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Ultrastructural and Biochemical Characterization of Promastigote and Cystic Forms of *Leptomonas wallacei* N. Sp. Isolated from the Intestine of its Natural Host *Oncopeltus fasciatus* (Hemiptera: Lygaeidae)

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ABSTRACT. Promastigote forms of a trypanosomatid were isolated from the third and fourth ventricles of the midgut and from the hindgut of the phytophagous hemipteran *Oncopeltus fasciatus*. Some individuals had adhered to its anterior region, close to the flagellar pocket, or to the flagellum up to four rounded aflagellated forms known as straphangers cysts. Scanning electron microscopy revealed that the flagellated forms presented a twisted cell body and a long flagellum, and the cysts, smaller than the parental promastigote, had a nascent flagellum. Transmission electron microscopy showed that promastigotes were typical, while cystic forms were ovoid dense cells devoid of a cyst wall, but presenting a cell coat, a special subpellicular region limited by a membrane unit, and a condensed cytoplasm. The kinetoplast-DNA fibrils appeared as dense spots and the condensed chromatin was arranged in a labyrinthic structure. Desmosome-like structures, observed in the region of adhesion of the precystic forms to the parental promastigote, could explain how cysts remain attached to the mother cell during the encystation process. Release of membranes from the surface of promastigotes and cysts seems to be correlated with the condensation of the cytoplasm during encystment. Morphological and isozyme analyses indicated that this trypanosomatid belongs to the genus *Leptomonas*. The molecular karyotype of this isolate was compared with that of a strain of *Leptomonas oncopelti* obtained from *Oncopeltus varicolor* by contour-clamped homogeneous electric field (CHEF) electrophoresis and revealed similar DNA banding patterns between 2,200–825 Kb, but not in lower bands (825–225 Kb). This suggested that the isolate from *O. fasciatus* and that from *O. varicolor* were not identical. Based on our findings we are describing *Leptomonas wallacei* n. sp. for our isolate from *O. fasciatus*.

Key Words. *Leptomonas oncopelti*, lower trypanosomatids, PFGE, trypanosomatid cysts.

ONCOPELTUS fasciatus is a natural host of several species of lower trypanosomatids belonging to the genera *Crithidia* (Hanson and McGhee 1961; McGhee, Hanson, and Schmittner 1969), *Leptomonas* (McGhee and Hanson 1962; Noguchi and Tilden 1926), and *Phytomonas* (Holmes 1925; McGhee and Hanson 1964). Infected loci in *Crithidia acanthocephali* (Hanson and McGhee 1961) or *Leptomonas oncopelti* (Laugé and Nishioka 1977; McGhee and Hanson 1962) are in the intestinal tract, and in *Phytomonas elmassiani* (McGhee and Hanson 1964) in salivary glands and hemolymph.

Leptomonas oncopelti was first described by Noguchi and Tilden (1926) who obtained five isolates from three hemipterans of the family Lygaeidae: *Oncopeltus* sp., *O. fasciatus,* and *Lygaeus kalmii,* and from two plant hosts belonging to the family Asclepiedaceae: *Asclepias nivea* and *Asclepias syriaca.* Due to morphological and biochemical similarities, the five isolates were considered to be a single species and described as *Herpetomonas oncopelti.* McGhee and Hanson (1962), who made the first descriptive study of *L. oncopelti* in its natural host *O. fasciatus,* described promastigote and cystic forms and proposed a model for the formation of cysts.

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Table 1. Infection on adult *Oncopeltus fasciatus* by trypanosomatid flagellates.

Infected locus	Percentage of insects infected	Parasite forms	
		Flagellate	Aflagellate
Intestinal tract	100%	+++	++
Salivary glands	0%	–	–
Hemolymph	0%	–	–
Feces	100%	++	+

+, ++, +++: Semi-quantitative estimation of the presence of parasite per locus.

Cysts in trypanosomatids have been reported only in *Blastocrithidia* and *Leptomonas* and were first observed by Patton (1909) in *Leptomonas lygaei,* and by McCulloch (1915) in *Blastocrithidia leptocoridis.* Trypanosomatids can originate from true cysts or straphangers cysts (Wallace, 1966). True cysts were described by Gibbs (1951b) from *Leptomonas capsularis,* a parasite in the hindgut of the hemipteran *Cletus ochraceus.* The promastigotes adhered to the gut wall of the insect and differentiated into aflagellated forms that detached in the gut lumen where they stayed as free ovoid cells, called resistant bodies. Straphangers cysts from *L. oncopelti* were described in the midgut and hindgut of *O. fasciatus* by McGhee and Hanson (1962). In this encystment process, the promastigote was produced by an unequal cell division with up to four aflagellated forms that remained attached by their anterior ends, close to the flagellar pocket. Cyst formation did not depend on adhesion to the intestinal epithelium. Ultrastructural information about cell differentiation during the encystment process

Table 2. Occurrence of trypanosomatid parasites in the intestinal loci of *Oncopeltus fasciatus.*

Intestinal locus	Segments	Parasitism	
		Flagellated	Aflagellated*
Foregut	Pharynx	–	–
	Oesophagus	–	–
	Crop	–	–
Midgut	$Ventricle_1$	–	–
	$Ventricle_2$	–	–
	$Ventricle_3$	+++	+
	$Ventricle_4$	+++	+
Hindgut	Malpighian ampoule	+++	++
	Ileum	+++	++
	Rectum	+++	++

* Free aflagellated forms or adhered to its parental promastigote.

+, ++, +++: Semi-quantitative estimation of the presence of the parasite per locus.

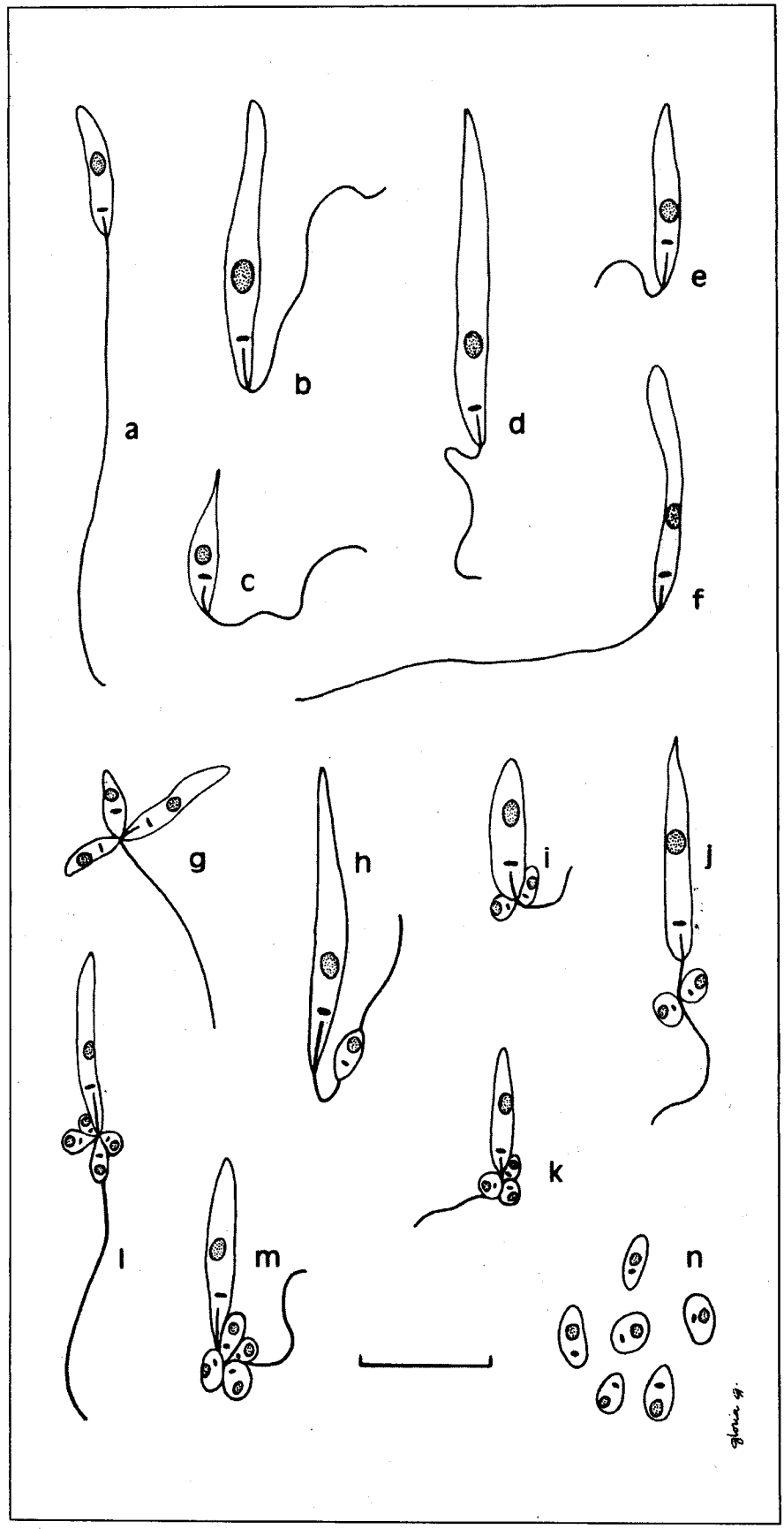
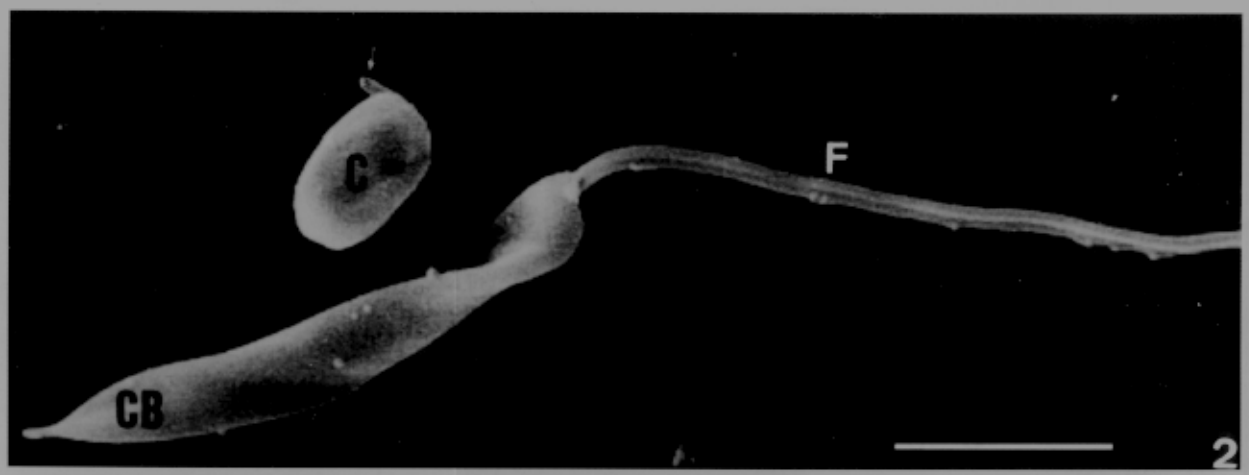


