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Evolution

Monophyletic origin of β-division proteobacterial endosymbionts and their coevolution with insect trypanosomatid protozoa *Blastocrithidia culicis* and *Crithidia* spp.

(endosymbiosis/kinetoplastida/ribosomal RNA gene/compensatory mutation/biased base transition)

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ABSTRACT **Some trypanosomatid protozoa (order Kinetoplastida) are well known to harbor bacterial endosymbionts. Their phylogenetic positions and evolutionary relationships with the hosts were deduced by comparing the rRNA gene sequences. Earlier, we observed that these symbionts from three *Crithidia* spp. are identical and are closely related to *Bordetella bronchiseptica*. We have now sequenced the genes of another endosymbiont and the host protozoan *Blastocrithidia culicis*. The 16S rRNA genes of the *Blastocrithidia* and *Crithidia* symbionts share ≈97% identity and form a distinct group, branching off the *B. bronchiseptica* lineage in the β-division of Proteobacteria. Comparison of their secondary structures in the stem regions suggests compensatory mutations of the symbiont sequences, contributing to their biased base transitions from G to A and C to T. Two putative genes encoding $tRNA^{Ile}$ and $tRNA^{Ala}$ are highly conserved in the otherwise variable internal transcribed spacer region. Comparisons of the host rRNA gene sequences suggest that the symbiont-containing *Crithidia* and *Blastocrithidia* are more akin to each other than to other trypanosomatids. The evidence suggests that *Blastocrithidia* and *Crithidia* symbionts descend from a common ancestor, which had presumably entered an ancestral host and thence coevolved with it into different species. We therefore propose naming the symbionts *Kinetoplastibacterium blastocrithidii* and *Kinetoplastibacterium crithidii*.**

Bacterial endosymbionts exist in diverse eukaryotes—e.g., insects, plants, and protozoa (1–3). These endosymbionts have gained attention because of possible relevance to origins of mitochondria and chloroplasts (4). The small subunit (SSU) rRNA gene has widely served as a phylogenetic marker for microorganisms with little fossil records and has proved especially useful for molecular taxonomy of noncultivable endosymbionts (5, 6).

Bacterial endosymbionts have been observed in some insect trypanosomatids (7, 8)—e.g., *Crithidia oncopelti* (9), *Crithidia deanei* (10), *Crithidia desouzai* (11), *Blastocrithidia culicis* (12), and *Herpetomonas roitmani* (13). The symbionts defy cultivation outside their hosts and are limited usually to one per protozoan. Nutritional analyses of hosts rendered permanently symbiont free (14) have demonstrated that the symbionts supply them with growth factors—e.g., heme, purines, various amino acids, and/or vitamins (15, 16). The symbionts and their hosts are thus intimately associated, suggestive of an ancient evolutionary origin of this endosymbiosis.

To better understand the phylogenetic positions of these symbionts and the symbiont–host evolutionary relationships, we have studied their gene sequences—i.e., those from *Crithidia* spp. (17). In the present study, we have obtained the rDNA sequences from the blastocrithidial symbiont and protozoan host. These sequences along with those of the crithidial symbionts were compared. The results suggest that the symbionts are of monophyletic origin within the β-division of Proteobacteria and have coevolved with their hosts into different species.

MATERIALS AND METHODS

Cells. *B. culicis* (ATCC catalogue no. 30268) was cultured and cloned in brain/heart infusion medium (BHI) (Bacto-Difco) (Detroit) as described for *Crithidia* spp. (17). Symbiont-free lines of *B. culicis* were obtained by treating cloned cells for 14 days with chloramphenicol at 800 μg/ml in BHI broth containing 0.5% erythrocyte lysates (18). Loss of symbionts in these lines was demonstrated by fluorescence microscopy and the absence of symbiont DNA was demonstrated by Southern blot analysis (17, 18).

