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# *Herpetomonas roitmani* (Fiorini et al., 1989) N. Comb.: A Trypanosomatid with a Bacterium-like Endosymbiont in the Cytoplasm

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ABSTRACT. The trypanosomatid previously described as *Crithidia roitmani* is characterized here at the ultrastructural and biochemical levels. The data indicates that the parasite belongs to the *Herpetomonas* genus, and we therefore suggest the flagellate to be denominated as *Herpetomonas roitmani* n. comb. Cladistic analysis of isoenzyme data generated by eight different enzymes showed that the parasite presented a distinct banding pattern and could be grouped with some *Herpetomonas* spp., but not with *Crithidia* spp., used as reference strains. Accordingly, when the parasites were grown for longer periods in Roitman's defined medium, expontaneous differentiation from promastigotes to opisthomastigotes (typical of the *Herpetomonas* genus) occurred. Transmission electron microscopy revealed the presence of bacterium-like endosymbionts in the cytoplasm of all evolutive forms of the parasite. All morphological alterations characteristic of endosymbiont-bearing trypanosomatids could be observed.

**Key words.** Differentiation, isozyme electrophoresis, taxonomy, trypanosomatid, ultrastructure.

SCREENING of the digestive tract of some diptera captured in Minas Gerais, Brazil led to the isolation of a trypanosomatid flagellate from the mid- and hindgut of the fly *Ornidia obesa* (Diptera: Syrphidae). A cloned strain was obtained, and according to the nutritional requirements and the morphological characterization in Giemsa-stained preparations, it was named *Crithidia roitmani* [9]. However, detailed examination of axenically cultivated parasites revealed in older cultures the presence of para- and opisthomastigote forms, typical of the genus *Herpetomonas*. Therefore, to characterize further the organisms, we studied the in vitro transformation process, and biochemically analysed the parasites and compared them with others of the *Crithidia* and *Herpetomonas* genera by isoenzyme electrophoresis. Nutritional studies showed that the parasites could be maintained in a medium lacking hemin, adenine, and several amino acids and vitamins [9], a condition observed in endosymbiont-bearing trypanosomatids [7, 19]. Since these data suggested the presence of endosymbionts, the ultrastructure of the trypanosomatid was also examined. The results here described show that the parasite previously described as *C. roitmani* is in fact an endosymbiont-bearing *Herpetomonas*. We therefore propose that flagellate to be reclassified to *Herpetomonas roitmani* (Fiorini et al., 1989) n. comb.

## MATERIALS AND METHODS

**Parasites.** Isolation and cloning of *H. roitmani* were described in [9]. The organisms have been maintained axenically since then at 28° C in Warren's [29] or Roitman's [21] culture media supplemented with 10% fetal calf serum. For the isoenzyme electrophoresis analysis, the parasite was compared with the following trypanosomatids: *Crithidia deanei* (ATCC 30255), *Crithidia desouzai* [9, 18], *Crithidia fasciculata* (ATCC 11745), *Crithidia guilhermei* [26], *Crithidia luciliae* (ATCC 14765), *Crithidia oncopelti* (ATCC 30264), *Herpetomonas anglusteri* [9, 18], *Herpetomonas megaseliae* (ATCC 30209), *Herpetomonas muscarum muscarum* (ATCC 30260), and *Herpetomonas samuelpessoai* (ATCC 30252). All parasites were grown for 24 h in Warren's medium.

**Differentiation.** Parasites were inoculated into Roitman's defined medium and cultivated at 28° C. Cells were collected by centrifugation at 12-h intervals and stained for light microscopy with the Giemsa stain. The percentage of pro-, para-, and opisthomastigote forms was determined. Two hundred cells were analysed in each count, which were made in duplicate by two workers.

**Transmission electron microscopy.** Two-day-old culture forms of *H. roitmani* (at the exponential phase of growth) were collected by centrifugation at 1,500 *g* and fixed for 2 h with 1.5% glutaraldehyde and 4% paraformaldehyde in 0.05 M cacodylate buffer, pH 7.2, containing 1 mM $CaCl_2$. Cells were then rinsed in 0.1 M cacodylate buffer containing 3% sucrose and 1 mM $CaCl_2$ and post-fixed for 1 h with 1% osmium tetroxide-0.8% potassium ferricyanide in 0.1 M cacodylate buffer, pH 7.2, containing 1 mM $CaCl_2$. After rinsing in 0.1 M cacodylate buffer with 3% sucrose, the parasites were *en bloc* stained with 2% aqueous uranyl acetate, dehydrated in ethanol, and embedded in Epon. Ultrathin sections were briefly stained with uranyl acetate and lead citrate and observed in a Jeol 100CX transmission electron microscope.

