

CRITHIDIA RICARDOI SP. N. A NEW SPECIES OF TRYPANOSOMATIDAE ISOLATED FROM CULEX SALTANENSIS DYAR, 1928 (DIPTERA: CULICIDAE)

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A flagellate trypanosomatid was isolated in culture from the digestive tract of the mosquito Culex saltanensis Dyar, 1928. It grows exuberantly in liver infusion in the form of epimastigotes and choanomastigotes typical of the genus Crithidia. The trypanosomatid was compared to C. deanei, C. fasciculata, C. luciliae, C. oncopelti and C. guilhermei. The techniques used for comparison were electron transmission microscopy, isoenzymes and kDNA restriction profiles. No endosymbionts were found at electron microscopy. Results for the biochemical methods employed indicate that the trypanosomatid isolated from C. saltanensis is a new species of Crithidia. The name C. ricardoi sp. n. is proposed for this new species.

Key words: *Crithidia ricardoi* sp. n. – trypanosomatidae – *Culex saltanensis* – kDNA – isoenzymes – electron microscopy – cell sorting

Trypanosomatids parasitizing insects are very common. They include the subgenus *Leptomonas*, *Herpetomonas*, *Crithidia*, *Blastocrithidia* and *Rynchoidomonas*. Under the genus *Crithidia* Léger, 1902 many species or varieties have been described as parasites of insects (Wallace, 1966). In order to formalize new findings, biochemical analysis, as suggested by Wallace et al. (1983), should be used to give the appropriate taxonomic evaluation of the differences found. To correctly identify the flagellate isolated, five other *Crithidia* were used for comparison. Species used were isolated from Diptera and Hemiptera. *C. fasciculata* Léger, 1902 is the type-species for the genus *Crithidia*. It was isolated from the mosquito *Anopheles maculipennis*. *C. luciliae* (Strickland, 1911) Wallace & Clark, 1959 was isolated from *Phaenicia sericata*, a Calliphoridae. *C. oncopelti* (Noguchi & Tilden, 1926) Lwoff & Lwoff, 1931 was described as originating either from the milkweed *Asclepias syriaca* or from the milkweed bug *Oncopeltus fasciatus*. Posterior serological studies concluded that *C. oncopelti* did not come from a

plant or hemiptera and its origin remains unknown (McGhee & Hanson, 1968; McGhee et al., 1969). *C. deanei* Carvalho, 1973 was isolated from an insectivorous hemiptera *Zelus leucogramus*. Whether *C. deanei* is actually an intestinal parasite of *Z. leucogramus* or it was accidentally ingested within one of its insect prey is a fact to be clarified (Carvalho & Deane, 1974; Wallace et al., 1983). *C. guilhermei* Soares et al., 1986 was isolated from the Calliphoridae fly *Phaenicia cuprina*.

The mosquito found infected by *C. ricardoi* was *Culex (Culex) saltanensis* Dyar, 1928, a highly ornitophilic mosquito species. It is the natural vector of *Plasmodium juxtannucleare*, an avian malaria parasite (Lourenço-de-Oliveira, 1991) at Granjas Calábria, Jacarepaguá, Rio de Janeiro, Brazil. This mosquito has a wide geographic distribution in South America being recorded in Argentina (Salta Province, its type-locality), Venezuela, Panama and in Brazil (States of São Paulo, Rio de Janeiro and Bahia).

MATERIALS AND METHODS

The digestive tracts of *C. saltanensis* specimens caught at Granjas Calábria (23° 00'S, 43° 26'W), Rio de Janeiro, Brazil, dissected in aseptic conditions, were observed by light

Financial support was provided by CAPES and FAPERJ.

⁺Current adress: Departamento de Bioquímica e Biologia Molecular.

Received 5 January 1993.

Accepted 9 September 1993.

microscopy for flagellates. Positive tracts were diluted in sterile 0.9% NaCl solution, inoculated in 2 ml of NNN-LIT supplemented with 10% fetal calf serum and 100U penicillin. Tubes were kept at 27.5 °C and transferred at three to four day intervals.

For the measurement at the light microscopic level, smears of 3-day-old culture forms were fixed in methanol and stained in Giemsa.

For transmission electron microscopy, 3-day-old culture forms were collected by centrifugation at 1500G for 5 min. The pellet was fixed, embedded and ultrathin sections observed as described in Soares et al. (1986).