Cloning and Sequencing of SSU rRNA Genes. Standard methods were followed for isolation of total DNA and for molecular cloning and related techniques (19). The 16S rRNA gene plus the downstream internal transcribed spacer region of *B. culicis* endosymbiont was amplified from the total DNA of symbiont-containing cells by PCR using two pairs of eubacteria-specific primers (p1Seq, 5′-AGAGTTTGATCCTGGCTCAG-3′; p1100Rev, 5′-AACTAATGACAAGGGTTGCGC-3′; p3Seq, 5′-CCCGCACAAGCGGTGGATG-3′; p23sRev, 5′-TCCAAGGCATCCACCGTAT-3′) (see refs. 17 and 18). No PCR products were obtained from symbiont-free lines. Both PCR products were of the expected size and were cloned in pGEM PCR cloning T vector (Promega). For subcloning, the 1100-bp fragment (p1Seq-p1100Rev) and the 1200-bp fragment (p3Seq-p23sRev) were cut with *Sac* II and *Nco* I, respectively. These fragments were cloned into pBluescript SK+ (Stratagene) and completely sequenced as double-stranded DNA (United States Biochemical Sequenase, version 2.0) by the dideoxyribonucleotide chain-termination method using additional primers (p2Rev, 5′-AGCCGGTGCTTATTCTGCAG-3′; pITS100Seq, 5′-GTGCAGTCGGTATAGG-3′; pITS300Rev, 5′-GCTCTCCCAATTGAGCTACA-3′).

To clone the 18S rRNA gene of *B. culicis*, the total cell DNA was partially digested with *Pst* I and ligated into pBluescript SK+ for transformation of XL1-Blue competent *Escherichia coli*. The library was screened with the 2.1-kb coding region of the SSU rRNA gene PCR-amplified from *C. oncopelti* (18). Positive clones contained inserts, each with a

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Abbreviation: SSU, small subunit.

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2-kb *Pst* I fragment. Preliminary sequencing of this fragment revealed the coding region of the SSU rRNA gene minus the first 75 bp. Fragments cut with *Apa* I, *Hinc*II, *Hin*dIII, *Sac* II, and *Xho* II were subcloned to facilitate sequencing. The first 75-bp sequence missing from the clone was obtained from the genomic DNA by PCR amplification using the primer (p5Seq, 5′-GGAAGCTTATCTGGTTGATCCTGC-CAGTA-3′) specific to the 5′ conserved region of the SSU

