonas* sp., *L. seymouri* and *L. collosoma*)	−8165.35245	<0.0001[a]	1119 (2)	<0.0001[a]
Constraint 7 (Monophyly of *Leptomonas* Nfm, *Leptomonas* sp., *L. seymouri*)	−8158.86124	<0.0001[a]	1106 (1)	<0.0001[a]
Constraint 8 (Monophyly of all *Blastocrithidia*)	−8351.16272	<0.0001[a]	1199 (75)	<0.0001[a]
Constraint 9 (Monophyly of *B. triatoma* and *B. culicis*)	−8153.08363	<0.0001[a]	1111 (3)	<0.0001[a]
Constraint 10 (Monophyly of *B. triatoma* and *B. gerricola*)	−8190.37029	<0.0001[a]	1122 (1)	<0.0001[a]

[a] At $P < 0.05$, the difference between the best tree and the tree in question is statistically significant. Abbreviations: O, outgroup; Bt, *B. triatoma*; T, trypanosomes; NT, non-trypanosomes.

also included two isolates of *Leptomonas* (*Leptomonas peterhoffi* and *Leptomonas* sp. F6), as well as both species of *Wallaceina.* The endosymbiont-containing *Blastocrithidia culicis* was found within the 'endosymbiontic' clade. Most *Leptomonas* species were found within the paraphyletic clade, diverging after the separation of *Leptomonas* sp. Nfm. However, *L. collosoma* was a sister-group to the 'endosymbiontic' clade. So, *Blastocrithidia* and *Leptomonas* appeared polyphyletic in this reconstruction.

The same conclusion was also reached with parsimony, distance (paralinear, Kimura 3-parameter), and neighbor-joining methods (not shown). The differences among the trees obtained were mainly concerned with several taxa whose positions were not very stable on the likelihood tree either. For example, *Leptomonas* sp. Nfm, whose association with the 'slowly-evolving' clade was supported only at the 58% level (Fig. 3) was associated with the clade of endosymbiont-free *Herpetomonas* in the distance trees. Also using distance, *L. collosoma,* whose grouping with the 'endosymbiontic' clade is supported at the 66% level in the likelihood tree (Fig. 3), was the earliest branch instead of *B. triatoma.* It should be noted that by imposing the topological constraint for this position of *L. collosoma* in the likelihood or parsimony analyses (Table 2, constraint 3), we obtained trees that are only slightly different from the corresponding best unconstrained trees.

With parsimony, there was a reversal in the branching order of the *Leishmania-Endotrypanum* and *Leptomonas seymouri-Leptomonas* sp. clades, while other clades remained unchanged. However, as was revealed by the distance and parsimony bootstrap analyses, these alternative topologies were not supported, and the corresponding consensus trees (not shown) were largely unresolved.

Regardless of the exact position of the above lineages, the polyphyly of the genera *Leptomonas* and *Blastocrithidia* was well established in the above analysis. In addition, we directly addressed this question by comparing the scores of trees with the enforced topological constraints vs. the best unconstrained trees. We have found that by enforcing monophyly for all taxa identified as *Leptomonas* (constraint 4), we would obtain significantly less likely or less parsimonious trees, and this remained so even after excluding *L. collosoma* (constraint 5) or several other isolates (Table 2, constraints 6 and 7). Similarly, by grouping together all *Blastocrithidia* species (constraint 8), two epimastigote isolates (constraint 9), or two endosymbiont-free species (constraint 10), we obtained significantly inferior tree scores (Table 2).

The polytomy in the well-supported terminal group composed of both species of *Wallaceina,* two species of *Leptomonas* (*L. peterhoffi, Leptomonas* sp. F6), and *B. gerricola* could not be resolved by either likelihood or parsimony even after excluding most of the taxa and aligning the remaining sequences along their entire lengths (2041 alignable characters). Only the distance (Fig. 4) and neighbor-joining (not shown) methods separated the branch of *Leptomonas* sp. F6 from the remaining four taxa. The rest of the terminal group was resolved identically by all the methods used: there was a highly supported clade of *Leptomonas* sp. and *L. seymouri* and a weakly supported clade of *C. fasciculata* and *Leptomonas* sp. Cfm (Fig. 4).