**Isoenzymes.** Each culture of the 11 trypanosomatids studied was homogenized by repeated freezing and thawing in liquid nitrogen in a hypotonic solution of 1 mM dithiothreitol, 1 mM aminocaproic acid, 1 mM EDTA. Lysates were frozen and analyzed electrophoretically the following day.

Isoenzyme patterns of the species were obtained by horizontal 12.5% starch gel electrophoresis using standard procedures for this group of organisms [16]. The sole difference was that 6-mm thick gels were used, producing five 1.2-mm slices per gel. Staining solutions and enzyme nomenclature follow Brewer [2] and Harris & Hopkinson [11]. Eighteen enzyme systems were assayed but only eight gave reproducible results: acid phosphatase (ACP, E.C. 3.1.3.2), glucose 6-phosphate dehydrogenase (G6PD, E.C. 1.1.1.49), hexokinase (HK, E.C. 2.7.1.1), malate dehydrogenase (MDH, E.C. 1.1.1.37), malic enzyme (ME, E.C. 1.1.1.40), 6-phosphogluconate dehydrogenase (PGD, E.C. 1.1.1.44), phosphoglucose isomerase (PGI, E.C. 5.3.1.9), and phosphoglucomutase (PGM, E.C. 2.7.5.1). After staining, gels were scored and preserved in 7% v/v acetic acid.

Electrophoretic patterns were interpreted conservatively by eliminating from the analysis bands that might be due to heterohybridization (both within heterozygotes and between loci) in the dimeric and tetrameric enzymes. In this way, the bands (allozymes) could be attributed to different gene products. A matrix of presence and/or absence for each allele in each species was built, and analyzed both phenetically and cladistically. The phenetic approach used the Jaccard similarity index, and the

