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The Genus *Crithidia*: Genotypic Diversity amomg Species

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ABSTRACT

Six species of insect trypanosomatids of the genus Crithidia were analyzed using different biochemical and molecular biology techniques, such as kinetoplast DNA (kDNA) restriction pattern analysis, kDNA cross-hybridizations, zymodeme analysis and, computer analysis of kDNA minicircle sequences for C. fasciculata and C. oncopelti. Our results indicate that C. deanei and C. oncopelti have widely diverged from the other species we analyzed, since their kDNA did not cross-hybridize with that from the other species, indicating that a great divergence of kDNA sequences has occurred during the evolution of this genus. Comparison of kDNA minicircle sequences of C. fasciculata and C. oncopelti from the literature, showed no significant sequence homology corroborating some of our data. Restriction profiles of kDNA from the different species showed no genotypic similarity thus supporting the status of separate taxa. Furthermore, no cross-hybridization was detected between kDNA from parasites of the genus Crithidia and kDNA from parasites of different genera of monogenetic and digenetic trypanosomatids such as Herpetomonas, Blastocrithidia, Trypanosoma, Leishmania, Endotrypanum and Phytomonas.

INTRODUCTION

The flagellates kinetoplastids that constitute the family Trypanosomatidae have long attracted curiosity. Digenetic members of this family are being intensively studied as they are causative agents of very serious and widespread diseases in man and animals, such as leishmaniasis and African and South-American trypanosomiasis (Vickerman 1979). Trypanosomatids of the genus Phytomonas are associated with diseases of coffee, coconut and oil palm, being transmitted to the plant host by hemipteran insects. In recent years, the interest in cellular and molecular biological studies of trypanosomatids has also increased because of the presence, in these organisms, of the kinetoplast DNA which possesses some very unique features (see Simpson 1986 and 1987 for reviews). The kDNA is organized by catenation of thousands of circular DNA molecules (maxicircles and minicircles). The minicircles are responsible for 80% of the mass of the network, varying in size from

about 400 base pairs (bp) in Trypanosoma vivax to 1400 bp in Trypanosoma cruzi and to 2500 bp in Crithidia fasciculata. The maxicircles are equivalent to mitochondrial DNA and are present in about 50 copies per network. It has been recognized that kDNA minicircles contain between one and four conserved regions of about 100-200 bp (mini repeats) per molecules, depending on the species. These sequences are not conserved between the different genera of trypanosomatids. However, each conserved sequence contains a 12 bp sequence (GGGGTTGGTGTA) which has been recognized in all minicircle sequences from all trypanosomatid species and has been pointed out as the origin of replication (Birkenmeyer et al. 1987). The kDNA of C. fasciculata contains around 5000 molecules of minicircles. Sugisaki and Ray (1987) described a strain (CF-C1) of this species containing a nearly homogeneous population of kDNA minicircles presenting two conserved sequence regions in different kDNA minicircle classes, each containing a single copy of the 12 bp universal sequence. Other very unique features like DNA bending and RNA editing have further stimulated research on these organisms.

The monogenetic or "lower" trypanosomatids are parasites of many different orders of arthropods such as Diptera, Hemiptera and also Hymenoptera, Lepdoptera, Orthoptera and Siphonaptera. The species of "lower" trypanosomatids described so far belong to the genera Herpetomonas, Crithidia, Blastocrithidia and Leptomonas (Mc ghee and Cosgrave 1980). These parasites offer rich material for the study of the evolutionary processes of parasitism and the phylogenetic relationships.

The taxonomy of "lower" trypanosomatids has been based mostly on cell morphology, host range, life cycle and growth condition in cultures (Wallace 1979). None of these criteria, however, are infallible and they can lead in some cases to the misidentification of isolates. It is not uncommon for trypanosomatids to be reclassified in different genera after further studies (Roitman et al. 1976). Biochemical methods have been useful both for identification and comparison of different isolates or for species characterization in cases where classical criteria prove to be insufficient (Camargo et al. 1982).