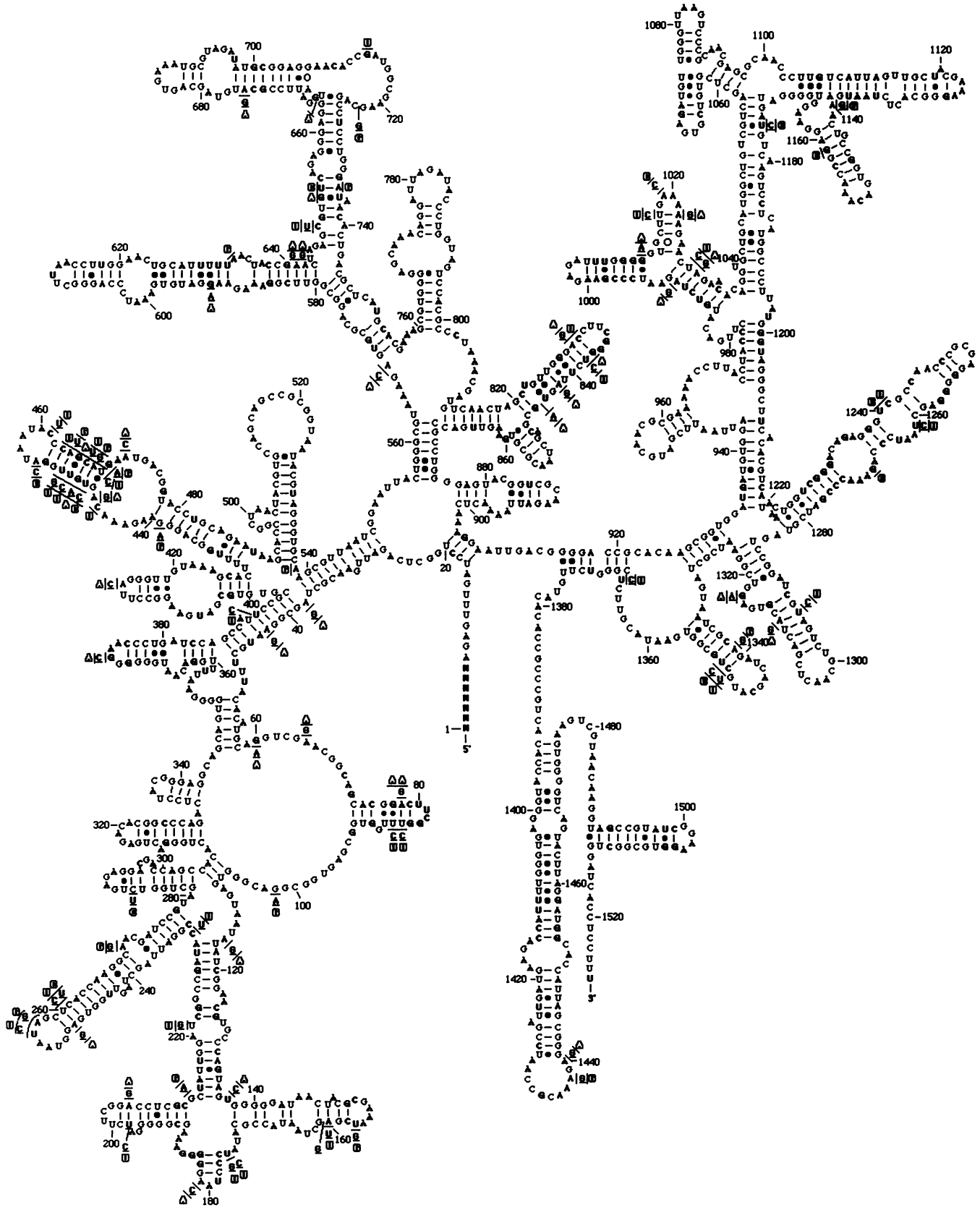

FIG. 1. Secondary structure of 16S rRNA gene of *B. culicis* endosymbiont. Backbone, *B. culicis* symbiont sequence; underlined and outlined bases, substitutions in *B. bronchiseptica* and *C. oncopelti* symbiont sequences, respectively. Secondary structure is constructed after that of the *E. coli* 16S rRNA gene (24).

Table 1. Evolutionary distances (above the diagonal) and percentage similarities (below the diagonal) of some β-division Proteobacteria

	Eik.	*N.g.*	*P.t.*	*R.g.*	*C.* endo	*B.b.*	*A.f.*	*B.* endo
Eik.		0.0601	0.1599	0.1443	0.1470	0.1250	0.1323	0.1422
N.g.	0.9355		0.1697	0.1494	0.1354	0.1194	0.1411	0.1399
P.t.	0.8585	0.8551		0.0934	0.1492	0.1276	0.1477	0.1547
R.g.	0.8680	0.8659	0.9158		0.1127	0.0984	0.1197	0.1178
C. endo	0.8711	0.8803	0.8764	0.8988		0.0457	0.0743	0.0317
B.b.	0.8866	0.8933	0.8895	0.9140	0.9567		0.0530	0.0485
A.f.	0.8812	0.8738	0.8740	0.8876	0.9309	0.9456		0.0782
B. endo	0.8793	0.8772	0.8734	0.8945	0.9718	0.9533	0.9266	

Evolutionary distances are calculated by DNAdist in PHYLIP using Kimura's two-parameter model. Percentage similarities are based on Bestfit output of the Genetics Computer Group. *Eik.*, *Eikenella* sp.; *N.g.*, *N. gonorrhoeae*; *P.t.*, *P. testosteroni*; *R.g.*, *R. gelatinosus*; *C.* endo, *Crithidia* endosymbiont; *B.b.*, *B. bronchiseptica*; *A.f.*, *A. faecalis*; *B.* endo, *B. culicis* endosymbiont.

rRNA gene (20) and another primer specific for the downstream sequence (p75Rev, 5'-GCGTTTCGCCAAGTTATC-C-3'). The amplified fragment was cloned in pGEM PCR cloning T vector and sequenced.

Phylogenetic Analysis. The rRNA gene sequences were initially aligned by using PILEUP in the Genetics Computer Group program package (University of Wisconsin, Madison). Alignments were further adjusted by using the SEQ. EDIT program (21) on the basis of alignments retrieved from the ribosomal data base project (22). Sequences were analyzed by maximum-likelihood analysis (FASTDNAML; ref. 22) as well as by a DNApars program (PHYLIP) (23). Bootstrapping analysis performed by using 500 replicas. Distance matrix was generated by the DNAdist program in the PHYLIP package with Kimura's two-parameter model (23).

