Morphological and biochemical characterization of the trypanosomatids *Crithidia desouzai* and *Herpetomonas anglusteri*

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Two species of trypanosomatids, *Crithidia desouzai* and *Herpetomonas anglusteri*, were recently isolated from Diptera in Minas Gerais, Brazil. The *Crithidia* species was found to harbor bacterium-like endosymbionts in the cytoplasm. To biochemically characterize these two species of trypanosomatids, and to try to verify the evolutionary meaning of the presence of endosymbionts, an electrophoretic study was undertaken whereby the two species were compared with eight other species in the same family. Horizontal 12.5% starch gel electrophoresis was used to resolve the isozymes of eight enzyme systems: acid phosphatase, glucose-6-phosphate dehydrogenase, hexokinase, malate dehydrogenase, malic enzyme, 6-phosphogluconate dehydrogenase, phosphoglucose isomerase, and phosphoglucomutase. Ten other enzyme systems were assayed without yielding any reproducible activity. The isozymes observed were conservatively interpreted as being due to the activity of 44 different alleles. All species studied differed in at least one enzyme system. The phenetic (Jaccard similarity index, UPGMA grouping) analysis produced a tree in which the species of *Crithidia* and *Herpetomonas* clustered separately, forming monophyletic groupings. All the endosymbiont-bearing species formed a monophyletic cluster, indicating that the presence of bacterium-like endosymbionts may be a synapomorphy of that group, and may represent, therefore, a unique event in the evolution of the genus.

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Deux espèces de trypanosomatidés, *Crithidia desouzai* et *Herpetomonas anglusteri*, ont été trouvées récemment chez des Diptères de Minas Gerais (Brésil). Le *Crithidia* contenait des endosymbiontes de type bactérien dans le cytoplasme. Une analyse des enzymes par électrophorèse a permis de déterminer la composition biochimique des deux espèces de trypanosomatidés, d'évaluer l'importance évolutive de la présence des endosymbiontes et de comparer les deux espèces à huit autres espèces de la même famille. L'électrophorèse horizontale sur gel d'amidon 12.5% a permis d'identifier les isoenzymes de huit systèmes d'enzymes (phosphatase acide, glucos-6-phosphate déshydrogénase, hexokinase, malate déshydrogénase, enzyme malique, 6-phosphogluconate déshydrogenase, phosphoglucose isomérase et phosphoglucomutase). L'analyse de 10 autres systèmes d'enzymes n'a révélé aucune activité décelable. Une interprétation consevatrice permet d'affirmer que les isoenzymes observés sont probablement dus à l'activité d'au moins 44 allèles différents. Toutes les espèces étudiées diffèrent par au moins un système d'enzymes. L'analyse phénétique (coefficient de Jaccard, groupements selon l'association moyenne) donne lieu à un dendrogramme dans lequel les espèces de *Crithidia* et d'*Herpetomonas* sont séparées, formant des regroupements monophylétiques. Toutes les espèces porteuses d'endosymbiontes forment un groupe monophylétique, ce qui indique que la présence d'endosymbiontes de type bactérien constitue peut-être un caractère synapomorphe de ce groupe et reflète donc un événement unique dans l'évolution du genre.

[Traduit par la rédaction]

Introduction

Two species of flagellate Protozoa of the family Trypanosomatidae, *Crithidia desouzai* and *Herpetomonas anglusteri*, were recently isolated from Diptera caught in Minas Gerais, Brazil (Fiorini et al. 1989). Nutritional studies showed that *Crithidia* grew in a culture medium that lacked hemin and adenine. This is also the case for the trypanosomatids *Crithidia deanei* (Mundim et al. 1974), *Crithidia oncopelti* (Newton 1957), and *Blastocrithidia culicis* (Chang and Trager 1974), all of which bear bacterium-like endosymbionts in their cytoplasm.