Isoenzyme and kDNA analyses were made comparatively with other *Crithidia* species: *fasciculata* ATCC # 11745, *oncopelti* ATCC # 30264, *deanei* ATCC # 30255, *luciliae* ATCC # 14765 and *guilhermei* (Universidade Federal do Rio de Janeiro – UFRJ) which was used only for the kDNA analysis, jointly with the above species.

For the isoenzyme characterization, agarose gel electrophoresis was performed for 11 *loci*: malate dehydrogenase (MDH E.C.1.1.1.37), malic enzyme (ME E.C.1.1.1.40), isocitrate dehydrogenase (IDH E.C.1.1.1.42), 6-phosphogluconate dehydrogenase (6PGDHE.C.1.1.1.44), glucose-6-phosphate dehydrogenase (G6PDH E.C.1.1.1.49), phosphoglucomutase (PGM E.C.2.7.5.1), esterase (EST E.C.3.1.1.1), peptidase 1 (PEP1 E.C.3.4.11), aconitase (ACON E.C.4.2.1.3), fumarase (FUM E.C.4.2.12) as described in Momen et al. (1985). Results were used to calculate the Jaccard's coefficient of similarity (Sneath & Sokal, 1973).

Clones were made from 0.5 ml of two 3-day-old *C. ricardoi* isolates using a fluorescent cell sorter (Epics/Coulter). Two cloned samples of the two isolates and the isolates themselves, in a total of four samples, were comparatively analyzed with the other species of *Crithidia*.

Extraction and analysis of restriction enzyme profiles of kinetoplast DNA were as follows: parasites were lysed using Sarkosil and digested by Pronase at 60 °C. kDNA was collected by centrifugation, extracted by phenol/chloroform and precipitated by ethanol. Purified kDNA (2 µg) was digested with the restriction enzymes Msp I, Rsa I and Hae III in appropriate buffers. The products of diges-

tion were separated by electrophoresis in 5-10% linear gradient polyacrylamide gels and stained with ethidium bromide (Pacheco et al., 1990).



Fig. 1: transmission electron microscopy of an ultrathin section of *Crithidia ricardoi* sp. n. Longitudinal section of a parasite, showing the mitochondrion (M), nucleus (N), glycosomes (G), kinetoplast DNA (K) and the flagellum (F). Bar = 1 µm.

TABLE

Input data matrix of the allelic presence/absence for eleven isoenzymatic loci in five *Crithidia* species

<i>Crithidia</i> Species/Enzymes	PGM	G6P	6PG	GPI	ME	FUM	PEP1	EST	IDH	MDH	ACON
<i>C. ricardoi</i> (c1)	010	01	10	1	100	0111	1	10000	001	01110	10
(c2)	010	01	10	1	100	0111	1	10000	001	01110	10
(c3)	010	01	10	1	100	0111	1	10000	001	01110	10
(c4)	010	01	10	1	100	0111	1	10000	001	01110	10
<i>fasciculata</i>	001	10	10	1	1000	0111	1	01100	001	01111	10
<i>deanei</i>	010	01	10	1	110	1111	1	10000	011	11110	00
<i>oncopelti</i>	100	01	01	0	101	0110	0	00011	100	10000	00
<i>luciliae</i>	001	00	10	1	100	0111	1	01000	001	01111	01

c1 - clone of isolate 1 of *Crithidia ricardoi*; c2 - total population of isolate 1 of *C. ricardoi*; c3 - clone of isolate 2 of *C. ricardoi*; c4 - total population of isolate 2 of *C. ricardoi*.

RESULTS

Crithidia ricardoi sp. n.

Type host: *Culex (Culex) saltanensis* Dyar, 1928 (Márcia Gonçalves de Castro ident.)

Location in host: gut, mainly hindgut.

Location: Granjas Calábria (23° 00'S, 43° 26'W), Jacarepaguá, Rio de Janeiro, Brazil.

DIAGNOSIS: *C. ricardoi*: total length: 9.5 to 18.0 µm (average: 13.0 µm); body length: 5.0 to 12.0 µm (avg 7.0 µm); free flagellum: 4.5 to 10.0 µm (avg 5.0 µm); body width: 1.5 to 3.0 µm (avg 1.5 µm); nucleus width: 1.0 to 1.5 µm (avg 1.0 µm). N = 50 cells.

