Acta Microscópica, Vol. 1, Nº 2, November 1992, pp 45-54

Stereological Study of Isolates of the Genus *Phytomonas*

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ABSTRACT

The cytoplasmic organelles of different isolates of the genus ***Phytomonas*** were comparatively analysed in relation to their relative volume in the cell. Isolates from ***Euphorbia hyssopifolia, E. characias*** and ***E. pinea, P. francai from Manihot esculenta***, and ***P. davidi*** were employed. Data were obtained for mitochondria, lipid inclusions, glycosomes (a peroxisome - like organelle), multivesicular bodies, nucleus, kinetoplast, vacuoles, dense bodies and other inclusions. Lipid inclusions were more frequent in biphasic medium grown isolates, while glycosomes were more conspicuous in the ***E. characias*** isolate. kinetoplastic DNA was more diffuse in the ***E. pinea*** and ***E. characias*** isolates. The mean cellular volume was measured in a ZBI Coulter-counter coupled to a channelizer; biphasic medium-grown cells were smaller than monophasic medium-grown ones. These observations, in association with others previously reported, indicate that each isolate has unique characteristics and could be considered distinct species.

KEY WORDS

Protozoa, Trypanosomatidae, ***Phytomonas***, electron microscopy, stereology.

INTRODUCTION

Members of the genus ***Phytomonas*** include all trypanosomatids which are found in plants. When found in the lactiferous tubes of euphorbs, these parasites are generally designated ***P. davidi***. In recent years, several investigators have obtained isolates of ***Phytomonas*** that can be axenically maintained ***in vitro***. In our laboratory, we are cultivating one isolate obtained from ***Euphorbia hyssopifolia*** by Attias and De Souza (1986), P. francai from ***Manihot esculenta*** isolated by Vainstein and Roitman (1986), and two isolates obtained from the euphorbs ***E. characias***, and ***E. pinea*** by Dollet ***et al*** (1982)

In previous papers, these isolates had been comparatively analysed in tems of general structure [3], isoenzyme profiles [21], and urea cycle [6]. The results obtained showed that differences exist among the various isolates. In the isolates from ***E. hyssopifolia*** and ***E. characias*** the glycosomes showed an organized array [3].

In the present work, we report data on a stereological analysis of the various ***Phytomonas*** isolates. The results obtained substantiate the existence of differences among the various isolates and suggest that they may represent different species.

MATERIALS AND METHODS

Parasites. ***Phytomonas davidi*** [12], ***Phytomonas*** sp. from ***Euphorbia hyssopifolia*** [2], ***Euphorbia characias*** and ***Euphorbia pinea*** [8] and ***Phytomonas francai*** from ***Manihot esculenta*** [20] were maintained as described previously [3]. ***Phytomonas davidi*** and the ***Phytomonas*** sp. isolated from ***Euphorbia hyssopifolia*** were grown in Roitman's complex medium [14] plus 5% (v/v) fetal calf serum. ***Phytomonas francai*** and the isolates from ***Euphorbia pinea*** and ***E. characias*** were maintained in biphasic medium with a blood agar base (5.2% BHI-agar) (w/v) and 20%

defibrinated rabbit blood and on overlay of Roitman's complex medium.

Electron Microscopy. The parasites were collected in the midlog phase of growth (48 hs) by centrifugation (2000g for five minutes), rinsed in phosphate-buffered saline (PBS), pelleted, fixed in 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer, postfixed in 1% OsO_4 plus 5mM $CaCl_2$ and 0.8% potassium ferricyanide in the same buffer [3], dehydrated in acetone and embedded in Polybed (Polysciences). Ultrathin sections were stained with uranyl acetate and lead citrate, and observed in a JEOL 100-CX electron microscope. For scanning electron microscopy (SEM), fixed cells were adhered to 0.1% poly-L-lysine coated coverslips, post fixed in 1% OsO_4 in cacodylate buffer, dehydrated in an ethanol series and critical point dried with CO_2 in Balzers apparatus. After gold sputtering in a Balzers device, the specimens were observed in a JEOL 100-CX electron microscope with ASID System.