To demonstrate the presence of endosymbionts in this isolate, parasites were processed for transmission electron microscopy. To further characterize the two species of trypanosomatids, using biochemical parameters, cloned strains were compared

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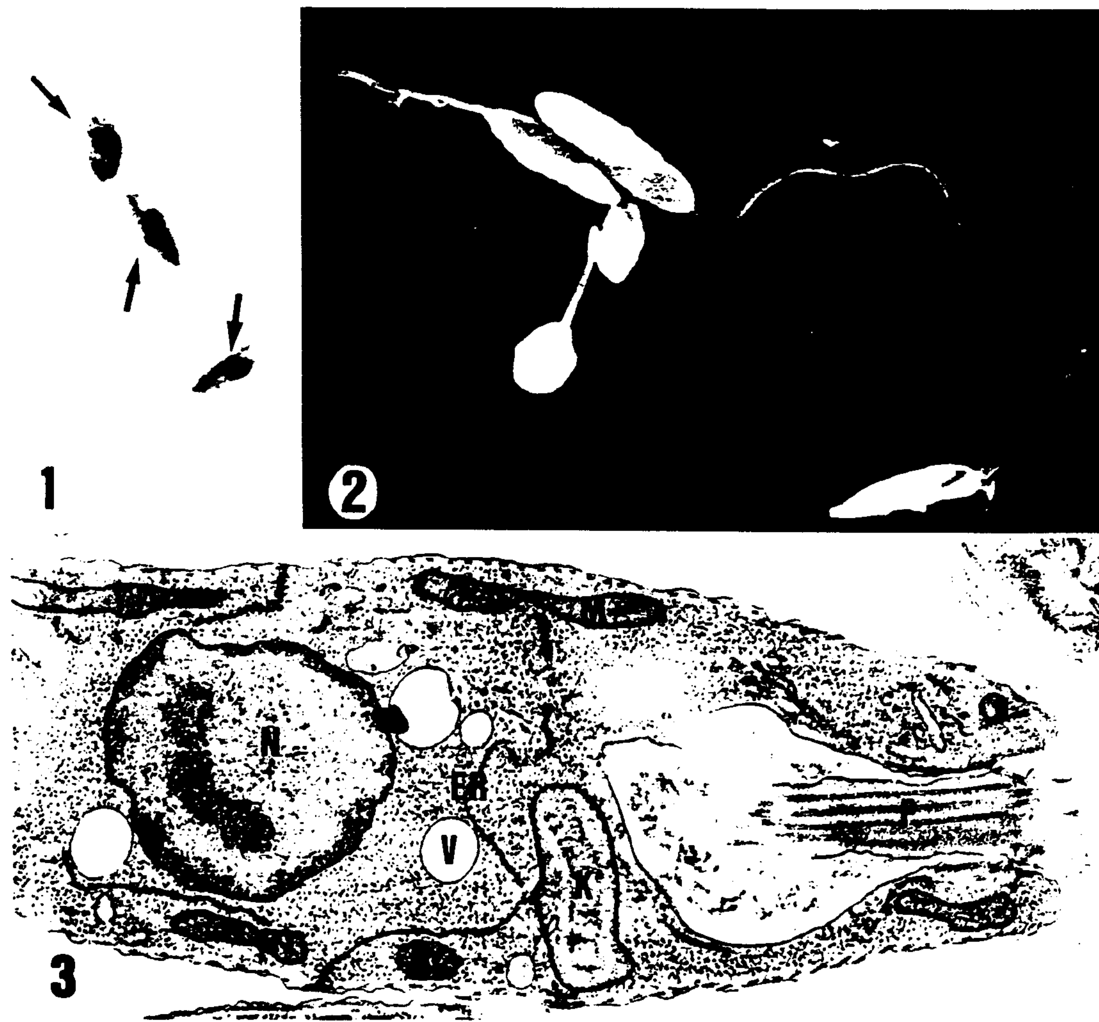

FIGS. 1–3. *Herpetomonas anglusteri*. Fig. 1. Pro-, para-, and opisto-mastigotes in the culture medium shown by light microscopy. The arrows indicate the kinetoplast. 1000×. Fig. 2. Scanning electron micrograph showing the elongated body with a long flagellum. 3200×. Fig. 3. Transmission electron micrograph of a typical promastigote form. The flagellum (F) arises from a thin kinetoplast (K) and emerges at the anterior tip of the cell. ER, endoplasmic reticulum; G, glycosome; M, mitochondrion; N, nucleus; V, membrane-bound vacuole. 20 000 ×.

with several other trypanosomatids of the genera *Crithidia* and *Herpetomonas* by isoenzyme electrophoresis. The results obtained are described in this paper.