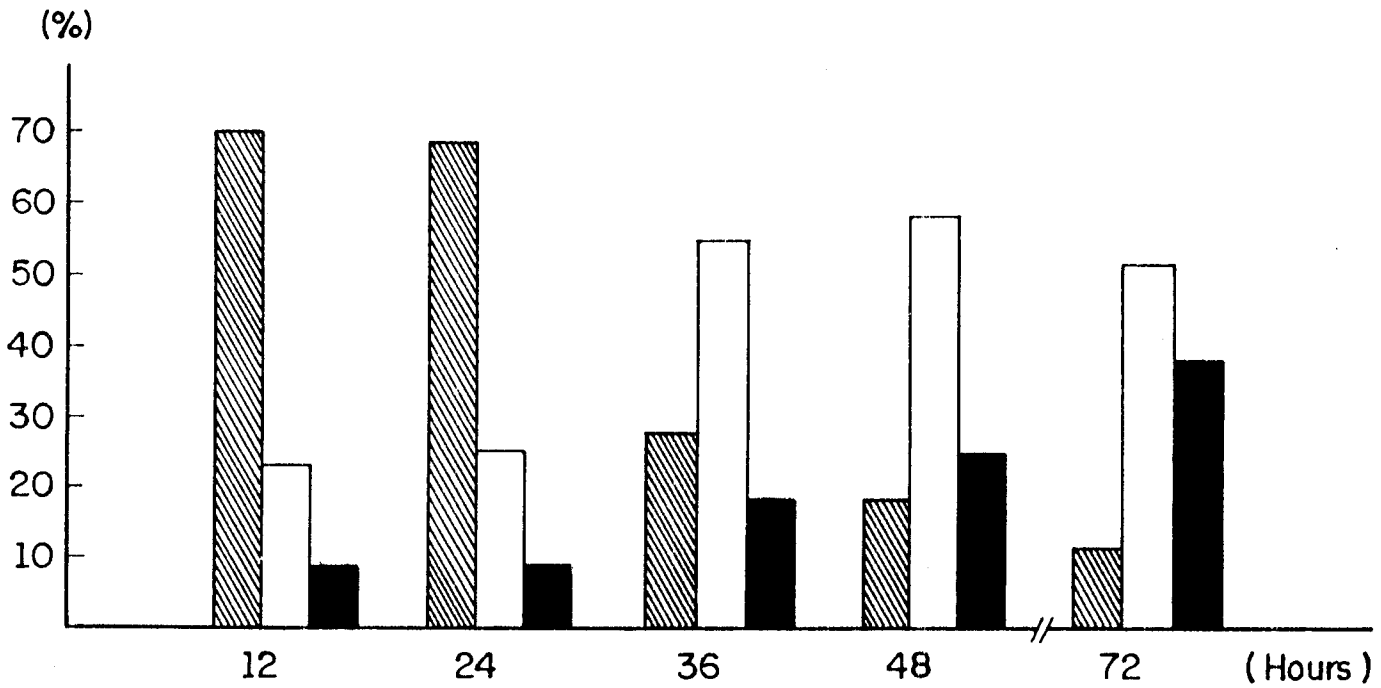


Fig. 1. Differentiation of *Herpetomonas roitmani* grown at 28° C in chemically defined medium (▨ = promastigotes, □ = paramastigotes, ■ = opisthomastigotes). Numbers in the X-axis represent hours in culture.

unweighted pair-group mean analysis [24] for building an overall similarity tree between the species. The cladistic analysis used a bootstrapped version of a Wagner algorithm [8]. Because of the lack of an available outgroup, rooting of the tree was done arbitrarily trying to minimize reversals. Rooting of the tree by this method coincided with the mean point of the longest branch and with the root calculated by the phenetic approach.

## RESULTS

Light microscopy of Giemsa-stained cells showed that the parasite had a slightly elongated body, 6.5-μm long, with the flagellum emerging at the anterior tip of the cell. When the parasites were grown at 28° C in Roitman's chemically defined medium, most cells (70%) appeared as small promastigotes up to the first 24 h of in vitro cultivation. However, the number of para- and opisthomastigote forms steadily increased in aging cultures and after 72 h these stages predominated in the growth medium; the opisthomastigotes represented 37% of the total number of cells (Fig. 1). No growth was obtained when the parasites were maintained at 37° C.

The presence of para- and opisthomastigote forms in the culture medium could also be observed by transmission electron microscopy (Fig. 2–5). The large kinetoplast DNA network, 0.9- to 1.0-μm long and 0.6- to 0.8-μm wide, was frequently observed beside or posterior to the centrally located nucleus. In both cases, the flagellum ran inside the cell encased in a long invagination of the cell plasma membrane, the flagellar pocket, and emerged at the anterior end of the trypanosomatid.

A bacterium-like endosymbiont was found in the cytoplasm of the flagellates. The symbionts were elongated organisms (1.5–2.0 × 0.5–0.8 μm), enveloped by two unit membranes, with an electron-dense inner matrix (Fig. 6). The organisms always appeared in good condition showing no signs of degeneration. They were found at the posterior region of the trypanosomatid near the nucleus, usually one organism per cell (Fig. 2–5), dividing synchronically with the host cell by constricting in the middle of the body (data not shown). In opisthomastigote forms, the nucleus, endosymbiont, and kinetoplast were usually in close proximity (Fig. 5). The host cell presented all the characteristic morphological alterations that occur in endosymbiont-bearing trypanosomatids: absence of the flagellar paraxial structure, a looser kinetoplast DNA network, and mitochondrial branching with absence of subpellicular microtubules in sites where the mitochondrial branches were apposed to the plasma membrane (Fig. 7). Treating the parasites with the antibiotic chloramphenicol produced aposymbiotic strains that maintained the same morphological organization (data not shown).

By isoenzyme electrophoresis 46 alleles could be observed (Table 1). The cladistic analysis of the electrophoretic data showed that the species of *Crithidia* and *Herpetomonas* formed separate monophyletic clades (Fig. 8). The parasite previously described as *C. roitmani* presented a distinct banding pattern and grouped with the *Herpetomonas* spp. Only one parsimonious tree was produced by the bootstrapped cladistic analysis indicating a high consistency for the tree. The number of homoplasies was relatively low (consistency index = 0.708). The tree was topologically identical with that produced phenetically (Fig. 9).