Giemsa-stained and electron microscopy prepared cells showed a choanomastigote form for *C. ricardoi* sp. n. (Fig. 1).

C. ricardoi sp. n. had the same profile for five out of eleven isoenzymes with *C. deanei* (PGM, G6P, 6PG, PEP1 and EST) and *C. luciliae* (6PG, ME, FUM, PEP1 and IDH). Compared to *C. fasciculata*, four isoenzymatic loci were the same (6PG, ME, PEP1 and ACON). G6P was the only enzyme that exhibited similarity when compared to *oncopelti*. The enzyme MDH can distinguish the new isolate from all 4 other species (Table; Fig. 2).

Marked differences in the kDNA minicircle regions were observed among all the species analyzed including the new one, thus supporting the status of distinct *taxon* (Fig. 3). Although kDNA analyses of the new isolate had shown a more complex genotypic profile suggesting the possibility of mixture of subpopulations or mixed infection, preliminary experi-

ments with two cloned populations of *C. ricardoi* revealed parental genotypic similarity in their kDNA restriction profile, e.g., no differences were detected among cloned and randomized populations. Two other insect trypanosomatids, *Herpetomonas samuelpessoai* ATCC # 30252 and *Blastocrithidia culicis* (UFRJ) also displayed distinct profiles (data not shown).