DISCUSSION

Ten isolates of monogenetic trypanosomatids from insects previously classified as representatives of four different genera were investigated here by means of molecular phylogenetic analysis of the SSU rRNA sequences. Although the ribosomal dataset has its limitations (Philippe 1998), it still remains the most comprehensive single gene dataset for trypanosomatids, justifying the use of this marker for our phylogenetic analyses. The use of different taxon sets, each serving a specific analytical goal, should have allowed us to keep the maximal amount

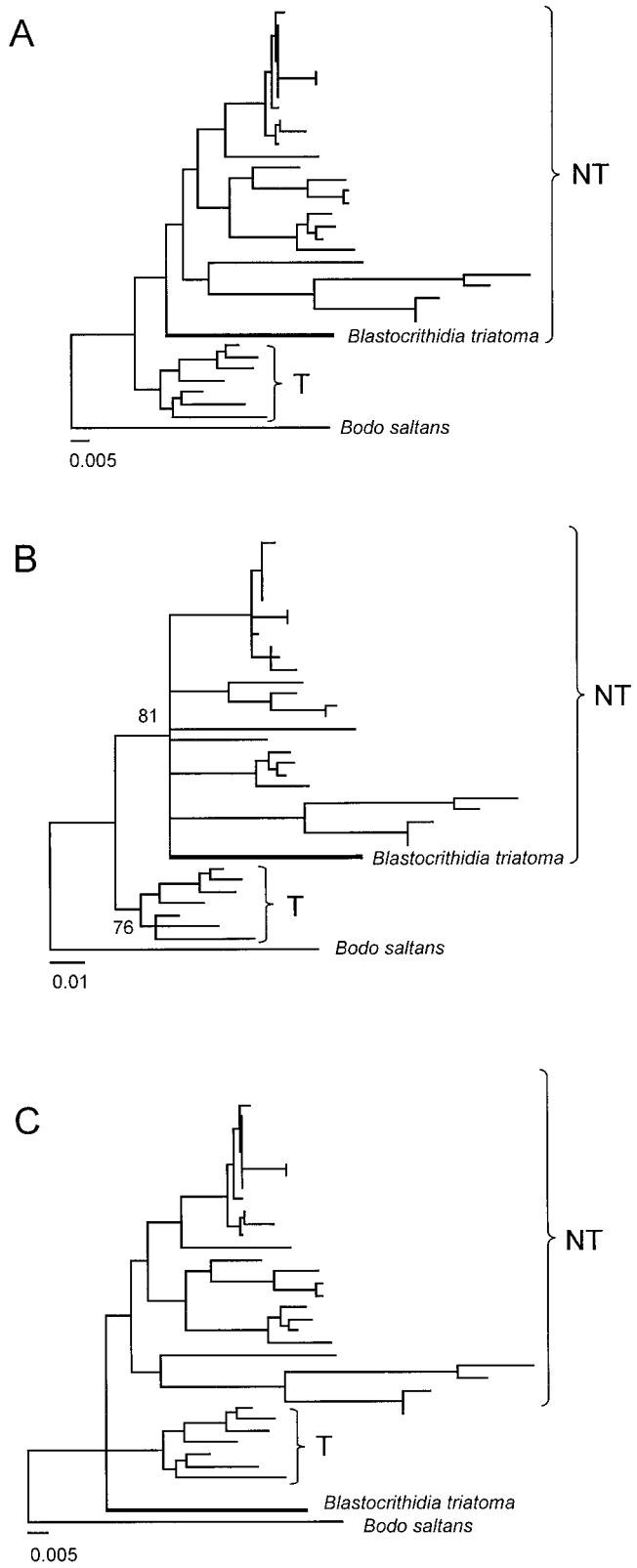

Fig. 2. Position of the root of the trypanosomatid small subunit rRNA tree as determined by maximum likelihood using taxon set 1 (see Table 2). (A) The best tree (unconstrained). The tree was obtained by heuristic search using empirical base frequencies, and the following parameters were estimated via likelihood: transition/transversion ratio (1.643619), value of proportion of invariable sites (0.199807), and value of γ-shape parameter (0.149852). (B) Majority consensus (50%) bootstrapped tree obtained for 100 pseudoreplicates with the same set of parameters as in A except that the same rate was assumed for all variable sites. (C) The tree obtained by using topological constraints 1 and 2 (see Table 2). The estimated parameters were as in A. Only the branches of *B. triatoma* and *B. saltans* are labeled on the tree, T—the clade of trypanosomes, NT—the clade of non-trypanosomes. The scale bars and numbers under the trees indicate the number of substitutions per site. *Ln*-likelihood of the tree B is −7410.91179, other likelihood values are given in Table 2.

of phylogenetic information that a given alignment can provide, and at the same time exclude the hypervariable sites that have a potential for mutational saturation (Philippe 1998). In order to minimize the influence of the unequal rate effects on the tree topology, we excluded the fast evolving taxa whenever possible. The same goal was approached by using the method of maximum likelihood with its low susceptibility to unequal rates, homoplasies, and a sampling bias (Swofford et al. 1996). Although the corresponding tree obtained for taxon set 2 (Fig. 3) is well resolved, it still contains several long branches (*B. triatoma, L. collosoma,* and *Leptomonas* sp. Nfm), and it is desirable to subdivide them in the future.