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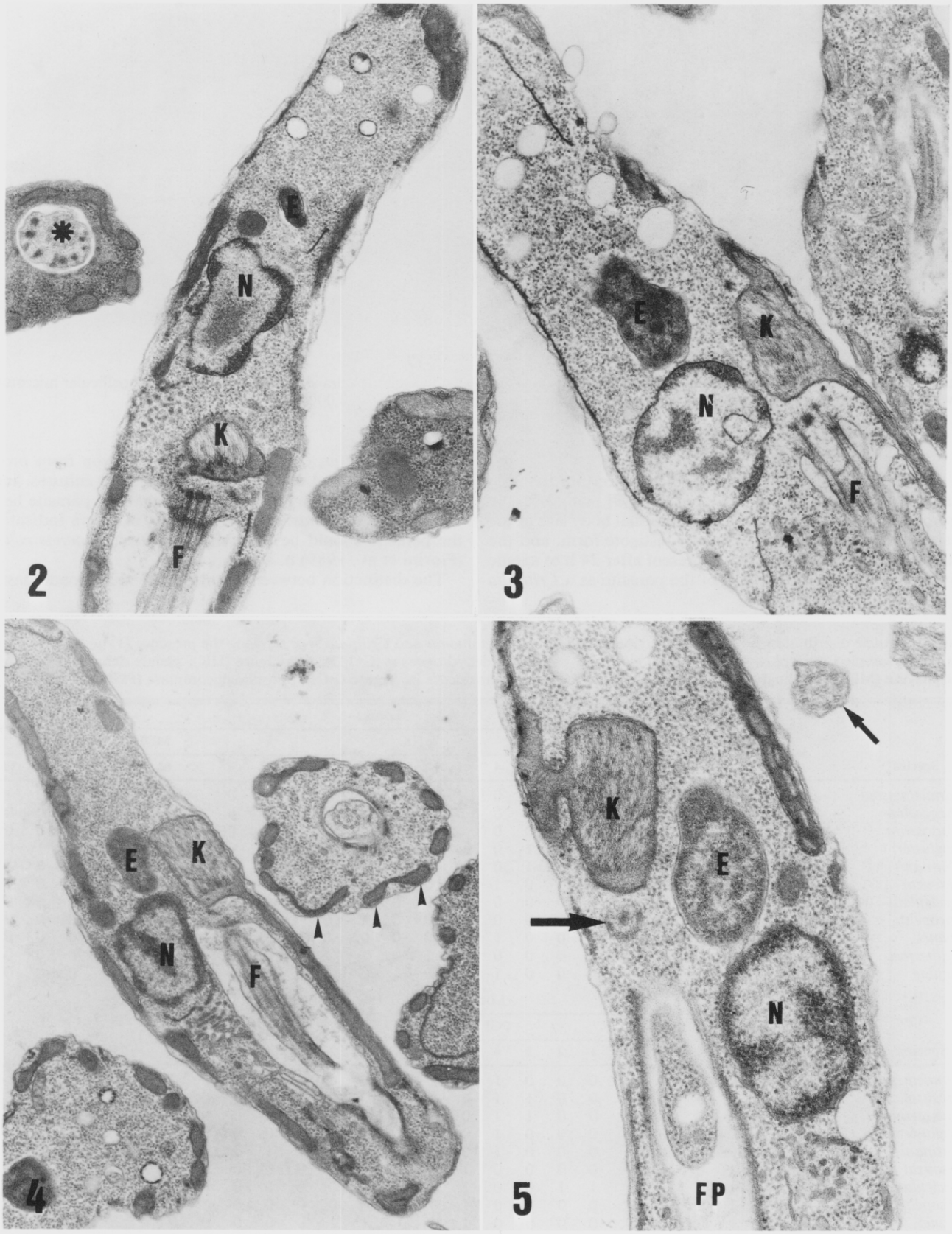
Fig. 2–5. Aspects of *Herpetomonas roitmani* during the differentiation process showing the migration of the kinetoplast (K). Fig. 2. Promastigote form. Dark structures in the flagellum (*) are the flagellum-cell body attachment regions (×21,000). Fig. 3. Paramastigote form (×21,000). Fig. 4. Intermediate stage between a para- and an opisthomastigote form. Arrowheads show the absence of subpellicular microtubules where the




mitochondrial branches are apposed to the cell plasma membrane (×15,000). Fig. **5**. Opisthomastigote form. Long arrow points to the basal body from where the flagellum originates. Short arrow shows the absence of the paraxial structure in the flagellum (×30,000). Endosymbiont (E), flagellum (F), flagellar pocket (FP), kinetoplast (K), nucleus (N).