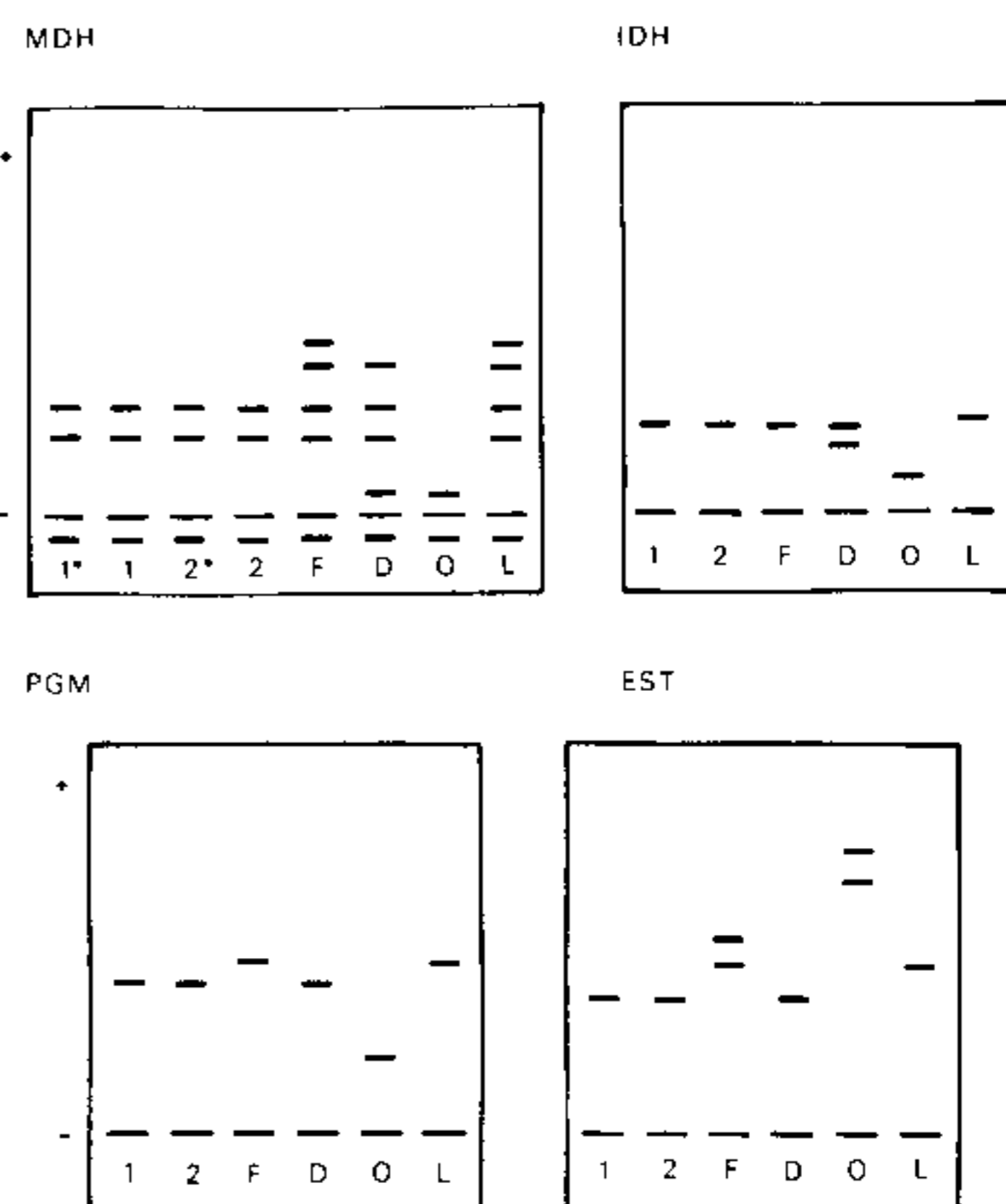


Fig. 2: diagram showing agarose electrophoresis gel profiles of isoenzymes: a - MDH (Malate Dehydrogenase); b - IDH (Isocitrate Dehydrogenase); c - PGM (Phosphoglucumutase); d - EST (Esterase). Lanes: 1 - Clone of isolate 1 of *Crithidia ricardoi*; 2 - Total population of isolate 1 of *C. ricardoi*; 3 - Clone of isolate 2 of *C. ricardoi*; 4 - Total population of isolate 2 of *C. ricardoi*; 5 - *C. fasciculata*; 6 - *C. deanei*; 7 - *C. oncopelti*; 8 - *C. luciliae*.