Stereology. The cell volume of living or fixed cell suspensions was evaluated with a ZBI Coulter-counter apparatus coupled to a channelizer. For morphometric analysis, electron micrographs were taken at random from ultrathin sections. In these micrographs, 30 cell profiles of each isolate were enlarged up to 30,000 diameters. All analyses were carried out with IBM PC-based three dimensional reconstruction system (HVEM-3D) [13]. The total area of all profiles for each organelle was compared with the total area of the cell (excluding the flagellum), and the results were expressed as the percentage of the volume of a determined organelle in relation to the total cell volume [23]. Statistical analysis was carried out using the t test in an IBM-PC (Statgraphics, Statistical Graphics System). Differences were considered significant at $P < 0.05$. Each sample was compared with all the others for the same organelle or cell structure.

RESULTS

Table I shows the mean cell volume for living or glutaraldehyde fixed cells as determined by using a Coulter-counter coupled to a channelizer. It could be seen that the isolates from *E. characias* and *E. pinea* were very similar to each other in relation to cell volume. The other isolates showed distinct average volumes, *P. davidi* being the smaller, and *P. francai* the largest of them. Cell size also varied according to the growth medium. Cells grown in biphasic medium were smaller than cells grown in Warren's medium plus 5% fetal calf serum. Glutaraldehyde fixation also altered the cell volume. The scanning electron micrographs showed that the isolates were very pleomorphic

Table I. Cell volume of the *Phytomonas* isolates*.

Isolate	Mean Cell Volume (μm^3) monophasic medium	biphasic medium	fixed
Phytomonas sp. isolated from ***E. hyssopifolia***	31.2	N.D.	22.63
Phytomonas sp. isolated from ***E. characias***	51.03	39.03	46.696
Phytomonas sp. isolated from ***E. pinea***	56.78	37.01	46.696
Phytomonas francai	66.4	53.5	38.25
Phytomonas davidi	28.6	N.D.	N.D.

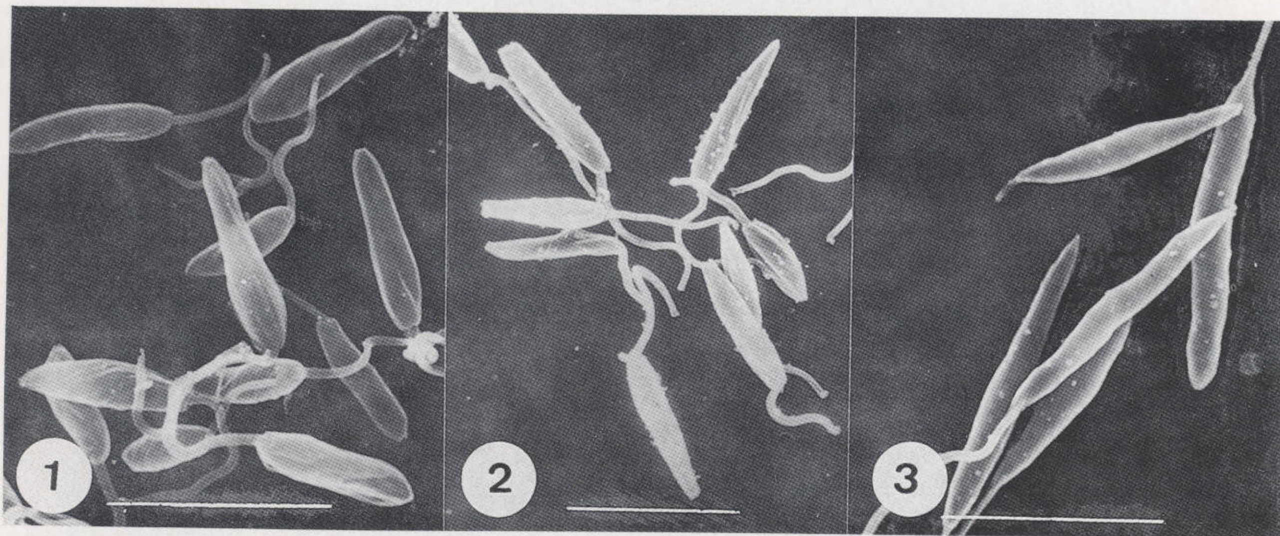
* Determined using a Coulter-counter coupled to a channalizer. Cell suspensions contained about 10^6 cells per ml.

ND - Not determined.

among themselves but still had some characteristics that indicated they were of different origin (Figs. 1-3).