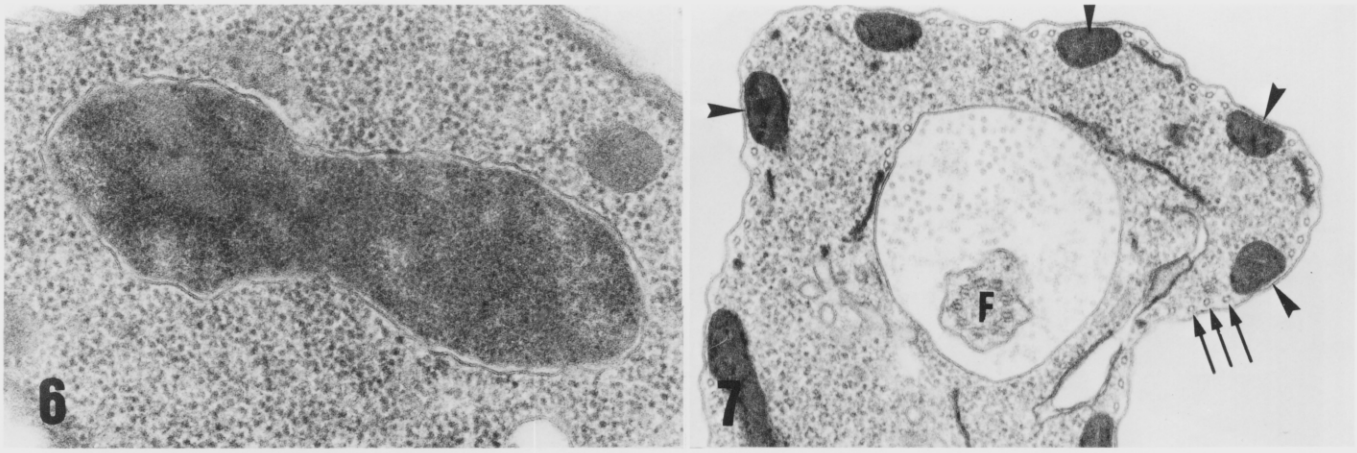



Fig. 6. High magnification of the endosymbiont of *Herpetomonas roitmani* (×36,500).

Fig. 7. Detail of the apposition of mitochondrial branches (arrowheads) to the cell membrane. Note the absence of subpellicular microtubules (arrows) in these regions and the absence of the paraxial structure in the flagellum (F) (×30,000).

## DISCUSSION

In this paper we present evidence demonstrating that the parasite previously described as *C. roitmani* [9] belongs to the *Herpetomonas* genus. We believe that the small body size of the parasite, easily mistaken for a choanomastigote form, and the small number of opisthomastigotes present after 24 h of axenic cultivation led to the description of the parasite as a *Crithidia* species. However, occurrence of differentiation from promastigotes to para- and opisthomastigotes in aging cultures, and the isoenzyme data clearly demonstrated that the parasite belongs to the *Herpetomonas* genus. Therefore, our data indicate that the parasite should be reclassified to *Herpetomonas roitmani* (Fiorini et al., 1989) n. comb.

The distinction between promastigote and choanomastigote

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.[a] Acid phosphatase (ACP); glucose 6-phosphate dehydrogenase (G6PD); hexokinase (HK); malate dehydrogenase (MDH); malic enzyme (ME); phosphogluconate dehydrogenase (PGD); phosphoglucose isomerase (PGI); phosphoglucomutase (PGM).

| | Locus | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ACP | | | | | | G6PD | | | | HK | | | | MDH | | | | | | | | | |
| Species | 1 | 2 | 3 | 4 | 5 | 6 | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| *H. samuelpessoai* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 0 |
| *H. megaseliae* | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 1 |
| *H. muscarum* | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 1 |
| *H. anglusteri* | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 0 |
| *H. roitmani* | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 0 |
| *C. desouzai* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *C. fasciculata* | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| *C. oncopelti* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *C. deanei* | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *C. guilhermei* | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *C. luciliae* | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |

| | Locus | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ME | | | | PGD | | PGI | | | | | | | PGM | | | | | |
| Species | 1 | 2 | 3 | 4 | 1 | 2 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| *H. samuelpessoai* | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| *H. megaseliae* | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| *H. muscarum* | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| *H. anglusteri* | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *H. roitmani* | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 |
| *C. desouzai* | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| *C. fasciculata* | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *C. oncopelti* | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| *C. deanei* | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 |
| *C. guilhermei* | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 |
| *C. luciliae* | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

[a] Numbers at each enzyme are for alleles in decreasing order of band mobility in the gel. All enzymes migrated cathodically.