Fig. 1. Camera lucida drawings of representative forms of the *Leptomonas wallacei* n. sp. wild type and its clones. **a–f.** Promastigotes. **g–m.** promastigotes with precystic forms (various stages) attached, **n.** detached cysts. Scale bar = 10 μm.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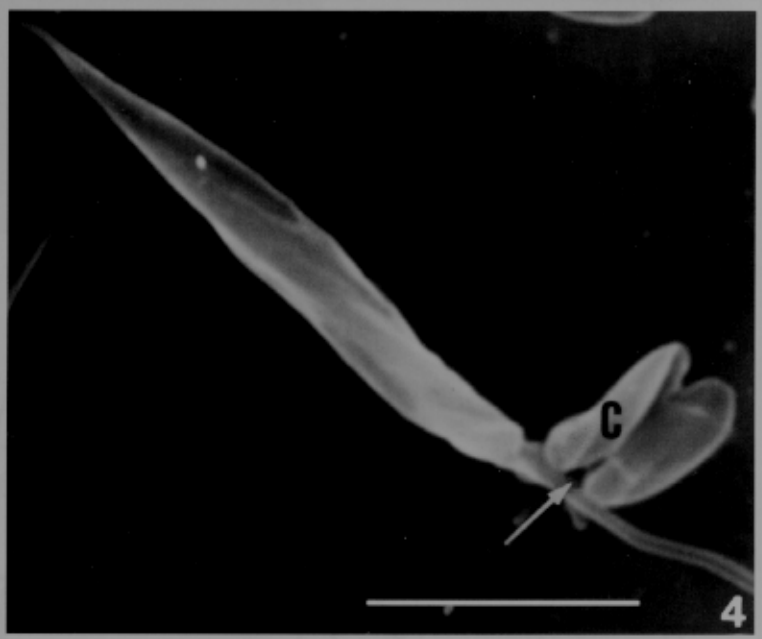
F
CB
2


P
PC
3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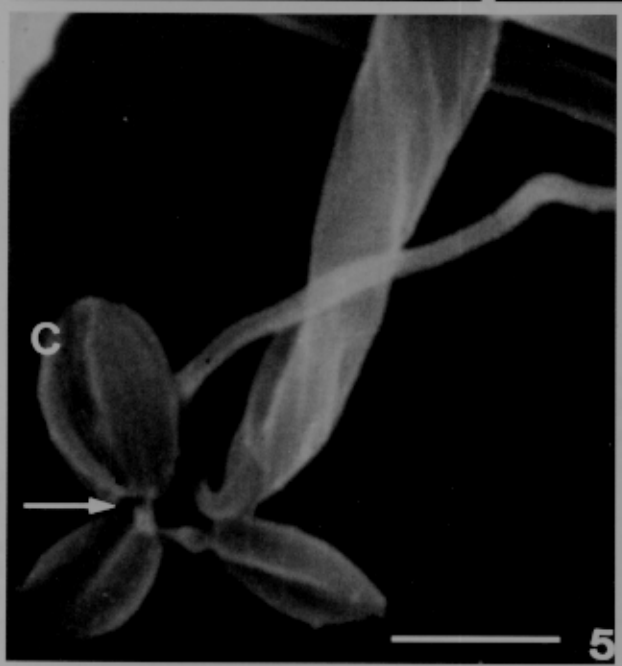
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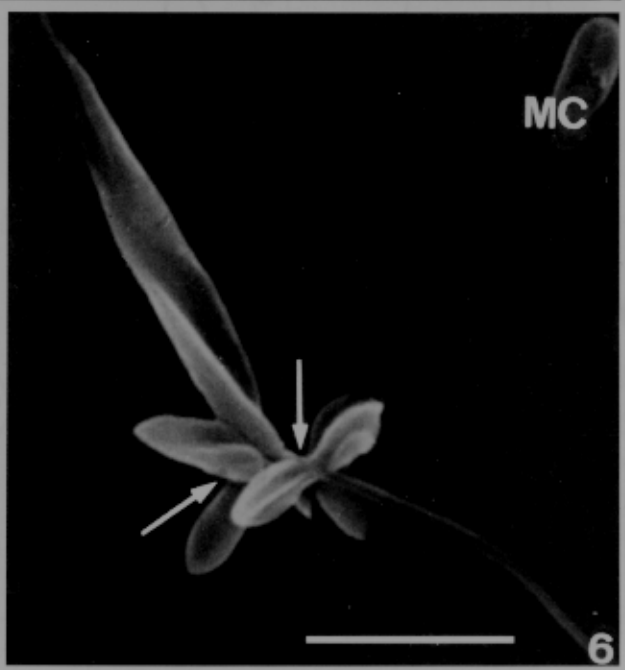
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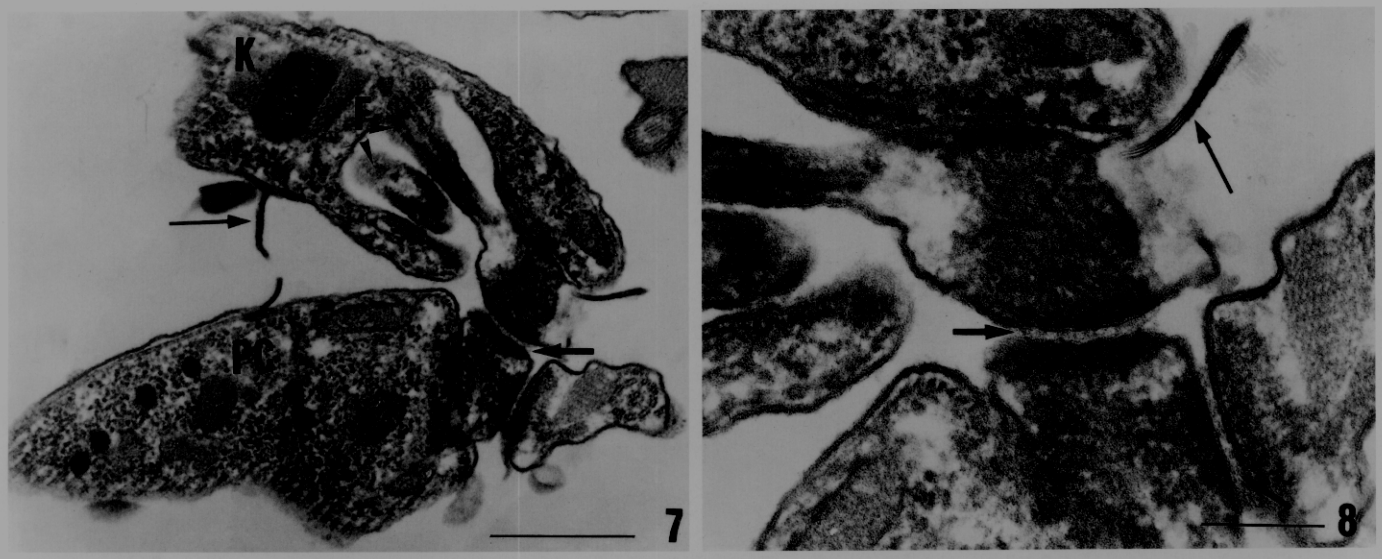

Fig. 7–8. Transmission electron micrographs of *Leptomonas wallacei* n. sp. clone B. **7.** Precystic cells (**PC**) adhered to each other and to a third flagellum, possibly from the parental promastigote (**F**). The flagella are enlarged and their membranes share dense desmosome-like structures (thick arrow). Filopodium-like structures (thin arrow) were frequently observed in precysts, bar = 2 μm. (**K**) Kinetoplast. **8.** Higher magnification of desmosome-like structures shown in Fig. 7 demonstrating the dense filaments in the intercellular space. Bar = 0.8 μm.

was published for *Blastocrithidia triatomae* (Mehlhorn et al. 1979; Peng and Wallace 1982), and *Leptomonas* spp. from Hemiptera (Laugé and Nishioka 1977; Takata et al. 1996), Lepidoptera (Abe 1980), and Siphonaptera (Molyneux and Croft 1980).