All five isolates exhibited ultrastructural characteristics that were present in all of them (Figs. 4 and 5) and others that permitted unique identification. One of the most prominent differences that could be observed was the arrangement of the kinetoplast DNA network (Figures 6-9). The compact DNA network of the kinetoplast (Fig. 6) was observed in *P. francai,* as well as in the *E. hyssopifolia* isolate and in *P. davidi.* The loose arrangement (Figs. 8-9) was observed only in E. *characias* and *E. pinea* isolates. The duplicated kinetoplast (Fig. 7) was observed in the *E. hyssopifolia* isolate and in *P davidi*; however, it was more common in the former, where it accounts for about 10% of the kinetoplasts observed. There was also a distinct pattern of the endoplasmic reticulum in the isolates. The E. *pinea* isolate had an especially well developed endoplasmic reticulum. Mitochondria, lipid inclusions and glycosomes also seemed to have distinct distribution and morphology in each isolate (Figs. 10-13). Glycosomes profiles in the *E. hyssopifolia* isolate were often long, fingerlike and sometimes branched structures (Figs. 10-11) while in the E. *characias* isolate they were observed as round or oval shaped structures forming stacks of several units (Fig. 12). In all isolates, glycosomes apposed to filamentous bundles were sometimes observed (Fig. 13). The presence or absence of mitochondrial cristae has already been signaled in Attias **et al.** (1988).

Table II shows the percentual relative volume occupied by the organelles in the different isolates as measured from randomly taken ultrathin sections (Fig. 4). It can be seen that large standard deviation values were obtained due to some of the organelles not being randomly distributed in the cell (for instance, the kinetoplast is located only at the anterior region) and the cell cultures not being synchronized, so that cells at different metabolic steps are caught in the same section. This explains why one cell profile has several lipid droplests and another has none. The analysed structures are shown in



Figures 1-3. Scanning electron micrographs of *Phytomonas* isolates. Fig. 1- Isolate from *E. hyssopifolia*. Fig. 2-Isolate from *E. characias*. Fig. 3- *P. davidi*. Cells have different sizes and shapes in each isolate. Bars = 1μm.