Recent work on biochemical analysis, such as isoenzyme electrophoresis, kDNA hybridization and kDNA restriction fragment patterns (Morel et al. 1980, Camargo et al. 1982, Lima et al. 1982, Momen et al. 1985, Pacheco et al. 1990a and 1990b, Kolesnikov et al. 1990, Motta et al. 1991) have shown the usefulness of such methods for the study of different trypanosomatids at the phenotypic and genotypic level. In addition, the finding of bacterium- like endosymbionts in the cytoplasm of C. oncopelti (Newton 1957) and C. deanei (Mundim et al. 1974) opens an interesting investigation field for the study of the interrelation of three distinct genomes in the same cell.

This paper shows the analysis of six different species, described as insect trypanosomatids of the genus Crithidia by biochemical and molecular methods and the evaluation of genotypic relationships among the different species. Moreover, available information on reported kDNA minicircle sequences of C. fasciculata and C. oncopelti has been analyzed by computer in order to extract common and divergent features.

MATERIALS AND METHODS

1. Parasites.

The monogenetic and digenetic trypanosomatids analyzed in this study were: Herpetomonas samuelpessoai (ATCC 30252), Blastocrithidia culicis (ATCC 30268), Crithidia fasciculata (ATCC 11745), C. deanei (ATCC 30255), C. luciliae (ATCC 14765), C. oncopelti (ATCC 12982), C. ricardoi (Sibajev et al. 1993), C. guilhermei (Soares et al. 1986), Leishmania mexicana (MNYC/BZ/62/M379), Trypanosoma cruzi (Y strain), Phytomonas davidii (ATCC 30287) and Endotrypanum schaudinni (IM 217). These strains were cryopreserved as well as maintained by serial passage in liquid cultures in the

Dept. of Protozoology of the Oswaldo Cruz Institute and at the Institute of Biophysics at the Federal University of Rio de Janeiro (UFRJ) and were kindly made available for the present study.

2. Extraction of kDNA and restriction digests.

Extraction and purification of kDNA and analysis of restriction profiles (schizodeme analysis) were carried out as previously described (Gonçalves et al. 1984; Pacheco et al. 1990a). The kDNA preparations were digested with the restriction enzymes Rsa I, Msp I and Hae III at 37°C for 3 h in the appropriate buffers. The restriction fragments were separated by electrophoresis in 5-10% linear gradient polyacrylamide gels and stained with ethidium bromide.

3. Hybridizations.

DNA samples were, prior to application in Nylon membranes (Zetaprobe, Biorad), denatured in 0.4N NaOH and applied under low vacuum using a manifold (Schleicher & Schuell Inc., Keene, NH, USA). kDNA was radiolabeled with alpha 32P dATP using the random primer method (FIOCRUZ, Brazil) to a specific activity of about 10^9 dpm per μg. Hybridization conditions were as follows: membranes were presoaked for 2 h in 0.6M NaCL, 0.12M Tris, 4mM EDTA, 0.75% non-fat milk and 0.1% SDS at room temperature, and then in hybridization solution (the same components above plus 50% formamide and 6% PEG) for 40 min at 42°C prior to the addition of the denatured probe. Hybridization was carried out at 42°C overnight and membranes were washed initially in 2X SSC for 20 min at room temperature and then 3 times in 0.1X SSC, 0.5% SDS at 56°C for 30 min. Autoradiography was done overnight with an intensifying screen at -70°C.

4. Zymodeme analysis.

Zymodeme analysis was performed as described previously (Momen et al. 1985). Eleven different enzymes were analyzed, respectively, PGM (2.7.5.1), G6PdH (1.1.1.49), 6PGdH (1.1.1.44), GPI (5.3.1.9), ME (1.1.1.40), FUM (4.2.12.1), PEP1 (3.4.11.1), EST (3.1.1.1), IDH (1.1.1.42), MDH (1.1.1.37), and ACON (4.2.1.30).

5. Numerical (cladistic) analysis.

The data obtained from isoenzymes electrophoresis in agarose gels permitted a phylogenetic analysis of the samples, using the algorithm of Wagner. The data matrix was based on the presence and absence of bands, and the most parsimonious tree with the smallest number of steps was constructed using the MIX program from the Phylip Software Package (Felsenstein 1989).