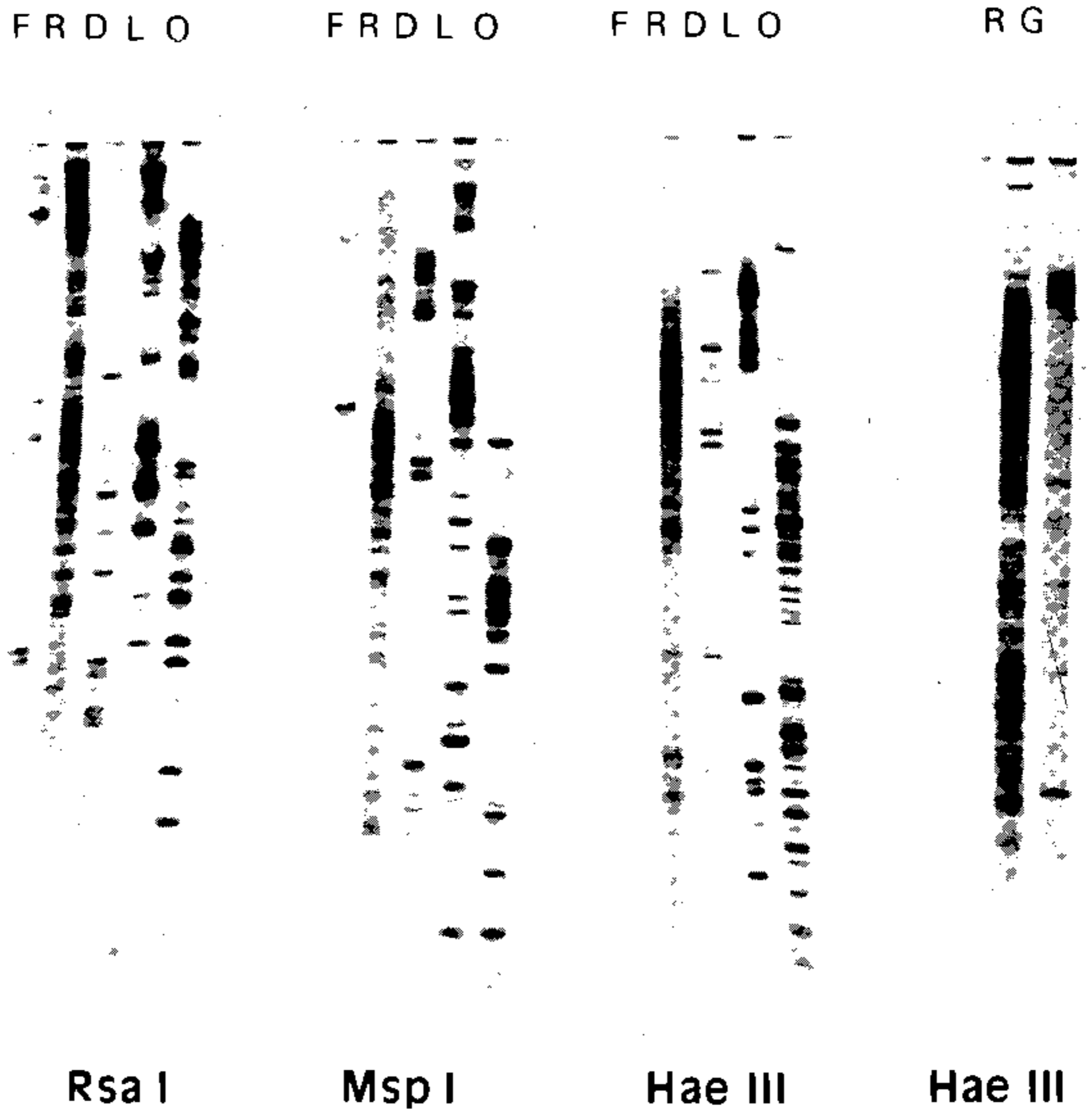


Fig. 3: kDNA minicircle fragments of different *Crithidia* species after digestion with the restriction enzymes *Rsa*I (A); *Msp*I (B) and *Hae*III (C) in a 5-10% gradient gel.

DISCUSSION

A flagellate trypanosomatid isolated from *C. saltanensis* was compared with five other species of *Crithidia*. Three different techniques were used: electron microscopy, isoenzyme electrophoresis and kDNA restriction analysis. Although in lower trypanosomatids, electron microscopy is likely to be uninformative, due to the homogeneity of the group, we believe that detailed examination of the fine structure of new isolates can show interesting findings that may produce results of taxonomic significance.

Similarity given by Jaccard's coefficient calculated from isoenzymatic data, indicates that: (a) the samples could be divided in two major groups separating *C. oncopelti* from other crithidias by a $S_j = 0.24$. A $S_j = 0.66$ separate the second group in two minor ones, the first containing *C. fasciculata* and *C. luciliae* and the second *C. ricardoi* and *C. deanei*. A $S_j = 0.85$ separate *fasciculata* from *luciliae* and *ricardoi* from *deanei*; (b) the four *C. ricardoi*

samples (two isolates and two cloned samples from them) had a $S_j = 1.00$ indicating total similarity (Fig. 4).

Wallace et al. (1983) pointed out that insect hosts can not be used as informative taxonomic data. It was previously believed that these parasites were narrowly limited in their host distribution and that each insect had its own peculiar parasite (names derived from insects shows this early belief). Based on host, one would expect that crithidias originating from common hosts such as Hemiptera for *luciliae*, *oncopelti* and *deanei* and Diptera for *fasciculata* and *ricardoi* should be grouped together in the isoenzymatic analysis. Nevertheless, *C. oncopelti* was grouped separately being the most dissimilar ($S_j = 0.24$). *C. luciliae* grouped with *C. fasciculata* and *C. deanei* with *C. ricardoi*. The analysis corroborates earlier suspicions that *deanei* originated from infected insect prey of *Z. leucogramus* (Carvalho & Deane, 1974). It also provides data on the eurixenic nature of the crithidias.