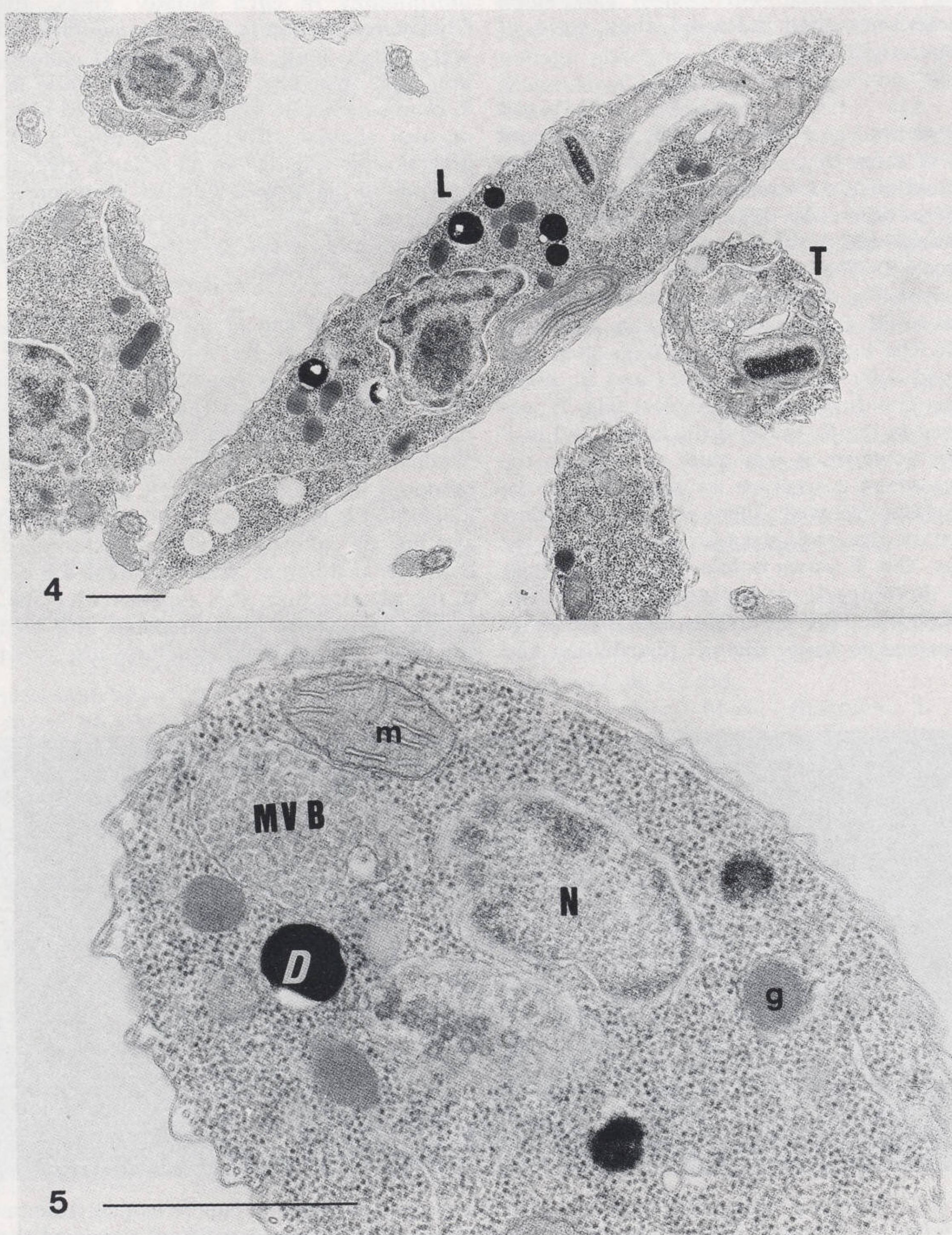
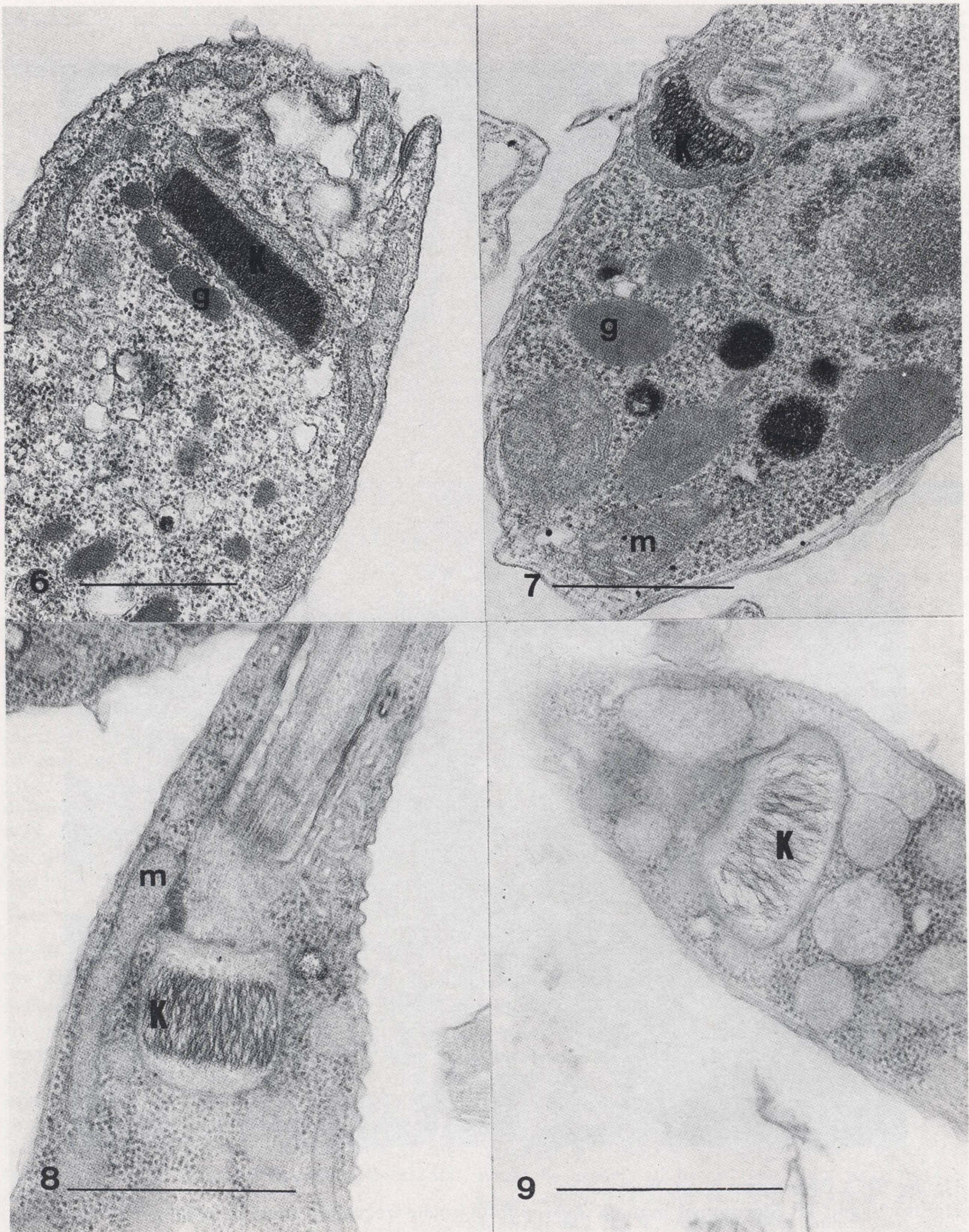