In the present paper, we report the isolation of a trypanosomatid from the intestinal tract of *O. fasciatus* and describe it as *Leptomonas wallacei* n. sp. This parasite produces cysts in insect gut or in culture medium. This isolate was studied under light and electron microscope, and their isozymes were compared with trypanosomatids belonging to different genera. Its molecular karyotype was also compared with that of a strain of *Leptomonas oncopelti* (sample 36/429TD) obtained from *Oncopeltus varicolor* (Sbravate et al. 1989; Takata et al. 1996).

MATERIALS AND METHODS

Insect colony origin and maintenance. Fifty adult *Oncopeltus fasciatus* (from the original colony established by Dr. N. Ratcliffe–Swansea University, UK) were kindly provide by Dr. Patrícia Azambuja Penna, Instituto Oswaldo Cruz, Rio de Janeiro, Brazil. Insects were kept in plastic pitchers cleaned with 70% (v/v) ethanol solution, fed with peeled, toasted sunflower seeds and fresh distilled water at 28 °C. Colonies were cleaned and fed weekly. The eggs were collected, washed with 2% (v/v) sodium hypochloride, and allowed to eclode in aseptic chambers. Newborn nymphs were maintained in the same way as adults.

Screening of infection in adult insects. Adult insects were submitted to fecal examination by pressing lightly on their abdomen against a glass slide. The feces were diluted in 20–50 μl of sterile, phosphate-buffered saline (PBS), pH 7.2, covered by a glass coverslip and observed under a Olympus BHB phase contrast light microscope. Insects infected with parasites were selected for further experiments. Twenty percent of the insects in the colony were screened for infections in the the hemolymph and the salivary glands, which required excision of the insect's head.

Isolation of the parasites. A group of 10 to 15 adult insects infected with parasites were starved, but given water, for 7 to 10 d to rid the intestinal tract of residual food and to reduce the intestinal fungal and bacterial flora. These insects were briefly washed in 70% (v/v) ethanol solution and transferred to a laminar flow chamber where they were carefully dissected.

Intact inteştinal tracts were placed in three changes of 5 mg/ml tetracycline in PBS, pH 7.2, and exposed to ultraviolet light over 20 min. The contents of intestinal tracts were inoculated into 5 ml of modified Warren's complex medium (MWCM) [brain heart infusion (BHI)-37 g/ml, hemin-10 mg/ml, folic acid-10 mg/ml] supplemented with 1 mg/ml tetracycline, gently suspended with a Pasteur pipette, and allowed to decant in a sterile environment for 1 h.

The upper portion of supernatant containing parasites was centrifuged at 1,400 *g* for 5 min and the pellet was resuspended in 5 ml of MWCM and 1 mg/ml tetracycline. Samples of 0.5 ml of the resuspended pellet were subcultured for 2 d in tubes containing 4.5 ml of MWCM supplemented with 10% (v/v) fetal bovine serum (FBS) and 1 mg/ml tetracycline, and were centrifuged at 1,400 *g* for 5 min to concentrate non-motile contaminants in the bottom and allow the swimming promastigotes to reach the top of the tube. These tubes were incubated at 28 °C for 48 h.

Subcultures were made each 48 h from the supernatant in the culture tubes and after twice repeating the process 5 ml of the culture were inoculated into one end of a W-shaped tube

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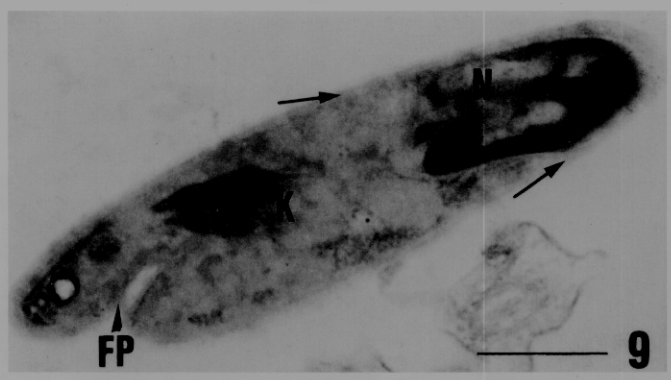
Fig. 2–6. Scanning electron micrographs of promastigote and cystic forms of parasites of *Leptomonas wallacei* n. sp. clone B. **2.** Cyst (**C**), showing the residual flagellum (small white arrow), close to a promastigote. Note twisted cell body (**CB**) and elongated flagellum (**F**), bar = 3.5 μm. **3–6.** Sequence of events of cyst formation. **3.** Parental (**P**) and precystic (**PC**) daughter cell. **4.** Two immature cysts (**C**) adhered to the flagellum (**F**) of the parental promastigote (white arrow), bar (**3, 4**) = 5 μm. **5, 6.** Almost mature cysts (**C**) are seen adhered to each other and to the flagellum of the parental cell. One mature cyst (**MC**) is also seen, bar (**5**) = 2.5 μm, bar (**6**) = 5 μm.



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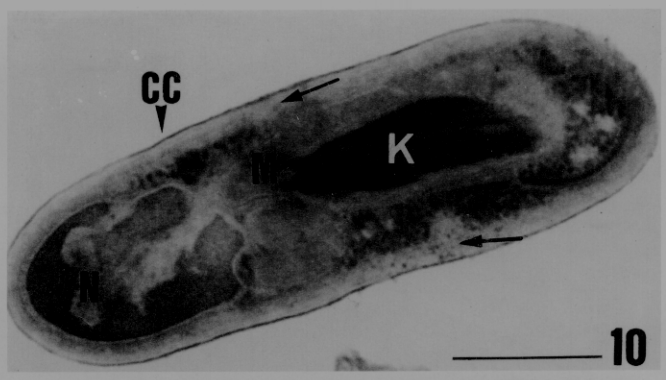

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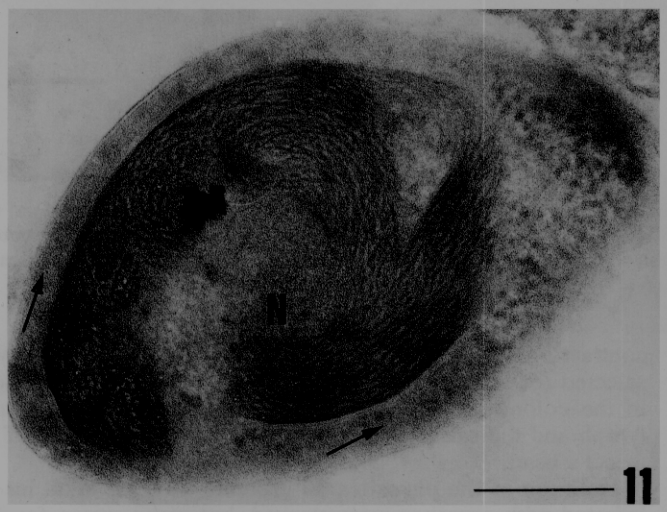

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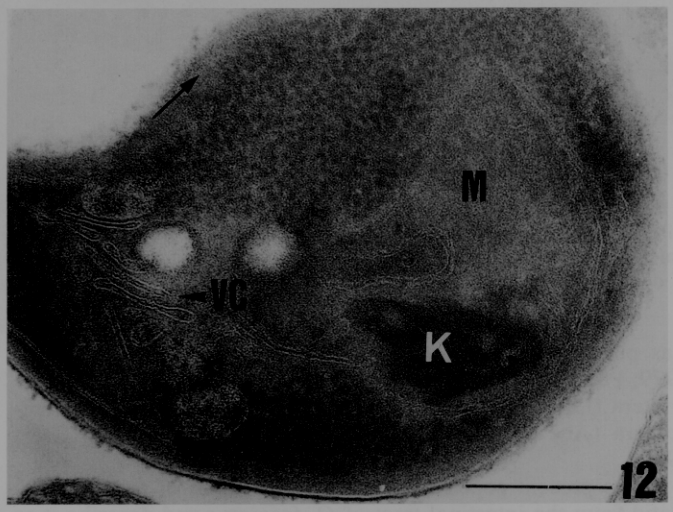