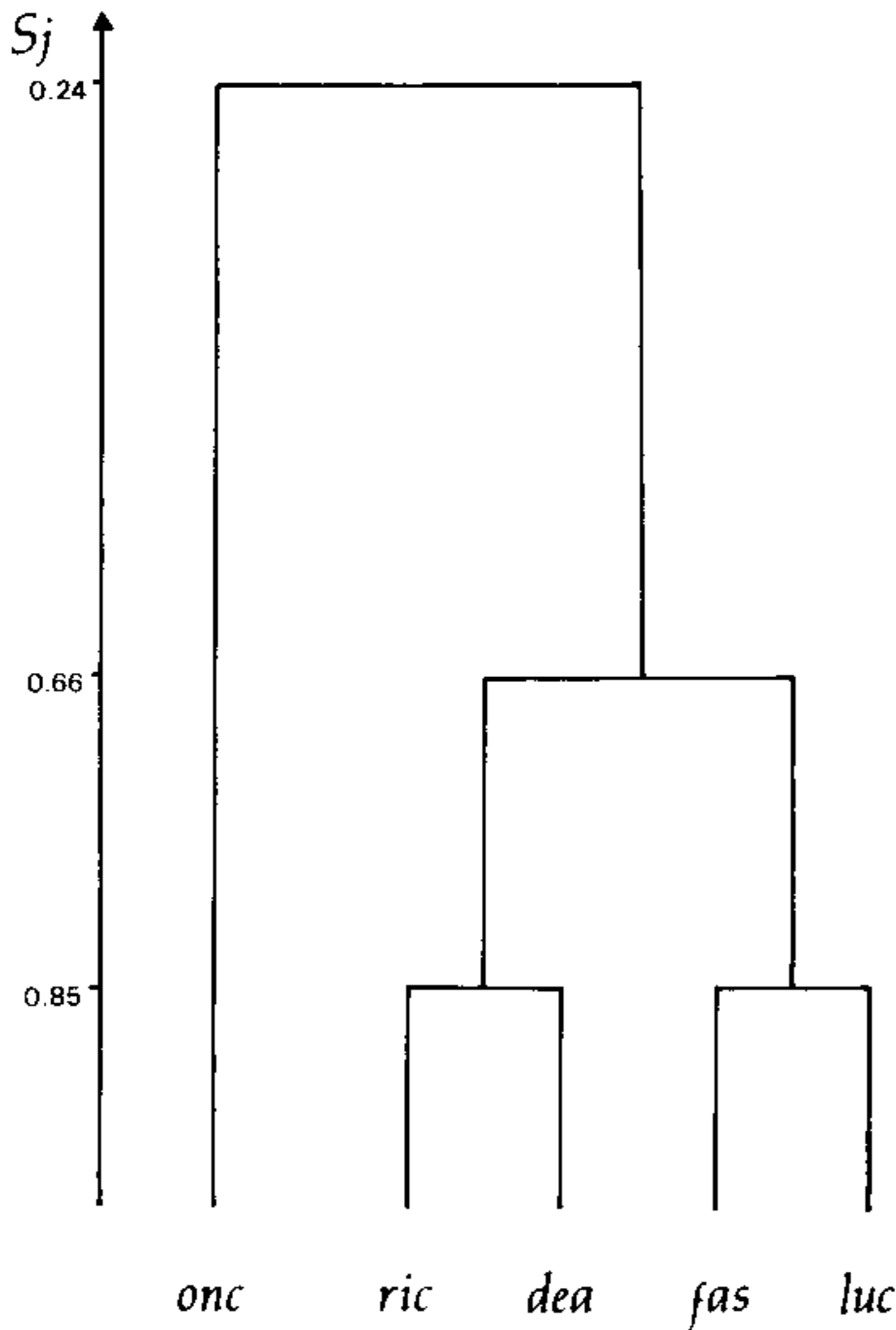


Fig. 4: dendrogram showing groups with different *Crithidia* species. The isolates and the clones are identical in *C. ricardoi*. That new species is by morphology more similar to *C. fasciculata* and *C. luciliae* but by isoenzymes dissimilar to them, grouped nearer to *C. deanei*. "Sj": Jaccard's Coefficient of similarity.

CONCLUSION

C. ricardoi sp. n. is a new species of the genus *Crithidia* named after Ricardo Lourenço-de Oliveira. It was isolated from *C. saltanensis* while this mosquito was being examined for oocysts and sporozoites of *Plasmodium juxtannucleare*. *C. saltanensis* is the natural vector of this plasmodium. *C. ricardoi* sp. n. abundantly parasitizes the gut, mainly the hindgut, of that mosquito species. It can be transmitted to *saltanensis* larvae by diluting cultured *C. ricardoi* in the water the larvae were being

bred in. *Crithidias* could be observed in the larval digestive tract and also adults after some days.

Combined results from electron microscopy, isoenzymes and kDNA analysis indicate that this isolate of *Crithidia* is a new species.

ACKNOWLEDGEMENTS

To Márcia Gonçalves de Castro for identification of the mosquitoes collected; to Maria Goreti Rosa Freitas-Sibajev for critical reviewing the manuscript; to Neide Thomaz for encouragement on the subject.

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