RESULTS AND DISCUSSION

***Blastocrithidia* and *Crithidia* Symbionts Share Common Features in Their rRNA Gene Sequences.** The rDNA sequence obtained from the *B. culicis* symbiont (GenBank accession no. L29265) shares several features in common with that of the *Crithidia* symbionts (GenBank accession no. L29303) (17). The G+C content is ≈53% for both. The two sequences share 97.3% identity in the SSU rRNA coding region, both being most closely related to *Bordetella bronchiseptica* (see below). Base substitutions vs. *B. bronchiseptica* in the *B. culicis* symbiont sequence are biased: 42 G to A and C to T transitions in a total of 72 substitutions (Fig. 1). Similar biased base transitions were reported previously in symbiont sequences from *Crithidia* and from other eukaryotes (ref. 17 and references therein). When the secondary structures of the SSU rRNAs constructed from the three genes in question are compared, compensatory mutations involving G·U intermediates are evident in the stem regions, which may contribute to the biased base transitions (Fig. 1). The internal transcribed spacer regions, presumably under less evolutionary pressure for conservation, differ by ≈20% overall between the two symbiont sequences. This region contains two putative genes encoding $tRNA^{Ile}$ and $tRNA^{Ala}$ deduced from their secondary structure analysis (data not shown). As expected, both genes are more conserved than the remaining internal transcribed spacer sequences between the two symbionts, differing by only 1 base.

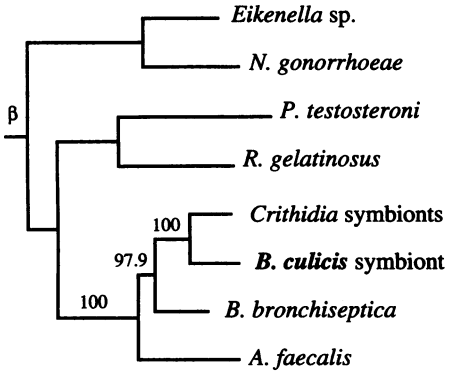

FIG. 2. Parsimony phylogenetic tree of the 16S rDNA sequences from symbionts and β-division purple bacteria. Confidence levels determined by bootstrap analysis for the endosymbionts and close relatives are shown above the branches. GenBank accession nos. are as follows: L06165, *Eikenella* sp.; X07714, *Neisseria gonorrhoeae*; M11224, *Pseudomonas testosteroni*; M60682, *Rodocyclus gelatinosus*; L29303, *Kinetoplastibacterium crithidii*; L29265, *Kinetoplastibacterium blastocrithidii*; X57026, *B. bronchiseptica*; M22508, *Alcaligenes faecalis*.

***Blastocrithidia* Symbiont Is Closely Related to *Crithidia* Symbionts, Both Being Proteobacteria in the β-Division.** As found previously with the *Crithidia* symbionts (17), the *Blastocrithidia* symbiont also belongs to the β-division Proteobacteria, according to the genetic distance analysis of its SSU rRNA gene together with those from 18 other representative bacteria (data not shown). The *B. culicis* symbiont was further compared with *Crithidia* symbionts and six other Proteobacteria in the β-division (Table 1). As shown in the consensus parsimony tree (Fig. 2), the *B. culicis* symbiont is grouped closest to the *Crithidia* symbionts. Bootstrap analysis supported the grouping of the two symbionts and their placement closest to *B. bronchiseptica* in 100% and 98% of the replicas, respectively (Fig. 2). This grouping was supported by the maximum-likelihood and distance analyses.

From the SSU rDNA sequence data, it is evident that the genetic distance between *Crithidia* and *B. culicis* symbionts (Table 1) is equivalent to those among different *Bordetella* spp. (25). Coupled with other known biological properties of the endosymbionts, this difference seems to justify the consideration of these symbionts as two new species of bacteria within a new genus. We propose naming the symbionts of *B. culicis* and *Crithidia* spp. *Kinetoplastibacterium blastocrithidii* and *Kinetoplastibacterium crithidii*, respectively. Members of the genus may be described as noncultivable, cell wall-deficient, intracellular Gram-negative Proteobacteria of the β-division, symbiotically associated with insect trypanosomatid protozoa (1, 3).

The SSU rRNA Gene Sequence Places *B. culicis* Closer to the Three Symbiont-Containing *Crithidia* spp. Than to Other Trypanosomatids. The *B. culicis* 18S rRNA gene was completely sequenced (GenBank accession no. L29266) (G+C content, 53.9%). Genetic distance analyses of the 18S rRNA genes suggest that *B. culicis* is more closely related to symbiont-containing *Crithidia* spp. (GenBank accession no. L29264) (26) than to all other trypanosomatid protozoa sequenced so far (Table 2). Evolutionary trees were constructed previously for these organisms by using *Bodo caudatus* (27) or *Trypanoplasma borreli* (28) as the outgroup. A consensus parsimony tree including representative members of the group and the symbiont-containing species is presented in Fig. 3. Bootstrapping analysis suggests that *B. culicis* is