The position of *B. triatoma* near the root of the tree was intriguing, since a monogenetic blastocrithidia-like organism was hypothesized to be among the ancestral trypanosomatid forms (Baker 1963; Hoare 1972). Analysis of the constrained trees indicated that the rRNA data are consistent with the various scenarios of the early divergence of *B. triatoma* within the Trypanosomatidae, suggesting a possibility of the origin of trypanosomatids from a monogenetic epimastigote.

The analysis has shown that four major clades of non-trypanosomes identified earlier (’*Phytomonas*’, ’*Herpetomonas*’, ‘Endosymbiontic’ and ‘Slowly-evolving’) (Hollar, Lukeš, and Maslov 1998) represent only the tip of the iceberg. Three isolates, *Leptomonas* sp. Nfm, *L. collosoma,* and *B. triatoma,* apparently do not belong to any of these clades. It is obvious that the set of investigated cultures falls short of adequately representing the diversity of insect trypanosomatids. The current work, by introducing ten isolates from new geographical loci, has in part filled this gap.

Previous analyses have shown that trypanosomatid taxonomy does not generally agree with phylogeny (Du, Maslov, and Chang 1994; Hollar, Lukeš, and Maslov 1998). Even when a monophyletic clade included all investigated representatives of a certain genus, suggesting it was a natural taxon, such as *Phytomonas* (Hollar and Maslov 1997; Marche et al. 1995) or *Trypanosoma* (Haag, O’hUigin, and Overath 1998; Lukeš et al.1997; Stevens et al. 1999), one could never be sure that the addition of new isolates would not change this situation. The most obvious disagreement between phylogeny and taxonomy, reported earlier, is the polyphyly of the *Herpetomonas* and *Crithidia* (Du and Chang 1994; Du, Maslov, and Chang 1994; Hollar, Lukeš, and Maslov 1998), in which the endosymbiont-bearing members formed a well-defined ‘endosymbiontic’ clade, while their endosymbiont-free counterparts formed separate clades. The ‘endosymbiontic’ clade also included a representative of *Blastocrithidia,* clearly demonstrating that the morphotypes used to define the trypanosomatid genera are of limited value for determining genetic relatedness among these organisms.