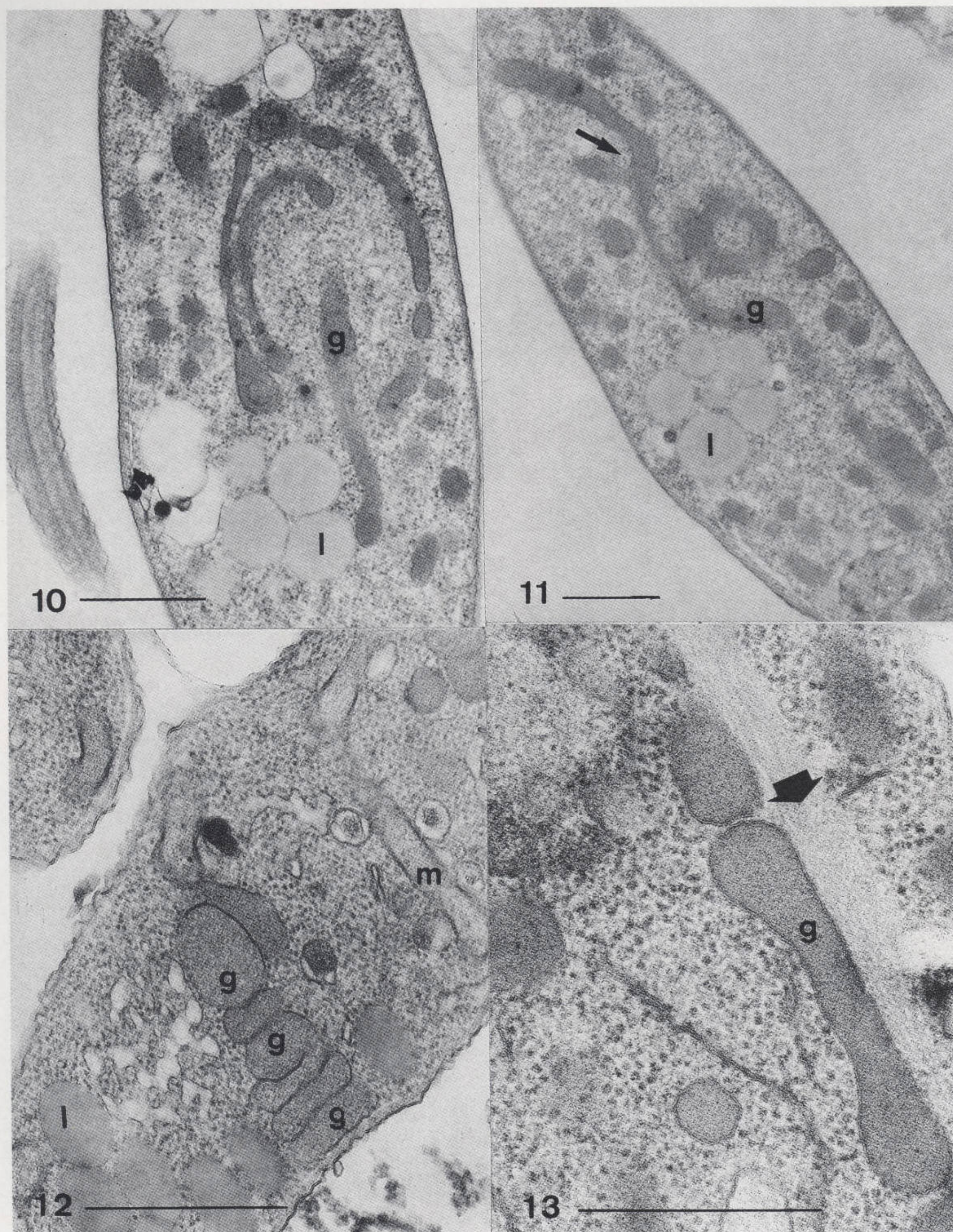