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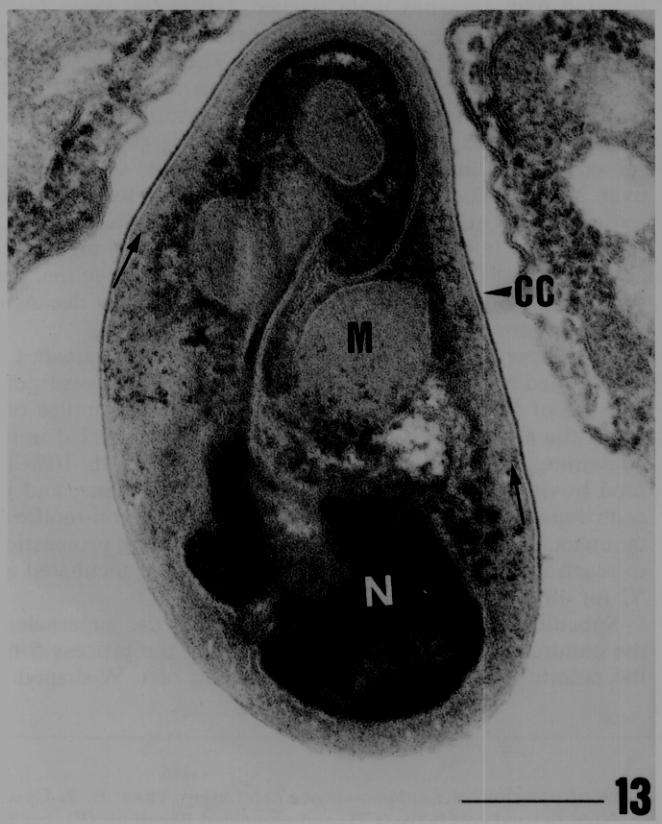

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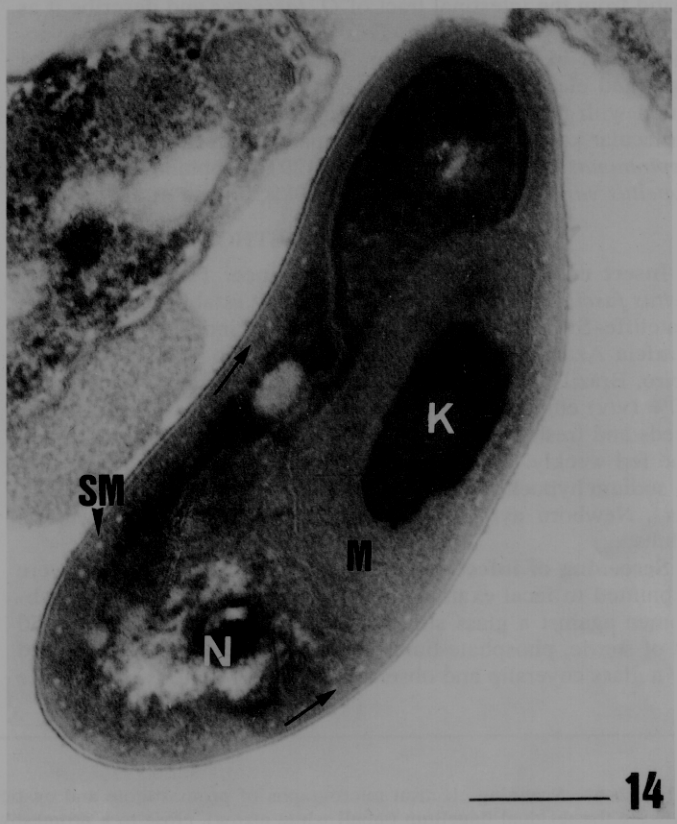

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Table 3. Measurements of promastigote trypanosomatids of *Leptomonas wallacei* and *Leptomonas oncopelti.*

Sample	Origin	Cell body (μm)		Flagellum (μm)	
		Range	Average ± SE	Range	Average ± SE
L. wallacei wild type	Culture	6.25–23.75	13.25 ± 0.57	7.81–40.62	20.88 ± 1.06
L. wallacei clone A	Culture	8.86–28.48	14.20 ± 0.49	6.96–37.97	17.21 ± 0.93
L. wallacei clone B	Culture	7.80–22.50	15.11 ± 0.43	10.00–35.62	23.38 ± 0.76
L. oncopelti[a]	Culture	6.25–23.75	15.29 ± 0.56	7.50–31.25	19.81 ± 0.80
L. oncopelti[b]	Insect	12.00–25.00	*	10.00–25.00	*
L. oncopelti[c]	Culture	11.50–17.00	*	6.00–18.00	*

[a] *L. oncopelti* (Takata et al. 1996) isolated from *O. varicolor* (Sbravate et al. 1989).
[b] *L. oncopelti* (Noguchi and Tilden, 1926) in the digestive tract of *O. fasciatus.*
[c] The same parasite as in [b] in culture conditions.
SE: standard error, N = 50.
* Measurements were not provided by the authors.

containing 27 ml of MWCM supplemented with 10% (v/v) FBS and 1 mg/ml tetracycline. The W tube was incubated at 28 °C for 7 d. After this incubation, aliquots of 0.5 ml were collected from the opposite side of the W tube and were subcultured in tubes containing 4.5 ml of MWCM supplemented with 10% (v/v) FBS. Tubes were incubated at 28 °C until establishment of an axenic parasite culture, which was named *Leptomonas wallacei* wild type. Cell growth and viability were estimated daily by cell counting in a Neubauer chamber under a Olympus BHB phase contrast light microscope.

Cell cloning and depositing in cell culture collection. *Leptomonas wallacei* wild type was cloned by cell limiting dilution. Two clones were obtained and designated *L. wallacei* clone A and *L. wallacei* clone B. The *L. wallacei* clone B was deposited in the Collection of Oswaldo Cruz Institute (CT-IOC)—Coleção de Tripanosomatídeos, Departamento de Protozoologia, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, Avenida Brasil, 4365, Manguinhos, 21045-900, Rio de Janeiro, Brazil—and registered as CT-IOC 194.

Evaluation of cell growth and measurement of parasites. After establishment of *Leptomonas wallacei* wild type and clonal cultures, parasites were grown in 4.5 ml of MWCM supplemented with 10% (v/v) FBS for periods varying from 1 to 7 d. About 5×10^5 cells/ml from a five-day-old culture were seeded and further incubated at 28 °C. Cell growth was determined daily using a Neubauer haemocytometer. Smears of 5-day-old cultures were Giemsa-stained after HCl treatment (Carvalho 1973). At least 50 randomly chosen organisms of each culture were measured, and drawn in camera lucida attached to a Zeiss Standard-20 light microscope. The measurements of parasites were made as recommended by Wallace et al. (1983). A strain of *Leptomonas oncopelti* (sample 36/429TD) (Takata et al. 1996) was grown under identical culture condition and measured for comparison.

Transmission and scanning electron microscopy. Parasites from 5- and 10-day-old cultures of *Leptomonas wallacei* clone B were fixed in a solution containing 2.5% (v/v) glutaraldehyde, 4% (w/v) freshly made formaldehyde, and 5 mM $CaCl_2$ in 0.1 M cacodylate buffer, pH 7.2, and processed for scanning and transmission electron microscopy as described elsewhere (Attias et al. 1988).

Isozyme analysis. The original culture of the isolate of *Oncopeltus fasciatus* was compared through isozyme electrophoresis with the following kinetoplastids: *Bodo* sp. (kindly provide by Dr. R. C. Vommaro, Federal University of Rio de Janeiro, Brazil), *Herpetomonas* sp. (Brazil et al. 1990; Sousa, M. A., Costa, K. C. F., Camargo, A. C., Santos, S. M., Ziccardi, M., Freschi, J. M.& Attias, M. 1995. Characterization of a trypanosomatid isolated from *Phthia picta.* Abstract. Mem. Inst. Oswaldo Cruz, **90**, Suppl. I: 246) *Herpetomonas anglusteri* (CT-IOC 167), *Herpetomonas mcgheei* (CT-IOC 181), *Herpetomonas megaseliae* (ATCC 30209), *Herpetomonas muscarum* (ATCC 30260), *Herpetomonas samuelpessoai* (ATCC 30252), *Leptomonas collosoma* (ATCC 30261), *Leptomonas samueli* (ATCC 30971), *Leptomonas seymouri* (ATCC 30220), *Leptomonas oncopelti* (sample 36/429TD) isolated from *Oncopeltus varicolor* (Sbravate et al. 1989; Takata et al. 1996) (CT-IOC 094), *Phytomonas* sp. isolated from *Chamaesycae thymifolia* (dos Santos, R. F. M., Romeiro, A., Attias, M. & de Souza, W. 1994. Reisolation of a *Phytomonas* sp from *Chamaesyce thymifolia.* Abstract. Mem. Inst. Oswaldo Cruz, **89**, Suppl. I: 219) (CT-IOC 213), and *Phytomonas davidi* (ATCC 30287). All organisms were cultivated in MWCM supplemented with 10% (v/v) FBS for 3 to 5 d at 28 °C. Each culture was washed 3× in PBS, pH 7.2, and concentrated to 10^8—10^9 cells/ml in hypotonic solution of 2 mM caprilic alcohol, 1 mM EDTA, 1 mM DTT, 100 μg/ml leupeptin, and 1 mM PMSF. Cells were sonicated in an ultrasonic breaker W-380 (Heat Systems, Farmingdale, U.S.) in the same solution at 0–4 °C and frozen at −70 °C until use.