Table 2. Evolutionary distances (above the diagonal) and percentage similarities (below the diagonal) of trypanosomatid protozoa

	C.o.	*B.cu.*	*L.d.*	*L.a.*	*E.m.*	*C.f.*	*Lepto.*	*T.c.*	*T.b.*	*B.ca.*
C.o.		0.0972	0.1101	0.1095	0.1126	0.1075	0.1069	0.1378	0.1635	0.1976
B.cu.	0.9185		0.1499	0.1487	0.1510	0.1509	0.1491	0.1769	0.1856	0.2185
L.d.	0.9140	0.8998		0.0033	0.0195	0.0272	0.0263	0.1293	0.1728	0.1896
L.a.	0.9134	0.8959	0.9967		0.0171	0.0263	0.0248	0.1280	0.1728	0.1876
E.m.	0.9133	0.8919	0.9803	0.9827		0.0347	0.0303	0.1257	0.1692	0.1885
C.f.	0.9179	0.9013	0.9787	0.9794	0.9705		0.0195	0.1327	0.1720	0.1834
Lepto.	0.9115	0.8946	0.9780	0.9808	0.9747	0.9808		0.1278	0.1727	0.1825
T.c.	0.9079	0.8815	0.9110	0.9096	0.9147	0.9142	0.9091		0.1622	0.2011
T.b.	0.8818	0.8914	0.8828	0.8786	0.8835	0.8829	0.8786	0.8990		0.2253
B.ca.	0.8621	0.8473	0.8658	0.8635	0.8638	0.8615	0.8650	0.8596	0.8574	

Evolutionary distances are calculated by DNAdist in PHYLIP using Kimura's two-parameter model. Percentage similarities are output of Bestfit of the Genetics Computer Group. *C.o., C. oncopelti; B.cu., B. culicis; L.d., L. donovani; L.a., L. amazonensis; E.m., E. monterogei; C.f., C. fasciculata; Lepto., Leptomonas* sp.; *T.c., T. cruzi; T.b., T. brucei; B.ca., B. caudatus.*

monophyletic with the symbiont-containing *Crithidia* spp. in nearly 80% of the replicas. This grouping holds in all phylogenetic trees generated by a variety of methods (e.g., distance, maximum likelihood), although the topology in other parts of the trees is less robust, especially within the clade that includes *Leishmania, Endotrypanum, Crithidia fasciculata*, and *Leptomonas*, as indicated by the low bootstrap values (Fig. 3). Our finding of the similar symbionts in *B. culicis* and *Crithidia* spp. lends additional credence to their phylogenetic closeness. *B. culicis* is morphologically much more similar to trypanosomes than to crithidias. The phylogenetic closeness of this species to the symbiont-containing *Crithidia* spp. is thus as unexpected as the finding of a distant relationship between the latter and *C. fasciculata* (26).

Monophyletic Origin of *Blastocrithidia* and *Crithidia* Symbionts: Their Coevolution with the Hosts. From their SSU rDNA sequences, *B. culicis* and *Crithidia* symbionts are phylogenetically most closely related, and so are their hosts. It is thus likely that this symbiosis might have been established in a single event (Fig. 3, arrow) between ancestral bacterium and ancestral host, which had subsequently coevolved into the extant symbiont and host species. However, the evolutionary distance between the two symbionts (0.0317) is 3-fold less than that of the two hosts (0.0972) (Tables 1 and 2). Evolution of symbiont and host sequences

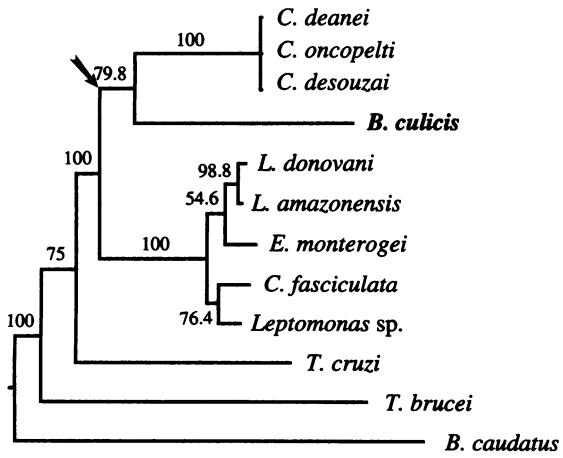

FIG. 3. Parsimony phylogenetic tree of the SSU rDNA sequences from kinetoplastid protozoa. Confidence levels according to bootstrap analysis for the symbiont-containing protozoa are shown above the branches. Arrow, hypothetical event of single entry for symbiosis. GenBank accession nos. for the organisms are as follows: L29264, *C. oncopelti, C. desouzai*, and *C. deanei*; L29266, *B. culicis*; X07773, *Leishmania donovani*; X53912, *Leishmania amazonensis*; X53911, *Endotrypanum monterogei*; X03450, *C. fasciculata*; X53914, *Leptomonas* sp.; M31432, *Trypanosoma cruzi*; M12676, *Trypanosoma brucei*; X53910, *B. caudatus*.