Figure 4. ***Phytomonas davidi***. Random ultrathin section where cells in various orientations were caugth. Measurements for morphometry were made in sections like this, from cells transversally (T), as well as longitudinally (L) sectioned. Bar = 1 µm.

Figure 5. Ultrathin section of ***Phytomonas davidi*** showing its typical organelles. Nucleus (N), mitochondrion (m), multivesicular body (MVB), glycosomes (g), dense granules (d), and endoplasmic reticulum (ER). Bar = 1 µm.



Figures 6-9. Kinetoplast-DNA pattern diversity in ***Phytomonas***. Fig. 6. Compact disposition of the K-DNA in the *E. **hyssopifolia*** isolate. Fig. 7. Duplicated disposition of K-DNA in the *E. **hyssopifolia*** isolate. Notice paramastigote like position of kinetiplast. Fig. 8 and 9. Diffuse pattern of K-DNA in the *E. **pinea*** (fig. 8) and *E. **characias*** (fig. 9) isolates. (m), mitochondria. Bar = 1 µm.



Figures 10-13. Glycosomes (g) in *Phytomonas.*
Figures 10 and 11. Glycosomes in the *E. hyssopifolia* isolate frequently seem to be long and branched (arrow). Bar = 1µm.

Figure 12. Glycosomes (g) in the *E. characias* isolate often show a stacked disposition. Mitochondria (m) in this isolate also do not show typical cristae. Bar = 1 µm.

Figure 13. *P. francai* Glycosomes (g) apposed to a bundle of filaments (arrow) are sometimes seen in all isolates. Bar = 0.5 µm.

Table II. Percent of cell volume occupied by organelles in the isolates of ***Phytomonas.***

Structure	*Phytomonas davidi*	*Phytomonas francai*	*Phytomonas* sp. isolates from		
			Euphorbia pinea	*Euphorbia characias*	*Euphorbia hyssopifolia*
	(isolate 1)	(isolate 2)	(isolate 3)	(isolate 4)	(isolate 5)
Lipid inclusions [a]	0.62 ± 1.19	2.80 ± 4.16	3.13 ± 3.40	1.43 ± 2.04	
Glycosomes [b]	2.85 ± 2.15	3.83 ± 2.35	6.81 ± 7.33	8.63 ± 6.98	3.86 ± 2.39
Mitochondria [c]	10.97 ± 7.96	7.16 ± 3.09	12.65 ± 9.44	8.63 ± 9.14	8.75 ± 3.71
K-DNA [d]	0.45 ± 0.72	0.29 ± 0.65	0.80 ± 1.58	0.80 ± 2.75	0.40 ± 0.61
Vacuole [e]	1.20 ± 1.94	1.39 ± 1.60	0.55 ± 0.61	1.59 ± 2.51	0.94 ± 1.06
Multivesicular body	1.50 ± 2.64		0.19 ± 0.42		
Dense bodies	0.24 ± 0.53				0.94 ± 1.18

Significant differences (p 0.05) were observed for the structures (a to e) in the isolates (1 to 5) indicated below: (a) between 1 and all others; (b) between 1 and 3 and 4; between 2 and 3 and 4; between 5 and 3 and 4; (c) between 1 and 2 and 4; between 2 and 3; between 4 and 5; (d) between 1 and 2 and 3; between 5 and 3 and 4; (e) between 3 and 1 and 5.

the ultrathin section of a typical cell (Fig. 5). The isolates that were maintained in biphasic medium were richer in lipid inclusions. In the isolates from *E. pinea* and *E. characias* the glycosomes occupied a comparatively larger volume of the cytoplasm. *Phytomonas francai* was the isolate which showed the least developed mitochondrion. The mitochondrion was more developed in the isolate from *E. pinea.*

Our analysis showed that there were significant differences in the volume occupied by the kinetoplast-DNA network between some isolates (Table II). The values were higher for the isolates from *E. characias* and *E. pinea,* intermediate for *E. hyssopifolia,* and *P. davidi,* and lower for *P. francai.* The kinetoplast of *E. pinea* and *E. characias* isolates were larger and presented a looser DNA structure, as compared with the other isolates. Measurements of the width and length of the kinetoplast network are shown in Table III.