Materials and methods

Organisms

Crithidia desouzai and *Herpetomonas anglusteri* were kept at 28°C in Warren's medium (Warren 1960) supplemented with 10% fetal calf serum. For the isoenzyme analysis the following parasites were used as reference strains: *Crithidia deanei* (ATCC[2] 30255) and *Crithidia oncopelti* (ATCC 30264) with symbionts, *Crithidia fasciculata* (ATCC 11745), *Crithidia guilhermei* (Soares et al. 1986), *Crithidia luciliae* (ATCC 14765), *Herpetomonas megaseliae* (ATCC 30209), *Herpetomonas muscarum muscarum* (ATCC 30260), and *Herpetomonas samuelpessoai* (ATCC 30252). All parasites were grown for 1 day at 28°C in Warren's medium supplemented with 10% fetal calf serum.

Scanning electron microscopy

The cells were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.2 for 2 h. The parasites were then left for 10 min to adhere to cover slips previously coated with poly-L-lysine (mol. wt. 70 000), briefly postfixed in 1% OsO_4, dehydrated in ethanol, and then critical-point dried with CO_2. The cover slips were coated with gold in a Balzer's sputtering device and observed under a Jeol 25S scanning electron microscope.

Transmission electron microscopy

The parasites were fixed for 2 h with 2.5% glutaraldehyde – 4% paraformaldehyde in 0.05 M cacodylate buffer, pH 7.2, with 1 mM $CaCl_2$. After fixation the cells were washed in 0.1 M cacodylate buffer and postfixed for 1 h with 1% OsO_4 – 0.8% potassium ferricyanide in 0.1 M cacodylate buffer, pH 7.2, with 1 mM $CaCl_2$. The cells were then washed again, dehydrated in ethanol, and embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate and observed in a Jeol 100CX transmission electron microscope.

Isoenzymes

Each of the cultures of the 10 trypanosomatids studied was homogenized by repeated freezing and thawing in liquid nitrogen in a hypotonic solution of 1 mM dithiothreitol, 1 mM aminocaproic acid, and 1 mM EDTA. The lysates were frozen and analyzed electrophoretically the following day.

The isoenzyme patterns of the species were obtained by horizontal

[2]American Type Culture Collection.

12.5% starch gel electrophoresis, using standard procedures for this group of organisms (Miles et al. 1980). The only difference was that 6 mm thick gels were used, yielding five 1.2-mm slices per gel. Staining solutions and enzyme nomenclature follow Brewer (1970) and Harris and Hopkinson (1978). Eighteen enzyme systems were assayed, but only 8 yielded reproducible results. These were acid phosphatase (ACP, EC 3.1.3.2), glucose-6-phosphate dehydrogenase (G6PD, EC 1.1.1.49), hexokinase (HK, EC 2.7.1.1), malate dehydrogenase (MDH, EC 1.1.1.37), malic enzyme (ME, EC 1.1.1.40), 6-phosphogluconate dehydrogenase (PGD, EC 1.1.1.44), phosphoglucoisomerase (PGI, EC 5.3.1.9), and phosphoglucomutase (PGM, EC 2.7.5.1). After staining, the gels were scored and preserved in 7% v/v acetic acid.

The electrophoretic data were interpreted conservatively by eliminating from the analysis those bands that might be due to heterohybridization (both within heterozygotes and between loci) in the dimeric enzymes. In this way the bands could be attributed to different gene products. A matrix of presence/absence for each allele in each species was built, and analyzed phenetically. The phenetic analysis used the Jaccard similarity index, and the unweighted pair-group mean analysis (Sneath and Sokal 1973) for building an overall similarity tree between the species.

Results

Light microscopy of Giemsa-stained parasites and scanning electron microscopy showed that *Crithidia desouzai* possesses a typical small choanomastigote form, with a body length of 4.7 µm. Near the nucleus of the parasites, one to three dense bodies could be seen by light microscopy. *Herpetomonas anglusteri* usually presents the promastigote form, 15.0 µm in body length, although para- and opisto-mastigote forms, typical of the genus *Herpetomonas*, could be seen in the culture medium (Figs. 1 and 2).

The fine structure of the flagellates was analyzed by transmission electron microscopy. *Herpetomonas anglusteri* did not show any structural features that could distinguish it from other members of the genus *Herpetomonas*. A typical promastigote form is shown in Fig. 3.

Ultrastructural analysis showed the presence of bacterium-like endosymbionts in the cytoplasm of *Crithidia desouzai*. The endosymbionts were enclosed by two unit membranes and were morphologically similar to those found in the other symbiont-bearing trypanosomatids (Fig. 4). These flagellates presented all the morphological differences typical of endosymbiont-bearing trypanosomatids: absence of the flagellar paraxial rod, a looser kinetoplast DNA network, and absence of subpellicular microtubules in regions where the mitochondrial branches were apposed to the plasma membrane (Fig. 4).