The current work extends these observations by showing that the genera *Blastocrithidia* and *Leptomonas* are also artificial taxa. The polyphyly of *Blastocrithidia* is shown by a separation

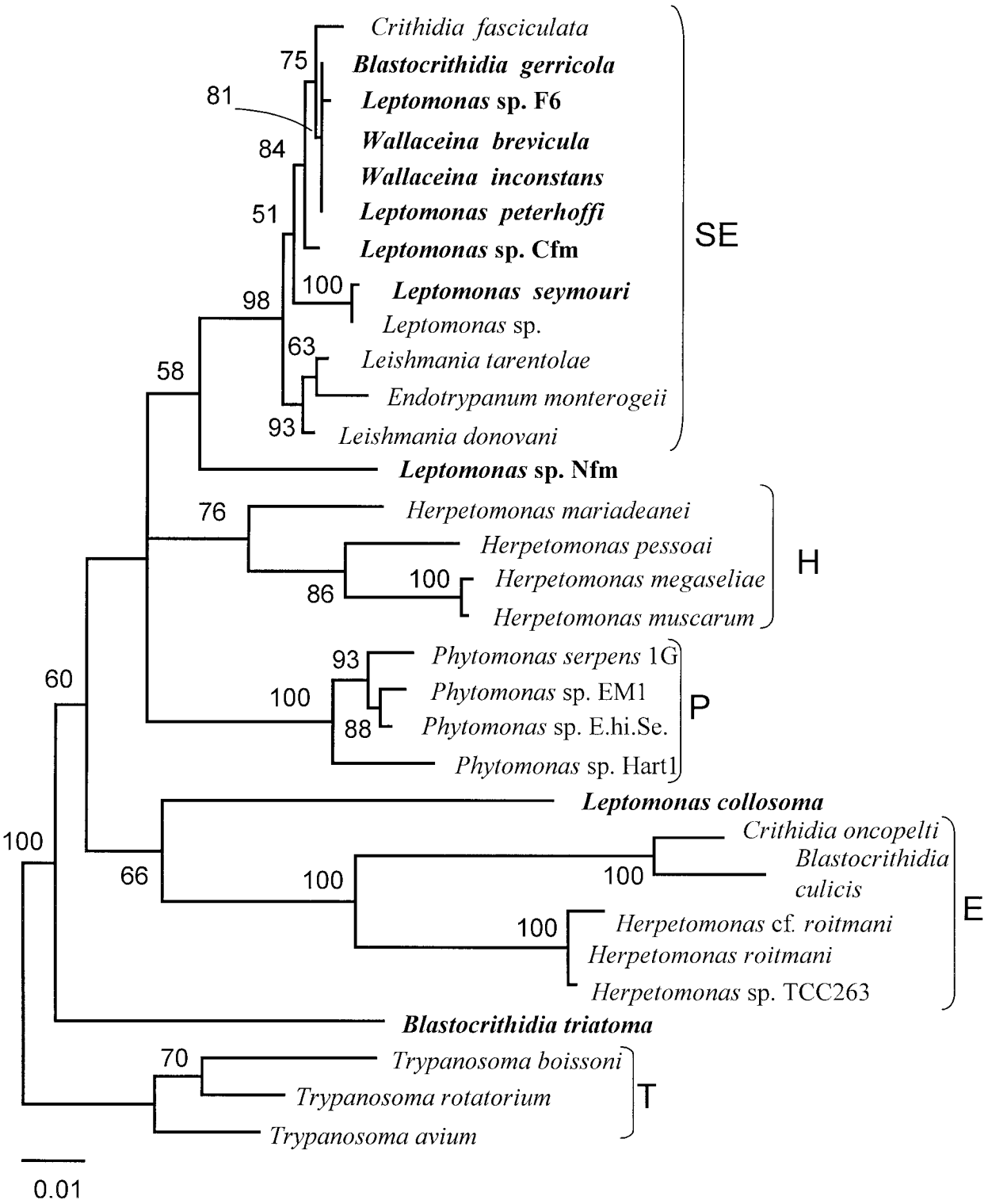

Fig. 3. Phylogenetic relationships within the clade of non-trypanosomes. The maximum likelihood consensus bootstrap tree for the small subunit rRNA was inferred from 100 pseudoreplicates of the alignment for taxon set 2 (Table 2) by heuristic searches using empirical base frequencies. The following parameters were estimated via likelihood: transition/transversion ratio (1.655089), and value of proportion of invariable sites (0.717556). All variable sites were assumed to evolve with the same rate. *Ln*-likelihood value is −8053.36975. The sequences determined in this work are indicated with boldface. T—trypanosomes, E—endosymbiont-containing trypanosomatids, P—*Phytomonas* spp., H—endosymbiont-free *Herpetomonas* spp., SE—trypanosomatids with slowly-evolving SSU rRNA sequences.

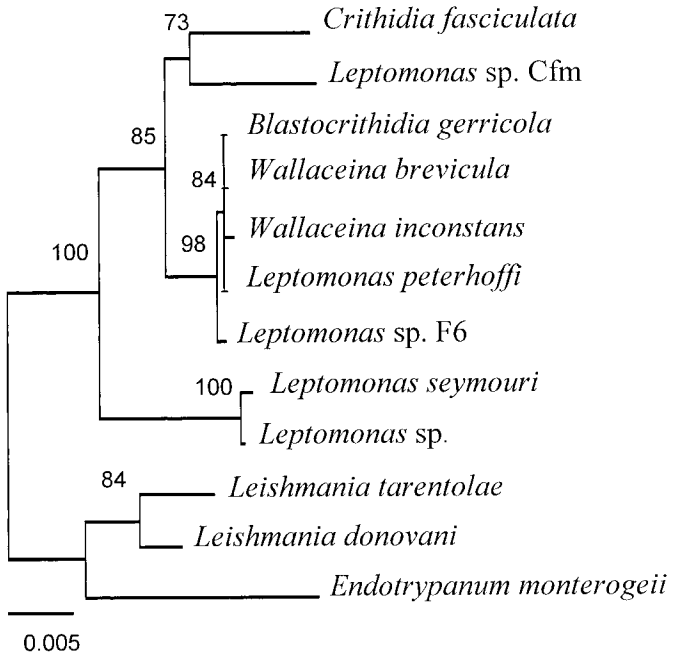

Fig. **4**. Relationships within the 'slowly-evolving' clade of non-trypanosomes (see Fig. 3). The paralinear distance bootstrapped (500 pseudoreplicates) consensus tree was inferred from the alignment constructed for taxon set 3 (Table 2). The scale bar under the tree corresponds to the number of substitutions per site.

of *B. triatoma* from *B. culicis*. The topological constraint bringing them together produced an inferior tree (Table 2). The case of *B. gerricola* needs a special discussion. This organism was originally identified as a blastocrithidia by observing epimastigotes in the bug intestine (Podlipaev 1985). At the same time, rare promastigotes were also noticed. It was suggested that the original isolate was a mixed infection and that another (not a *Blastocrithidia*) organism might have been present. The culture obtained from that source contained only promastigote- and choanomastigote-like cells (Fig. 1B). The phylogenetic analysis demonstrates that this isolate is, indeed, very close to *Wallaceina* (see discussion below). Therefore, in *B. gerricola,* the original description and our culture may correspond to different organisms (see also Bulat, Mokrousov, and Podlipaev 1999).