Only in *Phytomonas davidi* and *Phytomonas* sp. from **E. pinea,** multivesicular bodies were prominent. Dense bodies were seen most often in *Phytomonas* sp. from *E. hyssopifolia. P. davidi* was the only isolate where a contractile vacuole could be clearly detected, although in the *E. hyssopifolia* isolate this organelle was occasionally seen. However, small vacuoles were present in all isolates.

DISCUSSION

Attias **et al.** (1988) described some ultrastructural characteristics that can be used to distinguish different isolates of trypanosomatids of the genus *Phytomonas*. The *E. pinea* isolate, for instance, showed no typical mitochondrial cristae, as well as a greatly expanded endoplasmic reticulum. Its kinetoplast DNA network was diffuse in most cells observed. The *E. characias* isolate also had the same kinetoplast-DNA pattern, but could be distinguished from that of *E. pinea* by the absence of the profuse endoplasmic reticulum and by the occurrence of "stacked" glycosomes. Although these "stacks" could be associated to cell age, they were not observed in the other isolates. *Phytomonas francai* and the *E. hyssopifolia* isolate had a similar degree of compactness of the kinetoplast-DNA network, but only in the latter it would appear in the duplicated form in some of the cells. *P francai* and the *E. pinea* isolate are the isolate where lipid inclusions were most often

Table III. Mean lenght and width of the kinetoplast.

Isolate	Width (µm)	Lenght (µm)
Phytomonas sp. from *Euphorbia hyssopifolia*	0.23 ± 0.05	0.72 ± 0.13
Phytomonas sp. from *Euphorbia pinea*	0.32 ± 0.07	0.83 ± 0.27
Phytomonas sp. from *Euphorbia characias*	0.39 ± .08	0.93 ± 0.29
Phytomonas davidi	0.12 ± 0.02	0.66 ± 0.10
Phytomontas francai	0.20 ± 0.02	0.87 ± 0.24
Crithidia fasciculata	0.23	ND*

*ND, not determined

observed. *P. davidi* was the only isolate where a contractile vacuole could be clearly detected [1] although in the *E. hyssopifolia* isolate this organelle was occasionally seen.

We find the obtained relative volumes are consistent despite of the large standard deviation values obtained probably due to the fact that the organelles are not randomly distributed in the cell. The K-DNA network for example has a define location in the cell that makes it absent from most cell profiles randomly taken. The fact that cultures were not synchronized and cells at different metabolic stages are caught in the same section also influenced the standard deviation values obtained.

Cell volume does not seem to be a good parameter to distinguish any isolate since culturing conditions as well as chemical fixation are seen to induce volumetric alterations. This was also observed by Kastelein and Parsadi (1988) in several Phytomonas sp. isolates obtained from Euphorbs.

Our stereological data confirmed many of the previous morphological observations (review in De Souza and Attias, 1991). The kinetoplast-DNA network observed in the *E. pinea* and *E. characias* isolates occupied a larger volume of the cytoplasm, as compared with that of the others. The kinetoplsat-DNA network of the *E. hyssopifolia* isolate and *P. davidi* presented a larger volume than the one observed in *P. francai*. It is possible that this is due to the occurrence of the duplicated kinetoplast frequently observed in these isolates. In his review, Simpson (1972) analysed the kinetoplast-DNA network morphology of several species of trypanosomatids. He actually measured the width and calculated the circumference of the DNA rod from ultrathin longitudinal sections. Using the same method, we obtained the values listed in Table III. It is interesting to notice that the linear dimensions of kinetoplast-DNA are about one half, and the relative volume of the network is about 10 times smaller in *Phytomonas* than in other trypanosomatids, namely in *T. cruzi* [17] However, since linear duplication of kinetoplast-DNA network in *T. cruzi* leads to an eight fold increase in volume [17], the results are in agreement.