6. Computer analysis of kDNA minicircle sequences.

Sequence analysis and alignment were carried out on an VAX MX-750 computer using the programs Compare, Dotplot, Bestfit, Gap, Pretty and Pileup from the Wisconsin Genetic Computer Group (GCG) package (Devereux et al. 1984). The Crithidia kDNA minicircle sequences used in this study were as following: C. fasciculata 11 and 12: C. fasciculata strain CF-C1 major sequence class (complete minicircle sequence, 2515 bp, GB accession number M19266, clones pCFK128 and 120 respectively, Birkenmeyer et al. 1985, Sugisaki and Ray 1987). C. fasciculata 21 and 22: C. fasciculata isolate (partial minicircle sequence, clone pmcfl, Maslov et al. 1988). C. fasciculata 31 and 32: C. fasciculata strain CF- C1 minor sequence class (partial minicircle sequence, clone pCFK3, Sugisaki and Ray 1987). C. fasciculata 41 and 42: C. fasciculata strain CF-C1 minor sequence class (partial minicircle sequence, clone pCFK23, Sugisaki and Ray 1987). C. oncopelti strain SO68.4 (complete or nearly complete minicircle sequence, 1848 bp, GB accession number X17109, Pestov et al. 1990). Other published C. fasciculata minicircle fragments contained bent regions but no conserved sequences (M13681, Kitchin et al. 1986, Maslov et al. 1988).

RESULTS AND DISCUSSION

Schizodeme analysis.

Figure 1 shows the diversity of the kinetoplast genotypic pattern among different species of Crithidia as revealed by kDNA restriction analysis. Each species tested presented unique and very distinct profiles, thus suggesting the status of separate taxa. A more complex profile was observed in an isolate recently characterized as a new species C. ricardoi (Sibajev et al. 1993).

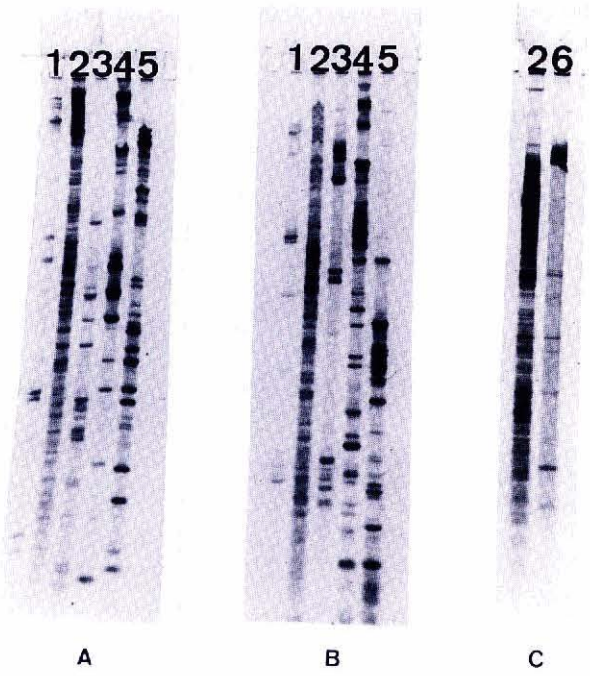

Fig. 1 Electrophoresis in a 5-10% polyacrylamide gradient gel showing kDNA restriction fragments of different Crithidia species after digestion with the restriction enzymes Rsa I (A), Msp I (B) and Hae III (C). 1) C. fasciculata, 2) C. ricardoi, 3) C. deanei, 4) C. luciliae, 5) C. oncopelti, 6) C. guilhermei. All species were also treated with Hae III (results not shown).

kDNA cross-hybridization.

Cross-hybridization experiments between kDNA of the different species yielded different intensities of hybridization signals, reflecting most probably degrees of nucleotide sequence homology, as shown in figure 2. If kDNA minicircle sequence homology reflects an evolutionary closer relationship, such would be the case with the species Crithidia fasciculata, C. luciliae, C. ricardoi and C. guilhermei. Surprisingly, kDNA from C. oncopelti and C. deanei did not hybridize with kDNA from any other species analyzed in this Crithidia group, indicating a much greater divergence from these two species. Moreover, no hybridization signal is detected using labeled kDNA from representative species of Crithidia when probing kDNA from other genera of monogenetic and digenetic trypanosomatids such as Herpetomonas, Blastocrithidia, Leishmania, Trypanosoma, Phytomonas and Endotrypanum.