Of several isolates of *Leptomonas* studied here, only *Leptomonas* sp. Nfm, *L. collosoma,* and *L. seymouri* maintained promastigote morphology in culture and could be regarded as bona fide representatives of this genus. However, their monophyly was not supported by our analysis, and this remained so even after exclusion of *L. collosoma.* The same was observed for any broader assembly of the isolates that showed a promastigote morphology in insect hosts (Table 2). This situation can be expected, since the promastigote morphotype is not unique for this genus but is shared by a variety of trypanosomatids. A high level of genetic heterogeneity within the current genus *Leptomonas* was also reported earlier (Camargo et al. 1992).

The genus *Wallaceina* (see also footnote to Table 1) was proposed for the monogenetic trypanosomatids with a characteristic endomastigote shape or a long curved intracellular part of the flagellum (Podlipaev, Frolov, and Kolesnikov 1990). Both species analyzed here were found within the 'slowly-evolving' clade forming an unresolved subgroup, which also included *B. gerricola* together with the three *Leptomonas* isolates, and which was associated with *C. fasciculata* and *Leptomonas* sp. Cfm (Fig. 3, 4). The close relationships of *Wallaceina* spp., *B. gerricola* and *L. peterhoffi* were previously supported by the universally-primed PCR/cross-hybridization (Bulat, Mokrousov, and Podlipaev 1999) and RAPD (S.A.P., Banuls, A., Gargani, D., Dollet, M., unpubl. data) analyses, and the similarity of *Wallaceina* spp. with *Leptomonas* sp. F6 was confirmed by restriction analysis of kDNA (Kolesnikov, Maslov, and Podlipaev 1990). Nevertheless, unlike the SSU rRNA sequences, these methods also revealed differences between the isolates in question. Some distinctions within this group can also be made based on morphology. The case of *B. gerricola* was discussed above. *Leptomonas peterhoffi* was originally described as a *Leptomonas* on the basis of promastigotes present in the host intestine (Fig. 1F) (Podlipaev 1985). Further investigation showed that in culture there were rounded cells with a varying position of the kinetoplast and a long curved flagellar pocket (Malysheva and Skarlato 1989). Similar shapes are seen in *Wallaceina,* but in *L. peterhoffi, Leptomonas* sp. F6, and also in *B. gerricola,* there were no typical endomastigotes characteristic for *Wallaceina.* From the morphological data we can conclude that *L. peterhoffi, Leptomonas* sp. F6, and *B. gerricola* represent close but separate entities with respect to *Wallaceina.* The association of *Leptomonas* sp. Cfm with *C. fasciculata* in the SSU tree does not conflict with the observed morphology as the boundary between the promastigote and choanomastigote shapes may be blurred. Investigations of faster evolving markers, such ribosomal spacers, may be helpful in obtaining a better resolution for these trypanosomatids.

With at least four genera of trypanosomatids being polyphyletic, the classification system may need a revision. Morphology can still be used as an auxiliary character, but cannot represent a basis for the future taxonomy. The genetic affinities of the organisms defined by molecular phylogenetic and cluster analyses will provide an alternative set of criteria. It has already become clear that some monophyletic groups, such as the trypanosomes, leishmanias, some phytomonads and endosymbiont-free herpetomonads, can represent the future natural taxa bearing the same names. Although the clade of leishmanias also includes two species currently classified as *Endotrypanum* (Croan and Ellis 1996; Croan, Morrison, and Ellis 1997; Noyes, Camps, and Chance 1996; Noyes et al. 1997), their identities have been questioned recently, and the very existence of a separate genus *Endotrypanum* has been questioned (Cupolillo et al. 2000). In addition, a new genus should be proposed for the clade of endosymbiont-containing trypanosomatids, as phylogenetic support for this clade is very strong (Hollar, Lukeš, and Maslov 1998). Most revisions will probably be required for the genera *Crithidia, Leptomonas, Blastocrithidia,* and *Wallaceina.* Application of additional markers and analysis of a broader range of isolates will be needed in order to reveal the true diversity of insect trypanosomatids.

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