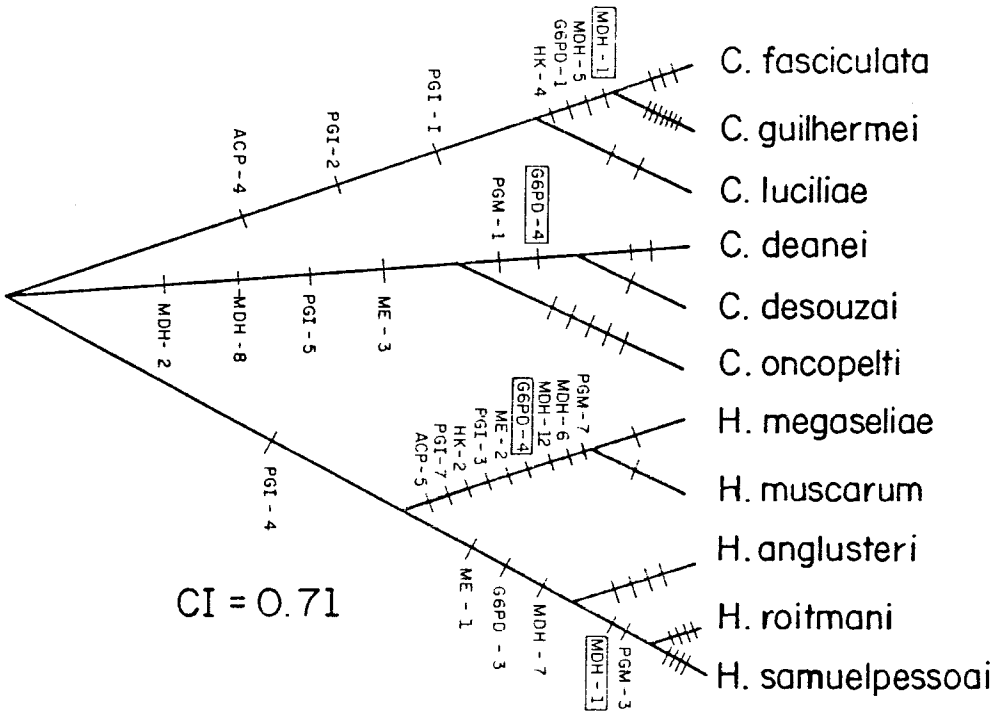



Fig. 8. Cladogram (Bootstrapped Wagner algorithm) of phylogenetic relationships between the 11 species of trypanosomatids studied. Only synapomorphies are shown. Autoapomorphies are just indicated. Abbreviations described in Materials and Methods.

(typical of the genus *Crithidia*) forms may be a quite difficult task in Giemsa-stained preparations of trypanosomatids. Indeed, the sole difference lies on the body form of the parasites, which is barleycorn-like in choanomastigotes and elongated in promastigotes [15, 28], since in both evolutive forms the flagellum emerges at the anterior extremity of the cell. Because of this similarity, recognition of parasites of *Crithidia, Herpetomonas,* and *Leptomonas* genera may be difficult [17, 22, 28]. Differentiation to para- and opisthomastigote forms are the characteristic remarks of parasites of the genus *Herpetomonas* [28], but these transformations occur usually in older cultures and the percentages of opisthomastigotes vary with strain and species [14, 23]. In this way, artificially induced differentiation has been used as an auxiliary tool in the systematics of parasites of the genus *Herpetomonas* [1, 4, 21]. The parasite previously described as *C. roitmani* expontaneously differentiated to para- and opisthomastigote forms when maintained in axenic cultures, thus indicating that it was a *Herpetomonas.*

Isoenzyme analysis allowed us to correctly identify the parasite. As assessed by isoenzyme electrophoresis analysis, the flagellate formerly described as *C. roitmani* presented a distinct banding pattern from that of the other *Crithidia* species. On the other hand, it formed a monophyletic group with the studied species of *Herpetomonas,* thus confirming its taxonomical identity as a *Herpetomonas* species. These findings also indicate that the electrophoretic analysis should be routinely used when assigning new species to any of these genera.