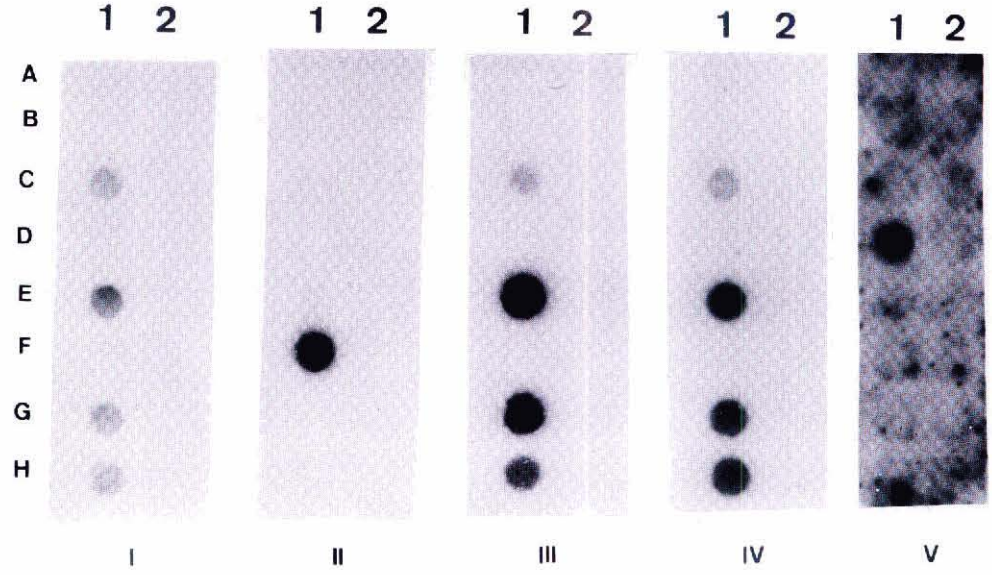

Fig.2 Dot blot hybridization showing the results of cross- hybridizations against distinct monogenetic and digenetic trypanosomatids using total kDNA of Crithidia species as probes.
Probes: C. fasciculata (I), C. oncopelti (II), C. ricardoi (III), C. guilhermei (IV) and C. deanei (V).

1A- Herpetomonas samuelpessoai
1B- Blastocrithidia culicis
1C- Crithidia fasciculata
1D- C. deanei
1E- C. luciliae
1F- C. oncopelti
1G- C. ricardoi
1H- C. guilhermei
2A- Leishmania mexicana
2B- Trypanosoma cruzi
2C- Phytomonas davidii
2D- Endotrypanum schaudinni

Numerical analysis from zymodeme data.

Figure 3 shows the isoenzymograms obtained when the Crithidia samples where analyzed by agarose gel electrophoresis.