Isozyme patterns of the species were obtained by horizontal starch gel electrophoresis as previously described (Milles et al. 1980; Motta et al. 1991). Biochemical assays and staining were conducted as established by Harris and Hopkinson (1978) and Manchenko (1994). Eight enzyme systems were used: 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

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Fig. **9–14**. Ultrathin sections of cystic forms of *Leptomonas wallacei* n. sp. clone B. All cystic forms are ovoid with a dense cytoplasm where ribosomes are seen (Fig. 10, 12, 13). A thin cell coat (**CC**) is evident (Fig. 10, 12–14) and the subpellicular microtubules (**SM**) are present in some sections (Fig. 13, 14) in the homogeneous subpellicular region (black arrow). The very condensed k-DNA (**K**) inside the mitochondrion (Fig. 9, 10, 12–14) and anteriorly located nucleus (**N**) are observed. In some sections the labyrinthic structure of nuclear DNA can be observed (Fig. 11). A complex of membrane-limited vesicles (**VC**) of unknown function are also present in the cytoplasm (Fig. 12). Bars (**9, 10**) = 1.7 μm and 1 μm, respectively, bars (**11–14**) = 0.7 μm.

Table 4. Measurements of parasite cystic forms of *Leptomonas wallacei.*

Sample	Longitudinal axis (μm)		Transverse axis (μm)	
	Range	Average ± SE	Range	Average ± SE
L. wallacei wild type	*	*	*	*
L. wallacei clone A	1.87–4.37	3.39 ± 0.37	1.25–1.87	1.52 ± 0.08
L. wallacei clone B	2.53–5.06	2.98 ± 0.20	1.26–2.53	1.97 ± 0.12

* Cysts not detected in Giemsa-stained slides. SE: Standard error, *L. wallacei* clone A, N = 22; *L. wallacei* clone B, N = 14.

(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 could be attributed to different gene products. A phylogenetic tree was obtained using the HENNIG 86 program with the exhaustive search option (Implicit Enumeration-IE). After tree construction, character states were marked on the knodes according to Solé-Cava et al. (Solé-Cava et al. 1994).

Molecular karyotype. Exponentially growing cells of the original isolate from *Oncopeltus fasciatus,* clones A and B, and *Leptomonas oncopelti* (CT-IOC 094) in MWCM supplemented with 10% (v/v) FBS were used to prepare intact chromosomal DNA in agarose plugs as described elsewhere (Tavares, Grimaldi, and Traub-Csekö 1992; Van der Ploeg et al. 1984). Counter-clamped homogeneous electric field (CHEF) electrophoresis was employed to comparatively analyze the chromosomal banding pattern of these strains. The pulse ramps were assayed: 220–80 s (24 h), 200–50 s (29 h), and 100–50 s (40 h), respectively, using 0.9, 1.0, and 1.5% agarose gels in the running buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.0). All runs were carried out in a CHEF DR III System (BioRad) at 5.5 V/cm, 12 °C, and electrode angle of 120°. *Saccharomyces cerevisae* chromosomal DNA was used as the molecular weight marker. Following electrophoresis, the gels were stained with ethidium bromide and photographed under UV transillumination.

RESULTS

Screening of infection in adult insects. All insects examined were positive for parasites in intestine and feces. Both promastigote or cystic forms were seen in both loci of infection. Salivary glands and hemolymph were never infected (Table 1). In the intestine, only the third and fourth ventricles of midgut and hindgut were infected. Foregut and the first and second ventricles of the midgut were negative for both forms of the parasite (Table 2).

Cell growth, light microscopy and measurement of parasites. Growth rates were similar for the *Leptomonas wallacei* wild type and its clones with the maximum numbers occurring on the fifth day.

Giemsa-stained smears of five-day-old cultures of the *L. wallacei* clones A and B had a vast majority of promastigotes and aflagellated cells either free in the culture medium or adhered (up to four cells) to the anterior end of a parental promastigote, similar to the straphanger cysts described for some *Blastocrithidia* and *Leptomonas* species. In aflagellated cells, both the nucleus and the kinetoplast were present. Promastigotes showed a certain degree of pleomorphism in all cultures examined (Fig. 1).

Promastigotes from *Leptomonas wallacei* wild type, clones A and B, and the 36/429TD isolate (Takata et al. 1996) did not show statistically significant differences (t test, $p > 0.05$) (Table 3). Likewise, no significant differences were found between the cyst stages of IOF clones (t test, $p > 0.05$) (Table 4).

Table 5. Allele distribution for the eight enzymes studied in 14 kinetoplastid isolates, showing the presence (1) or absence (0) of the allele in the sample. Alleles (bands) of each locus are numbered according to decreasing mobility in the gel.

Protozoan	Enzyme system[a]																		
	G6PD						HK				MDH				ME				
	1	2	3	4	5	6	1	2	3	4	1	2	3	4	1	2	3	4	5
Bodo sp.	?	?	?	?	?	?	0	0	0	0	1	0	0	0	1	0	0	0	0
Herpetomonas sp.[b]	1	0	1	1	0	0	0	1	0	0	0	1	0	1	0	1	0	1	0
H. anglusteri	1	0	1	1	0	0	?	?	?	?	0	1	0	1	0	0	1	0	0
H. mcgheei	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
H. megaseliae	0	1	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	1	0
H. muscarum	0	1	0	0	0	0	?	?	?	?	0	1	0	1	0	1	0	1	0
H. samuelpessoai	1	0	1	1	0	0	?	?	?	?	0	1	0	0	0	0	1	0	0
L. colosoma	0	1	0	0	1	0	?	?	?	?	0	1	1	0	?	?	?	?	?
L. oncopelti	0	0	0	0	0	1	0	0	0	1	?	?	?	?	0	0	0	0	1
L. samueli	0	0	0	1	0	0	?	?	?	?	?	?	?	?	?	?	?	?	?
L. seymouri	1	0	0	0	0	0	1	0	0	0	0	1	0	0	?	?	?	?	?
Phytomonas sp.[c]	1	0	1	1	0	0	?	?	?	?	1	0	0	0	?	?	?	?	?
P. davidi	0	0	1	0	0	0	0	1	1	0	0	1	0	1	0	0	0	1	0
L. wallacei wild type	0	0	0	0	0	1	0	0	0	1	?	?	?	?	0	0	0	0	1

[a] G6PD: glucose 6-phosphate dehydrogenase, HK: hexokinase, MDH: malate dehydrogenase, ME: malic enzyme, PGD: 6-phosphogluconate dehydrogenase, PGI: phosphoglucose isomerase, PGM: phosphoglucomutase.

[b] Trypanosomatid isolated from *P. picta* (Brazil et al. 1990; Sousa, M. A. et al. 1995. Abstract).

[c] Trypanosomatid isolated from *C. thymifolia* (dos Santos, R. F. M. et al. 1994. Abstract).

?: Enzyme not revealed in this species.

Scanning electron microscopy. As observed in light microscopy, promastigotes were elongated and had a slightly twisted cell body of variable size (Fig. 2–6). The free portion of the flagellum was two to three-fold longer than the cell body. Cystic forms, about 3 μm in its longitudinal axis (Fig. 2), were ovoid with a slight torsion and a very short flagellum, not seen in the light microscope. Up to four intermediate forms, called precystic stages (Takata et al. 1996), were seen adhered to the flagellum of the parental promastigote, in the opening of the flagellar pocket (Fig. 3–6).

Transmission electron microscopy. The ultrastructure of promastigotes was typical, except for the flagellar specializations between the parental individual and forming cysts. At the site of adhesion between the parental promastigote and the forming cyst, the flagella of both cells were enlarged, with a thicker cytoplasmic layer, apparently originating from the paraxial rod adjacent to it (Fig. 7) and regularly spaced dense filaments at the intercellular space (Fig. 8). Individuals fixed in the process of cyst generation showed a duplicated flagellum. Differentiating cystic forms showed a denser cytoplasm and filopodium-like membranes, indicating a reduction of cell volume and cytoplasmic condensation (Fig. 7, 8).

Mature (i.e. detached) cystic forms were strikingly different from promastigotes in size, shape, and density (Fig. 9–14). The cell surface was limited by a single unit membrane (Fig. 12) and covered by a cell coat (Fig. 10, 12–14). No cyst wall was present, but a subcortical homogeneous region limited by a membrane was observed in all cystic cells (Fig. 9–14). This membrane seems to belong to the endoplasmic reticulum. Subpellicular microtubules were seen in some cystic forms (Fig. 13, 14). Filopodium-like structures were not observed at this stage. The flagellar pocket (Fig. 9) was shortened with a small flagellum without paraxial rod confined to its lumen (not shown).