The presence of bacterium-like endosymbiont organisms has been morphologically assessed at the ultrastructural level in few trypanostomatids: *Crithidia oncopelti* [12, 13], *Crithidia deanei* [20], *Crithidia desouzai* [18, 27], and *Blastocrithidia culicis* [5]. A constant feature in these trypanosomatids, which was also observed in *H. roitmani,* is the presence of several alterations in the host cell morphology probably induced by the symbiont [10]. *Herpetomonas roitmani* is the first *Herpetomonas* and the fifth endosymbiont-containing trypanosomatid where such morphological alterations have been described. These alterations were maintained in parasites freed of the symbionts after treatment with the antibiotic chloramphenicol [6, 19]. Morphologically, the symbiont of *H. roitmani* is similar to those found in the other symbiont-bearing trypanosomatids [5, 12, 13, 18, 20, 25, 27]. Nutritional, biochemical, and ultrastructural

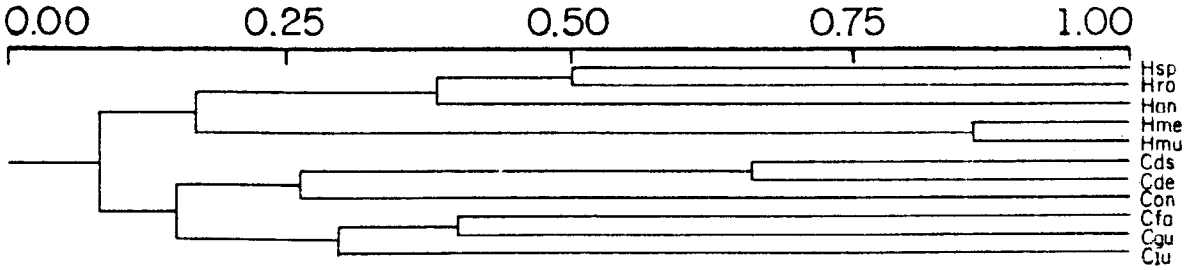



Fig. 9. UPGMA phenogram of Jaccard similarity indices between the 11 species of trypanosomatids studied.

data accumulated so far (reviewed in [18]) strongly suggest that they are of bacterial nature, probably Gram-negative bacterium-like microorganisms enveloped by two unit membranes and lacking a peptidoglycan layer [25]. Other evidence further demonstrates that the symbionts are not a moneran prey enclosed in a food vacuole: first, the monerans show no signs of degeneration and second, the parasites are maintained in axenic cultures so that they cannot uptake the bacterium-like organisms from the medium. Furthermore, there are usually only one or two moneran individuals in each host cell dividing synchronically with the protozoan. Uncontrolled multiplication of the monerans would result in the disruption of the flagellates and subsequent contamination of the culture medium, neither of which could be observed.

Bacteria-like structures were already described in *Herpetomonas* spp. other than *H. roitmani*. Wenyon [31] observed some cytoplasmic inclusions (also at the posterior end of the host cells) in *H. muscarum* obtained from the gut contents of house flies. Brun [3] studied later, by electron microscopy, the development of *H. muscarum* in *Chrysomyia chloropyga* and described two different kinds of bacterium-like organisms, which could only be observed in parasites located at the endoperitrophic space of the midgut of the insects. Interestingly, the kinetoplast of such flagellates appeared with a looser organization of the DNA fibrils (see his figures 5–10), as occurs in the endosymbiont-bearing trypanosomatids. The presence of cytoplasmic bacterium-like symbionts has been also suggested in a *Herpetomonas* sp. isolated from *Aedes* spp. [30], but as far as we know, no ultrastructural analysis was undertaken to demonstrate whether the rod-like structures described at the light microscopic level were symbiotic organisms in those flagellates.

It seems to us that *Herpetomonas roitmani* (Fiorini et al., 1989) n. comb. may constitute a good model for the study of the presence of an endosymbiont on the process of differentiation of a trypanosomatid.

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