Clusters of virus-like particles were also observed in the cytoplasm of *Crithidia desouzai*. The particles were usually found near the nucleus and the endosymbiont (Figs. 4 (and inset) and 5). The cytoplasmic virus-like particles were rounded, with a mean diameter of 44.8 ± 6.7 (SD) nm. The distance between particles was 34.95 ± 7.11 nm. The particles were clustered, usually in a hexagonal array. Smaller electron-dense particles with a mean diameter of 36.55 ± 5.84 nm were frequently seen inside the nucleus, dispersed in the nuclear matrix (Fig. 6).

Unique isoenzyme banding patterns were observed for each of the 10 species studied (Table 1), thus confirming the taxonomic status of the new trypanosomatid species. *Crithidia desouzai* was genetically very similar to *C. deanei*, but differed in its PGM pattern. The phenetic analysis produced a tree in which the species of *Herpetomonas* and *Crithidia* formed separate clades; within the genus *Crithidia* the endosymbiont-bearing species formed a separate group (Fig. 7).

TABLE 1. Allele distribution for the eight enzymes studied in *Herpetomonas* and *Crithidia* spp., showing the presence (1) or absence (0) of the allele in the sample. Alleles (bands) at each locus are numbered according to decreasing mobility in the gel

	MDH												G6PD			
	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4
H. samuelpessoai	0	0	0	0	0	0	1	1	0	0	1	0	0	1	1	0
H. megaseliae	0	0	0	0	0	1	0	1	0	0	0	1	0	1	0	0
H. muscarum	0	0	0	0	0	1	0	1	0	0	0	1	0	1	0	0
H. anglusteri	0	0	0	0	0	0	1	0	1	1	0	0	1	0	1	0
C. desouzai	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1
C. fasciculata	1	0	0	1	1	0	0	1	0	0	0	0	1	0	0	0
C. oncopelti	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
C. deanei	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1
C. guilhermei	1	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0
C. luciliae	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1

	PGM						PGI							ME			
	1	2	3	4	5	6	1	2	3	4	5	6	7	1	2	3	4
H. samuelpessoai	0	0	1	0	0	0	0	0	0	1	0	1	0	1	0	0	0
H. megaseliae	0	0	0	0	0	1	0	0	1	1	0	0	1	0	1	0	0
H. muscarum	0	0	0	0	0	1	0	0	1	1	0	0	1	0	1	0	0
H. anglusteri	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0
C. desouzai	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0
C. fasciculata	0	0	0	0	0	0	1	1	0	0	1	0	0	0	0	1	0
C. oncopelti	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	1	0
C. deanei	1	1	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0
C. guilhermei	1	0	0	1	0	0	1	1	1	0	0	0	0	0	0	0	1
C. luciliae	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0

	PGD		HK				ACP				
	1	2	1	2	3	4	1	2	3	4	5
H. samuelpessoai	0	1	1	1	0	0	0	0	0	0	0
H. megaseliae	0	1	0	1	0	0	0	0	1	0	1
H. muscarum	0	1	0	1	0	0	0	1	0	0	1
H. anglusteri	0	1	0	0	0	0	0	0	0	0	0
C. desouzai	0	1	0	0	1	0	0	0	0	0	0
C. fasciculata	0	1	0	0	0	1	0	0	0	1	0
C. oncopelti	1	0	0	0	0	1	0	0	0	0	0
C. deanei	0	1	0	0	0	0	0	1	0	0	0
C. guilhermei	0	1	1	1	0	1	1	1	0	1	0
C. luciliae	0	1	0	0	1	0	0	1	0	1	0

N
B
K
M
F
4
V
N
V
G
B
N
6

Discussion

In this study, two species of trypanosomatids were characterized by isoenzyme analysis and electron microscopy: *Herpetomonas anglusteri*, isolated from *Liophygia ruficornis*, and *Crithidia desouzai*, isolated from *Ornidia obesa* (Fiorini et al. 1989).