The kinetoplast showed a compact arrangement of k-DNA fibrils immersed in a translucent matrix. The k-DNA network lost the characteristic arrangement of its fibrils being present as a dense spot in the mitochondrial matrix (Fig. 9, 10, 12–14). A mitochondrion with few cristae (Fig. 12–14) filled most of the cytoplasm of the cyst.

The nuclear chromatin was very condensed (Fig. 9, 10, 13, 14) and almost entirely filled the nucleoplasm. In transverse sections, a labyrinthic arrangement of nuclear DNA fibrils (Fig. 11) was sometimes observed. In longitudinal sections, the nucleus was localized posteriorly (Fig. 9, 10, 13, 14). The nucleolus, evident in promastigote forms, could not be distinguished.

The cytoplasm of cysts was very condensed and entirely filled with dense particles that probably correspond to ribosomes (Fig. 12, 13).

Isozyme analysis. Of the eight enzyme markers used, six gave reproducible results (Table 5). Three most parsimonious trees were obtained that differed only in the relative position of *Leptomonas* species. A strict consensus tree (Fig. 15) established from these trees resulted in a polytomy for the relationship of *Leptomonas* spp., but maintained the monophyly of this genus, indicating that the trypanosomatid isolated from *Oncopeltus fasciatus* must be allocated to the genus *Leptomonas*.

PFGE analysis. The *Leptomonas wallacei* wild type strain and its clones were identical in chromosomal DNA banding pattern when analyzed by pulsed field gel electrophoresis (Fig. 16). The 36/429TD isolate presented a similar banding pattern regarding the largest chromosomes (Fig. 16), but this was clearly distinct for molecules ranging in size between 825–225 kb (Fig. 17).

Taxonomic summary. Since the original cultures of Noguchi and Tilden (1926) are unavailable for comparison and the trypanosomatid studied by McGhee and Hanson identified as *Leptomonas oncopelti* (1962) was neither isolated nor maintained in culture, it is very difficult to decide whether the trypanosomatid studied here actually belongs to this species. We believe it more appropriate to give a description of this isolate and consider it a new species, naming it *Leptomonas wallacei* n sp. in honor of Dr. Franklin G. Wallace. A taxonomic summary for the *Leptomonas wallacei* n. sp., according to Wallace (1966), is shown below.

Species name. *Leptomonas wallacei* n. sp. Romeiro, Solé-Cava, Sousa, De Souza and Attias, 1998.

Description. Elongated promastigotes twisted at the middle of the cell body measuring from 7.8–22.5 μm (cell body) and 10.0–35.62 μm (flagellum). About 5% of the individuals had

Table 5. Extended.

Enzyme system[a]																							
PGD					PGI							PGM											
1	2	3	4	5	1	2	3	4	5	6	7	1	2	3	4	5	6	7	8	9	10	11	12
?	?	?	?	?	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
?	?	?	?	?	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	1	1	1
?	?	?	?	?	0	0	0	1	0	0	0	0	0	1	1	0	1	0	0	1	0	0	0
?	?	?	?	?	0	0	0	0	0	1	0	0	0	1	1	0	1	0	0	1	0	0	0
0	0	0	0	1	0	0	1	0	0	0	0	0	0	1	1	0	1	0	0	1	1	1	1
?	?	?	?	?	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	1	1	1
?	?	?	?	?	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
?	?	?	?	?	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
0	1	1	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0
?	?	?	?	?	0	1	0	0	1	0	0	0	0	0	0	1	0	1	1	0	0	0	0
?	?	?	?	?	0	1	0	0	1	0	0	0	1	0	0	1	0	0	0	0	0	0	0
?	?	?	?	?	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
?	?	?	?	?	0	0	1	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0
1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0

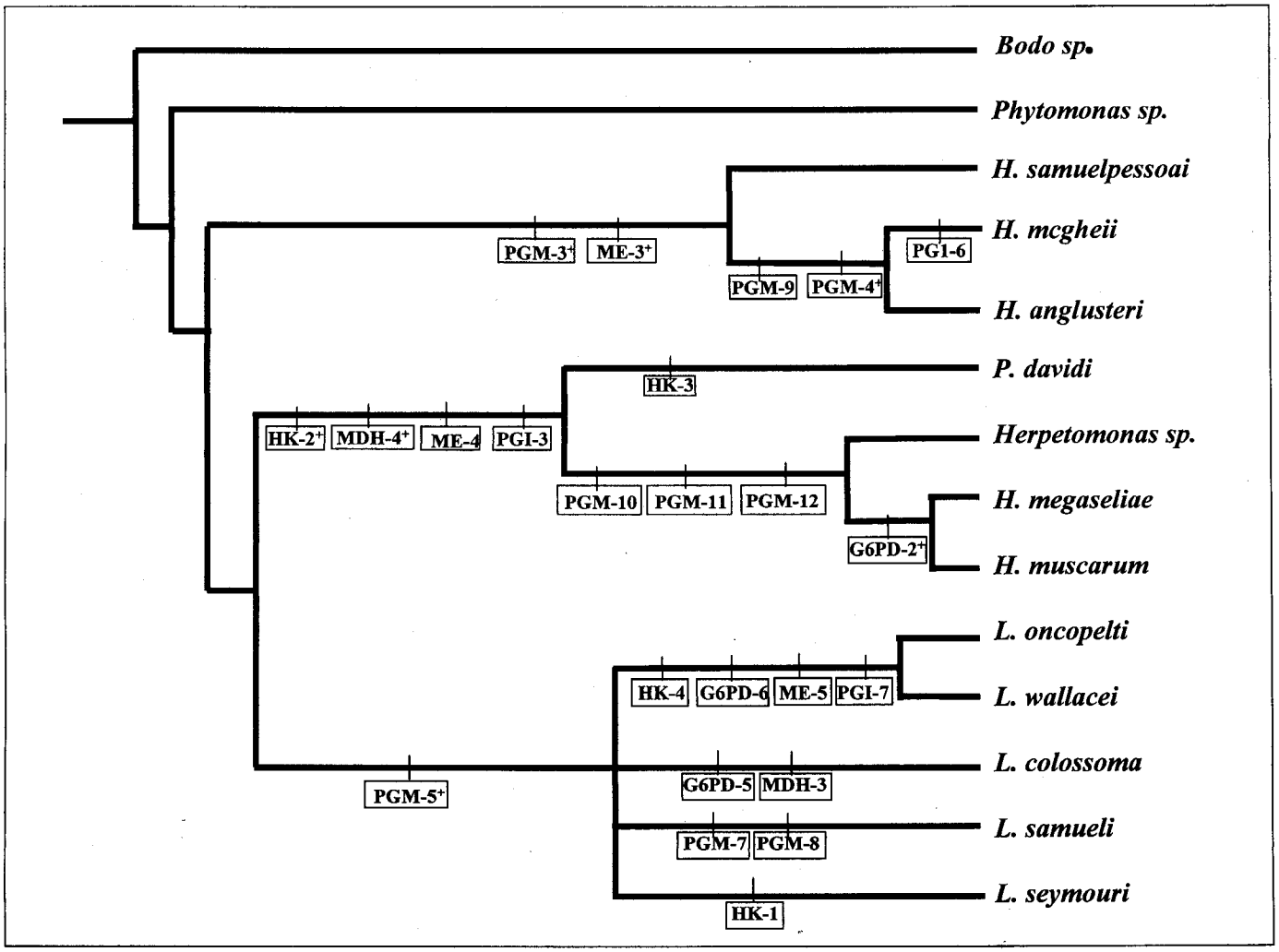

Fig. 15. A strict consensus tree showing phylogenetic relationships among kinetoplastids using isozyme locus as character. Synapomorphic and autopomorphic bands are indicated on tree branches. *—indicates homoplasic locus.

cystic or precystic forms adhered to the basal portion of the flagellum. Mature cystic forms detached and remained free in the medium (Fig. 1–14, 18).

Type host. *Oncopeltus fasciatus* (Hemiptera: Lygaeidae).

Other hosts. Unknown.

Location in host. Third and fourth ventricles of the midgut and the whole hindgut.

Type locality. Rio de Janeiro, Brazil.

Other localities. Unknown.

Prevalence. 100%, in all developing nymph stages, and adults of both sexes.

Material deposited. The *L. wallacei* clone B has been deposited in the Collection of the Oswaldo Cruz Institute (CT-IOC)–Coleção de Tripanosomatídeos, Departamento de Protozoologia, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, Avenida Brasil, Accession number CT-IOC 194.

Etymology. This species is named in honor of Franklin G. Wallace in recognition of his contributions to the field of taxonomy and biology of monoxenous trypanosomatids.

Remarks. Straphangers cysts are present adhered to the parental cell. These cysts can be single or in clusters of two to four individuals. They present a vestigial flagellum but do not show a cyst wall.