In the *E. characias* isolate the glycosomes occupied the largest relative volume among all the isolates. Comparing our results for

Phytomonas with the values found for other trypanosomatids [16], we can see that the mitochondrial volume. varies among the different trypanosomatids. This may reflect metabolic differences between isolates as well as different stages of the life cycle of a single isolate. In *Herpetomonas samuelpessoai* marked modifications in the mitohondrion were observed with the addition or omission of 2-deoxy-D-glucose to the culture medium [5], and ultrastructural and quantitative differences were also described during *Leishmania donovani* transformation [4]. Glycosome density, however, seems to be higher in *Phytomonas* than in most other genera, with the exception of *Leptomonas samueli* [18].

Statistical analysis of the morphometric data shown in Table II indicate that some isolates did not differ significantly on most of the parameters. This was the case between the *E. characias* and the *E. pinea* isolates as well as between the *E. hyssopifolia* isolate and either *P. davidi* and *P. francai*.. On the other hand, almost no correlation exists between the relative volume occupied by the organelles of *P. davidi* in relation to *E. pinea* and *E. characias* or between the *E. pinea* and *E. hyssopifolia* isolates. *P. francai* and the *E. pinea* isolates also differ in three parameters. These observations may be helpful for the establishment of species and strains in the future.

There exists some controversy on the taxonomy of *Phytomonas* spp. Among the *Phytomonas* isolates obtained so far, three species are designated: *P. davidi* from *E. heterophylla* [12]; *P. francai* from *M. esculenta* [21]; and *P. serpens* from *Lycopersicum esculentum* [10]. The isolates from *E. hyssopifolia*, *E. characias*, and *E. pinea* are still named *Phytomonas* sp. Several investigators were concerned in the past few years with the taxonomic position of these isolates ([3, 21, 6, 22]. Morphological data show differences among them and also in relation to *P. francai* and *P. davidi* [3].

The distribution of cationic particles and the surface charge was also peculiar for each isolate [9, 22]. The electrophoretic pattern for 6 different enzymes (aspartate-amino-transferas, alanine-amino-transferase, phosphoglucomutase, glucose-6-phosphate dehydrogenase, glucose phosphate isomerase and malate dehydrogenase) can distinguish all 5 isolates and, in some instances (e.g., *P. davidi* and *E. hyssopifolia* isolate) shows distinct patterns for all of them [21]. This evidence would suggest that each isolate here analysed would constitute a single species. However, the presence of ornithine cycle enzymes on *Phytomonas* isolates gave rise to some controversy [6]. It was observed that *Phytomonas* has an enzymatic profile close to the *Herpetomonas* genus in that it lacks arginase. Ornithyne carbomoyl-transferase was absent in the *Phytomonas* isolates except in *P. davidi* and the *E. hyssopifolia* isolate. There is also morphological similarity between some *Herpetomonas* isolates and *Phytomonas davidi*. This could suggest that *P. davidi* (and the *E. hyssopifolia* isolate) would not be "true" *Phytomonas* species. However, more recently, Teixeira and Camargo (1989), raised monoclonal antibodies against *P. francai* and *P. serpens* and showed that they were very specific for *Phytomonas* isolates while not reacting with other trypanosomatid genera. These authors also showed that *P. davidi* could not be detected by these antibodies and the *E. hyssopifolia* isolate was agglutinated only by one of them (against *P. serpens*) which discriminates between *P. davidi* and *E. hyssopifolia* isolate and keeps this isolate in the *Phytomonas* genus. There are some points that should be considered. First, our knowledge on the genus *Phytomonas* is still limited. It is not known if the insect host carries non-promastigote forms or if the same enzymes are expressed in the plant and in the insect stages second, since only a limited number of isolates of the genera *Herpetomonas* and *Phytomonas* were analysed, these observations cannot be considered consistent for any of them as yet. In view of the observations reported here and elsewhere [3, 6, 21, 22] we suggest that each isolate should be considered different species.

ACKNOWLEDGMENTS

The authors thank Mr. Luiz Herique Monteiro Leal and Mr. Celso Fassini for technical assistance, and Mrs. Alba Valéria Peres and Sandra Rocha for secretarial assistance. This work has been supported by CNPq, FINEP, FAPERJ and CEPG-UFRJ.

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