at an unequal rate must be assumed in order to accommodate the scenario of single-event symbiosis. A similar scenario was proposed for the endosymbionts in aphids, where host–symbiont coevolution was evident (29). It is less likely that trypanosomatid symbionts may be acquired independently by each host after the divergence of their ancestor into different species, although this possibility cannot be totally ruled out. The proposed single origin of endosymbionts and their coevolution with the hosts agree with the idea of synapomorphy of endosymbiosis deduced from phenetic analysis of the host protozoa alone (30).

Phagocytosis and Acquisition of Endosymbionts by Trypanosomatid Protozoa. It is known that the extant hosts are neither phagocytic nor susceptible to experimental infection by symbionts or other bacteria. Thus, the evolutionary antiquity of this symbiosis might date back to a time when the ancestral protozoa were still capable of these cellular activities to recruit endosymbionts. The presumptive ancestors are reminiscent of *Bodo* spp.—the only group of trypanosomatid protozoa known with certainty to remain phagocytic today. Conceivably, a *Bodo*-like ancestor may have acquired a bacterium by phagotrophy in a single event that has set the stage for the evolution of contemporary endosymbioses in trypanosomatid protozoa—e.g., *B. culicis* and *Crithidia* spp. The evolutionary descendance of these species from *Bodo* is, however, interrupted by several aposymbiotic groups—e.g., *Trypanosoma brucei, T. cruzi, Leishmania*, and *Leptomonas* according to the current view (Fig. 3). Our hypothesis must be tempered then with the consideration of either multiple losses of endosymbionts from the aposymbiotic groups or descendance of all the symbiont-containing species from another hitherto unidentified ancestral lineage; however, no current evidence is available to substantiate either possibility. How trypanosomatid protozoa acquired their endosymbionts awaits further investigation to better understand their evolution of phagocytosis and insusceptibility to bacterial infections.

The acquisition of symbionts is estimated to have occurred 40–120 million years ago. This time frame is deduced from the genetic distances of the relevant bacterial SSU rRNA genes according to their proposed evolutionary rate at 0.01–0.02 per site per 50 million years (29). The upper time limit is set tentatively by branching of the hypothetical ancestral bacterium toward the evolution of intracellular lifestyle—i.e., the divergence between the endosymbionts and their closest extracellular relative, *B. bronchiseptica* (0.0485); the lower time limit is set on the basis of the divergence between the two symbionts (0.0317) (Table 1). Divergence of the host protozoa presumably occurs after acquisition of the symbiont within the same time frame if the single-event hypothesis is correct.

The host protozoa under study were originally isolated from very different insects: *B. culicis* from a mosquito (*Aedes*

vexans), *C. oncopelti* from a plant sap feeding bug (presumably *Oncopeltus fasciatus*), *C. deanei* from a predatory bug (*Zelus leucogrammus*), and *C. desouzai* from a nectar-feeding fly (*Ornidia obesa*) (7–11). The association of these protozoa with so diverse a group of dipteran and hemipteran insects arouses wonder about what roles these insects may play in the evolution and/or spreading of the endosymbiosis in question. Whether the protozoa may acquire their symbionts from their insect hosts is unknown, but the extant Proteobacteria of the β-division are not outstandingly entomophilic (1). Indeed, insect endosymbionts studied so far belong to other divisions (see ref. 17 and references therein). The evolutionary and biological interrelationships of symbiont–protozoan–insect associations present many fascinating mysteries that await further elucidation.

In summary, SSU rRNA gene sequence analyses of the endosymbionts and their trypanosomatid hosts *B. culicis* and *Crithidia* spp. have led us to assume that the symbiotic associations originated from a single event, which involved the acquisition of β-division Proteobacterium by ancestral host followed by their coevolution into different species. Further analyses of additional symbiont and host genes will help us determine whether the same event may actually give rise to all endosymbioses observed in trypanosomatid protozoa (13, 31–34). Preliminary studies of *H. roitmani* and its endosymbiont yielded results consistent with this notion.

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