DISCUSSION

Taxonomic position of the trypanosomatid isolate from *Oncopeltus fasciatus*. The trypanosomatids isolated from the phytophagous hemipteran *O. fasciatus* were either promastigotes or rounded cysts that adhered to the anterior end of a parental promastigote or were free in the medium. These morphological features correspond to the genus *Leptomonas*. The ultrastructure of the flagellated forms of *Leptomonas wallacei* was similar to the promastigotes of other species of trypanosomatids (Attias et al. 1988; Roubaud 1908; Souto-Padrón et al. 1980). The phylogenetic analysis of isozymes provided most parsimonious trees, invariably grouping this parasite together with the other *Leptomonas* species tested and reinforcing its taxonomic position in this genus.

Samples of the original isolate obtained by Noguchi and Tilden (1926) are no longer available, but in Brazil, a *Leptomonas* sp. (sample 36/429TD) isolated from *Oncopeltus varicolor* by Sbravate et al. (1989) was suggested (Takata et al. 1996) to be the species *Leptomonas oncopelti* (McGhee and Hanson 1962; Noguchi and Tilden 1926). This isolate was compared with the *L. wallacei* wild type and its clones in terms of morphology, morphometry, and at the biochemical and molecular levels. The

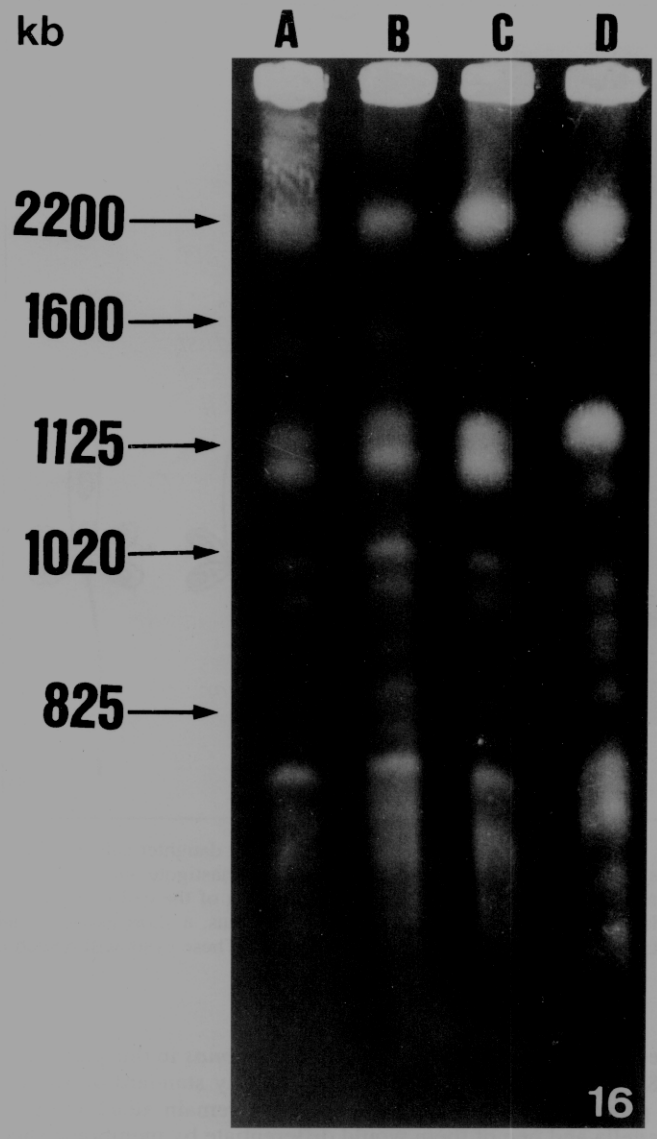

Fig. **16**. Separation of chromosome-size DNA molecules from the original strain isolated from *Leptomonas wallacei* n. sp. wild type isolated from *Oncopeltus fasciatus* (**A**), clone A (**B**), clone B (**C**) and the 36/429TD strain of *Leptomonas oncopelti* from *Oncopeltus varicolor* (**D**). CHEF electrophoresis in Materials and Methods.

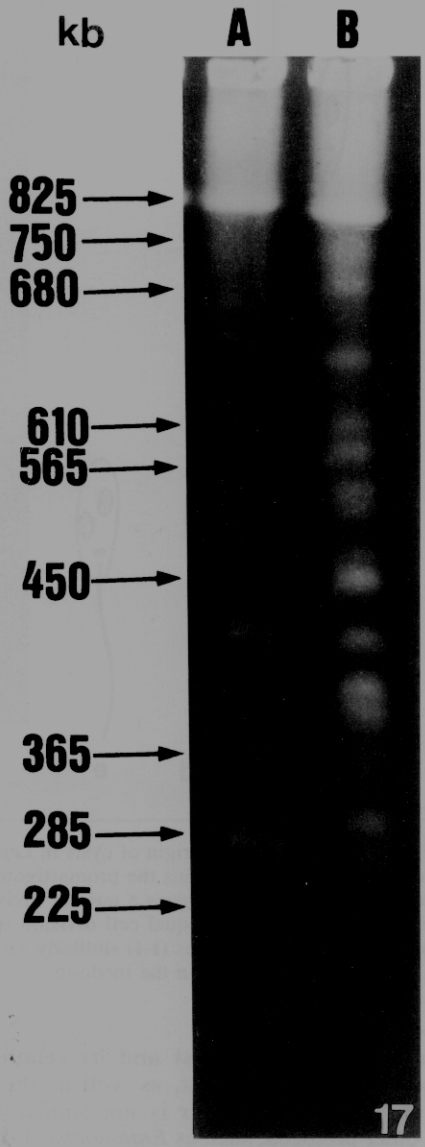

Fig. **17**. Separation of chromosome-size DNA molecules from the 36/429TD strain from the 36/429TD strain of *Leptomonas oncopelti* from *Oncopeltus varicolor* (**A**) and *Leptomonas wallacei* n. sp. wild type isolated from *Oncopeltus fasciatus* (**B**). CHEF electrophoresis in Materials and Methods.

morphological comparisons applied to a population of 50 randomly chosen individuals from the cultures of the *L. wallacei* wild type and 36/429TD strains (Table 3), showed that the two groups did not differ significantly. Thus morphology could place the two isolates (McGhee and Hanson 1962; Noguchi and Tilden 1926; Takata et al. 1996) and the present one under the species *L. oncopelti.*

The enzymatic profiles of the *L. wallacei* wild type and 36/429TD strains were also similar. The isozymes of 6-phosphogluconate dehydrogenase discriminated well between the two (Table 5). This enzyme was not used in the phylogenetic analysis because its activity was too low in the other species studied, except for *Herpetomonas megaseliae.* However, it remained a good diagnostic marker to distinguish *O. varicolor* (36/429TD) from *L. wallacei.*

The molecular karyotype analysis performed by CHEF electrophoresis discriminated the *L. wallacei* and the *Leptomonas* sp. obtained from *O. varicolor.* Using the same technique, one of us (M.A. Sousa, unpubl. data) verified that both isolates are also distinct from *Leptomonas* species: *Leptomonas colosoma* (ATCC 30261), *Leptomonas costoris* (ATCC 30262), *Leptomonas lactosovorans* (ATCC 30970), *Leptomonas mirabilis* (ATCC 30263), *Leptomonas pyrrhocoris* (ATCC 30974), *Leptomonas pulexsimulantis* (ATCC 50186), *Leptomonas samueli* (ATCC 30971), *Leptomonas seymouri* (ATCC 30220)—all these available in the American Type Culture Collection, Manassas, VA.

Unfortunately, neither of these isolates could be compared to Noguchi and Tilden's *L. oncopelti* by isozyme or PFGE analysis. In view of this, we would suggest that not only the present isolate constitutes a new species different from *L. oncopelti* (Noguchi and Tilden 1926), but the 36/429TD isolate from *O. varicolor* would also be a new and different species, still to be named by its authors (Takata et al. 1996).