Previous studies on the growth of *Herpetomonas anglusteri* in different culture media showed that this species is physiologically different from the other *Herpetomonas* species already described (Fiorini et al. 1989). At the ultrastructural level, this species presented the general morphological features described for other members of this genus analyzed previously in some detail (Brun 1974; Janovy et al. 1974; De Souza et al. 1976; Yoshida et al. 1978; Dedet et al. 1986).

Fiorini et al. (1989) have suggested that endosymbionts are present in *Crithidia desouzai*, because it can grow in a culture medium lacking hemin and adenine. This condition has previously been observed only in the trypanosomatids *C. deanei* (Mundim et al. 1974), *C. oncopelti* (Newton 1957), and *Blastocrithidia culicis* (Chang and Trager 1974), all of which have endosymbionts in their cytoplasm. The presence of such bacterium-like organisms in the cytoplasm of *C. desouzai* has now been confirmed by transmission electron microscopy.

As well as providing the host cell with nutrients such as hemin and purines (Chang and Trager 1974; Mundim and Roitman 1977; Salzman et al. 1985), the endosymbionts induce modifications in the host cell morphology (Freymüller and Camargo 1981), alter the composition of carbohydrates in the protozoan plasma membrane (Dwyer and Chang 1976; Esteves et al. 1982), and reduce the surface charge of the host cells (Oda et al. 1984). The effect of these modifications on trypanosomatid – host cell interactions (using nonpathogenic models) can be studied by comparing symbiont-free *Crithidia deanei*, *C. desouzai*, *C. oncopelti*, and *Blastocrithidia culicis* with normal strains, a field not yet fully explored. It was recently shown, for example, that the presence of an endosymbiont affects the interaction of *Crithidia deanei* with mouse macrophages. Endosymbiont-containing parasites were more likely to be ingested by mouse peritoneal macrophages but were more resistant than endosymbiont-free parasites (Rozental et al. 1987).

Different methods have been described for isolating pure fractions of endosymbionts (Tuan and Chang 1975; Alfieri and Camargo 1982; Novak et al. 1988), but until now few studies have used the intact organism to better understand its metabolism. The data obtained by Soares and De Souza (1988) in a freeze-fracture study of the endosymbiont of *Blastocrithidia culicis* support the idea that the symbiont is a Gram-negative bacterium-like microorganism enveloped by two unit membranes and lacking a peptidoglycan layer. The bacterial nature of the symbionts is also strongly suggested by its DNA organization (Spencer and Cross 1975), its sensitivity to antibiotics (Chang 1975), its metabolic pathways (Alfieri and Camargo 1982), and its fine structure (Gill and Vogel 1963; Gutteridge and Macadam 1971; Chang 1974; Mundim et al. 1974). The study of pure fractions of isolated symbionts from the four endosymbiont-bearing trypanosomatids might demonstrate whether all parasites are infected with the same bacterium-like organism.

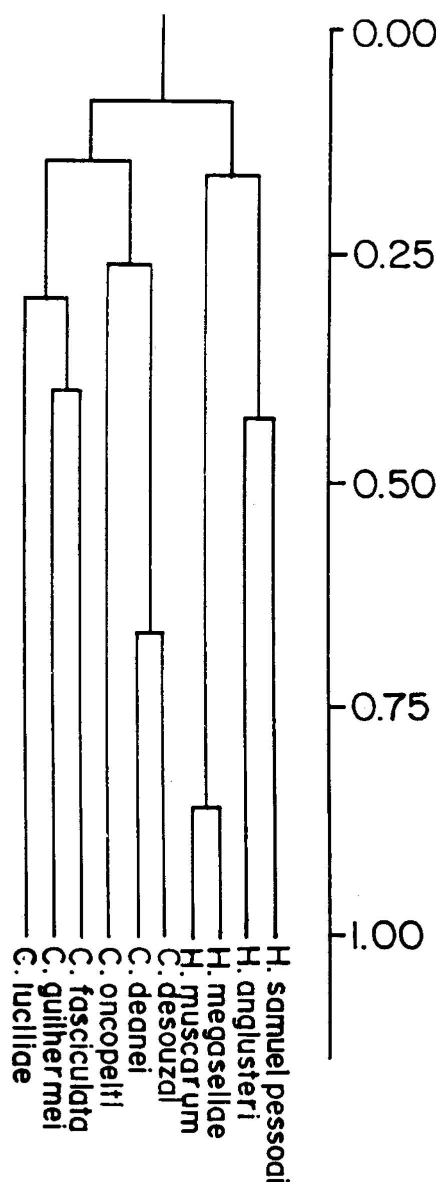

FIG. 7. UPGMA phenogram of Jaccard similarity indices between the 10 species of trypanosomatids studied.