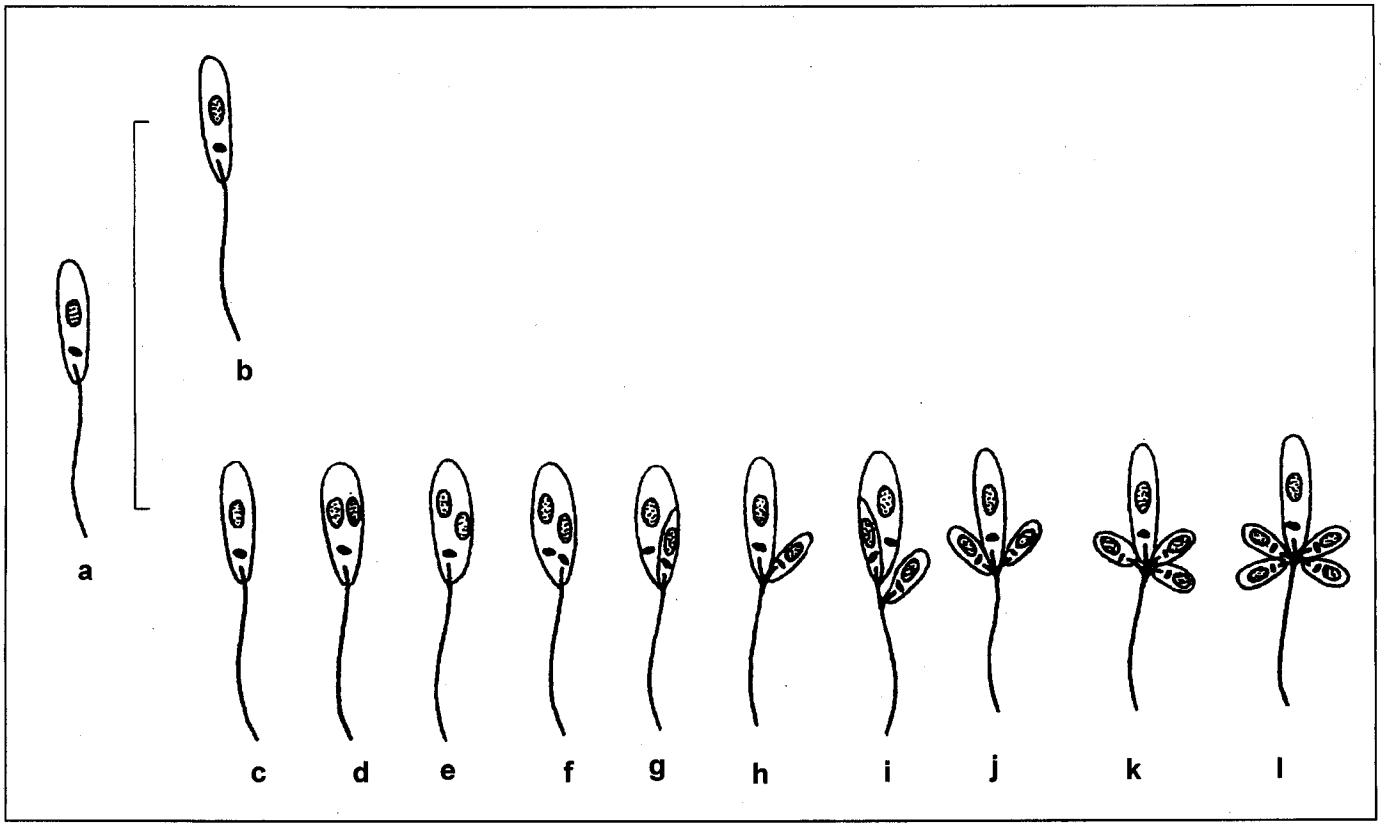

Fig. 18. Proposed model for the origin of cysts in *Leptomonas oncopelti*. The mother cell (**a**) originates two identical daughter cells by simple mitotic division. One of the cells retains the promastigote form (**b**), whereas the other (**c**) differentiates into a new promastigote and cysts. First, the cyst promastigote precursor undergoes a nuclear division (**d**). The new nucleus migrates towards the anterior region of the cell body (**e**) and the kinetoplast duplicates (**f**). An unequal cell division (**g**) occurs and produces the first straphanger cyst with a nucleus, a short axoneme and kinetoplast (**h**). Another three divisions (**i–l**) similarly give rise to other cysts that remain attached to the parental cell. These cysts will detach in clusters or as free, ovoid individuals in the medium.

The ultrastructure of the cyst and its relationship with the parasite life cycle. The cyst, as well as the encystment process, observed in *Leptomonas* is not similar to the cysts observed in other protozoa, such as *Entamoeba* (López-Romero and Villagómez-Castro 1993) or *Giardia* (Adam 1991; Gillin et al. 1987; Gillin, Reiner, and Boucher 1988). The encystment and excystment processes in trypanosomatids are not well-studied. Moreover, there are significant differences between the encystment process described by Gibbs for *L.capsularis* (1951b) and that described by McGhee and Hanson for *L. oncopelti* (1962). According to Gibbs (1951b), the protozoan, after its adhesion to the intestinal epithelium of the insect host, goes through a series of morphological changes that result in the cyst. According to McGhee and Hanson (1962), encystment does not depend on adhesion and the promastigote does not differentiate into a cyst. The parasite first divides, either by unequal cell division or by budding rounded forms that remain adhered to the parental individual. Later these detach and develop into cysts.

The encystment process we observed in *L. wallacei* is similar to that proposed by McGhee and Hanson (1962). However, it was not possible to discriminate between cysts originated by unequal cell division or by budding. However, in contrast to McGhee and Hanson's description (1962), we observed in several Giemsa-stained slides that the number of cysts adhered to the parental cell varied from one to four. Cyst division, as proposed by these authors, was not observed. So, we propose a new model for the encystment of *Leptomonas* in this paper (Fig. 18). Cysts would start from an apparently standard division of a parental cell. Both individuals would remain attached by the flagella and one of them would differentiate by membrane shedding as filopodia, cytoplasmic condensation, and reabsorption of the flagellum while the other would start a new division cycle (Fig. 1_g, $18_{g,h}$), originating another precystic form. Up to four cysts were seen attached to the flagellum of a parental individual (Fig. 1_{g-m}, 18_{h-k}). This may be due to a limit in the capacity of mother cell to hang cysts in its flagellum. Also, because encystment includes reabsorption of the flagellum of the cystic form and because adhesion occurs via this structure, it is quite possible that once the encystment process is complete, the cysts detach from the parental individual (Fig. 1_n). We believe that neither budding of cysts nor cell division of precystic individuals occurs during this process.

Typical cysts present a thick cyst wall, usually made of chitin that render the organism resistant to adverse conditions of food, temperature, humidity, and pH. In kinetoplastids, thin wall cysts have already been described in *Bodo caudatus* (Vickerman 1990). In trypanosomatids, different from other protozoa, cysts do not possess a cyst wall (Mehlhorn et al. 1979; Peng and Wallace 1982; Reduth and Schaub 1988; Takata et al. 1996; Tieszen et al. 1986). As expected, no cyst wall was observed in the cysts of *L. wallacei*. On the other hand, we observed just beneath the cell membrane a homogeneous subpellicular region, limited by a unit membrane, similar to that described in cysts

of *B. triatomae* (Peng and Wallace 1982; Reduth and Schaub 1988), *B. familiaris* (Tieszen et al. 1986) and *L. oncopelti* (Takata et al. 1996). The presence of subpellicular microtubules in this region complies with the descriptions of cysts of *L. oncopelti* (Takata et al. 1996). However, no subpellicular microtubules were observed in cysts of *B. triatomae* (Reduth and Schaub 1988).

In *Blastocrithidia,* the homogeneous subpellicular region shows amazing properties in the cyst physiology. This region is successively built up during encystment (Peng and Wallace 1982; Tieszen et al. 1986), ranging in thickness, from 80–90 nm, and may be used to estimate cyst age (Reduth and Schaub 1988). Freeze-fracture replicas of cysts of *B. triatomae* show that the homogeneous subpellicular region has proteins arranged in rows (Reduth and Schaub 1988). The limiting unit membrane between the homogeneous subpellicular region and cytoplasm of cysts may correspond to the endoplasmic reticulum.

In *Leptomonas,* the homogeneous subpellicular region, reported by Takata et al (1996), seems to correspond, by analogy, to the homogeneous subpellicular region of *Blastocrithidia.* In our observations, the occurrence of cysts with a homogeneous subpellicular region of variable thickness may represent different phases of encystment. The absence of subpellicular microtubules in some cystic forms is indicative of cyst maturation suggesting the engagement of the cytoskeleton in a more advanced phase of cellular differentiation of encystment. The homogeneous subpellicular region seems to confer on the cysts some resistance to physical agents (Porter 1910; Takata et al. 1996) just as the typical cyst wall does for other protozoa.

The condensation of cytoplasm characterizes cysts resistant to dehydration (Schaub and Pretsh 1981). During encystment of *B. sandoni* and *B. triatomae* the cytoplasm condenses after a series of unequal divisions of the parasite (Gibbs 1951a; Schaub and Pretsh 1981). In *L. wallacei,* the cytoplasmic condensation seems to be a consequence of shedding of cell membrane frequently observed in promastigotes and precystic forms. The membrane shedding would result in a reduction of cell surface and consequently in the decrease of total cell volume. As no loss of internal contents occurs, the cytoplasmic content will fill a smaller cell volume leading in consequence to its particles condensation.

Although subpellicular microtubules are absent in mature cysts, a residual flagellum, observed both by scanning and transmission electron microscopy, remains. This favors the rapid growth of the flagellum on excystment, favoring the mobility of parasites and their adhesion to the digestive tract of the host insect.

Desmosome-like structures were observed in the adhesion of precystic forms to its parental promastigote. These structures seem to confer resistance to detachment of these organisms from the mother cell and possibly avoid the detachment of cyst from the parental individual in the beginning of encystment. The filamentous structures linking parental cell and straphangers cysts were not previously described.

The occurrence of cysts in feces and their peculiar ultrastructure suggests that these organisms represent latent forms of the parasite able to act as proliferation agents. When eventually ingested by another insect, either by coprophagy or indirect contamination, they assure the dispersion of the protozoan among hosts.

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