Virus-like particles (VLPs) were seen in the cytoplasm of *Crithidia desouzai*. It is thus the only trypanosomatid parasite known to contain both bacterium-like organisms and VLPs in the cytoplasm. VLPs in trypanosomatids were first described in the cytoplasm (Molyneux 1974; Croft and Molyneux 1979) and

FIGS. 4–6. *Crithidia desouzai*. Transmission electron micrographs. Fig. 4. Portion of the cell showing the nucleus (N), the peripheral mitochondrion (M), the expanded kinetoplast (K), glycosomes (G), the bacterium-like endosymbiont (B), and the flagellar axoneme (F) lacking the paraxial structure. 26 400 ×. Inset: A cluster of virus-like particles (V) in the cytoplasm near the nucleus (N). 31 000 ×. Fig. 5. The clusters of virus-like particles (V) usually occur near the nucleus (N) and the bacterium-like endosymbiont (B). ER, endoplasmic reticulum; G, glycosome; M, mitochondrion. 30 000 ×. Fig. 6. Several electron-dense masses (arrowheads) are frequently seen inside the nucleus. These masses probably correspond to intranuclear stages of the virus-like particles. 52 000 ×.

nucleus (Croft 1979) of *Leishmania hertigi*. Different VLPs were also described in *Endotrypanum* spp. (Croft et al. 1980) and *Trypanosoma melophagium* (Molyneux and Heywood 1984). Single-stranded RNA viral particles were recently isolated from *Leishmania braziliensis guyanensis* (Tarr et al. 1988). The VLPs found in *C. desouzai* have an organization (paracrystalline array) and size (about 45 nm) similar to those of the cytoplasmic VLP of *Leishmania hertigi*. Although attempts to isolate this leishmanial VLP failed, it was suggested that they were reoviruses (Eley et al. 1987). The positive staining after EDTA treatment suggests that the VLPs of *C. desouzai* also consist of RNA (Soares et al. 1989). Periodic observations of the protozoan by transmission electron microscopy have shown that the VLPs have remained in the cytoplasm of the flagellates over a period of 2 years of in vitro passages in culture medium, with no apparent effects on protozoan ultrastructure and multiplication. *Crithidia desouzai* is thus an excellent experimental model for the study of interrelations among different genomes in the same cell, as four nucleic acids are present: the nuclear DNA, the mitochondrial kinetoplast–DNA network, the bacterium-like organism DNA, and the virus-like particle RNA.

The phenetic analysis of the electrophoretic data produced a tree in which the species of *Crithidia* and *Herpetomonas* clustered separately, forming monophyletic groupings. All species studied differed in at least one enzyme system. Our data (Fig. 7) agree with those of Lima et al. (1982): *C. fasciculata* and *C. luciliae* are grouped together and *C. oncopelti* is in a separate group. The phenetic analysis also suggests that *C. guilhermei* (although it differs from the other species by seven alleles) belongs to the same group as *C. fasciculata* and *C. luciliae*. These results contrast with those of Soares et al. (1986), who suggested the creation of a separate group for *C. guilhermei*.

The presence of bacterium-like endosymbionts in trypanosomatids may suggest convergent evolution throughout the group, but it may also be the result of unique evolutionary events within each genus. The autotrophy for hemin and adenine observed in endosymbiont-bearing trypanosomatids, for example (Newton 1957; Chang and Trager 1974; Mundim et al. 1974), might suggest selective advantages for such organisms, which would favor the rise of symbiotic relationships many times in the evolution of these genera. However, the relationship between endosymbionts and their hosts, as observed using electron microscopy, is very complex, and might require morphological specialization that is unlikely to have occurred more than once in the evolution of each taxon. If the presence of bacterium-like endosymbionts is the product of parallel or convergent evolution (homoplasy), it will not be correlated with other, independent genetic characteristics other than by chance alone. Alternatively, if it is the result of a unique event in the evolution of each genus, i.e., if it is a synapomorphy for a clade within the genus, it will co-vary within a monophyletic grouping, with other evolutionarily meaningful characters. The picture that emerged from the phenetic analysis suggests that the presence of bacterium-like endosymbionts is a synapomorphy of *C. deani*, *C. desouzai*, and *C. oncopelti*.

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