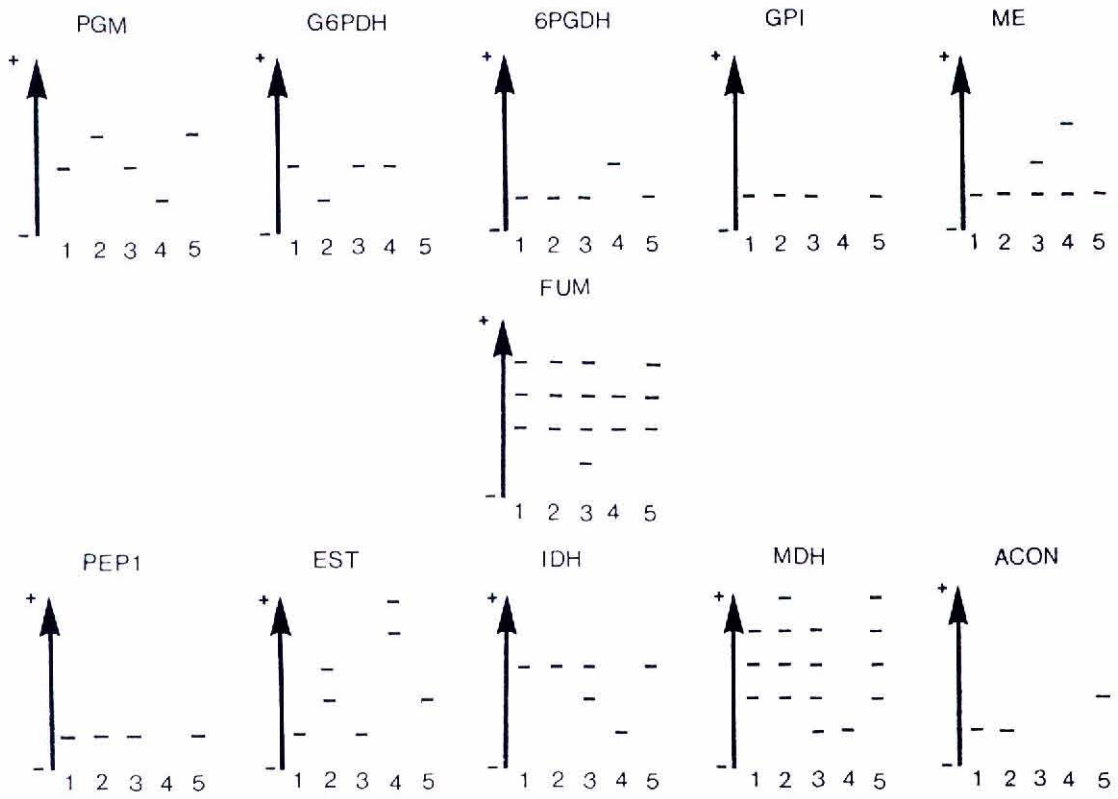

Fig. 3 Diagram showing agarose gel electrophoresis for 11 enzymatic systems. 1) C. ricardoi, 2) C. fasciculata, 3) C. deanei, 4) C. oncopelti, 5) C. luciliae.

Figure 4 shows the cladogram obtained using isoenzyme electrophoresis data from 11 different enzymes, as indicate in Materials and Methods. One can observe that Crithidia oncopelti forms a separate group from the other species, showing paraphyletic properties. C. fasciculata, C. luciliae, C. deanei and C. ricardoi form a monophyletic group. The isolate C.sp was recently typed as a new species C. ricardoi (Sibajev et al. 1993).

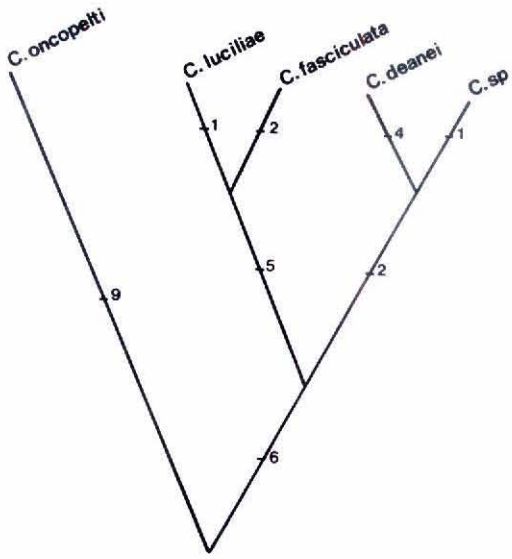

Fig.4 Cladogram obtained using isoenzyme electrophoretic results. The unrooted Wagner tree using, 30 steps, shows phylogenetic relationships between the 5 Crithidia species.

Computer analysis of kDNA minicircle sequences.

Figure 5 shows dotplot comparisons between two C. fasciculata minicircles, showing significant homology and the presence of two conserved regions (mini repeats) in each molecule (regions 11, 12 and 21, 22). On the other hand, comparison of the C. oncopelti minicircle nucleotide sequence with the most complete C. fasciculata sequence shows a lack of sequence homology. This region and the corresponding C. fasciculata repeats have been aligned, as shown in figure 6.

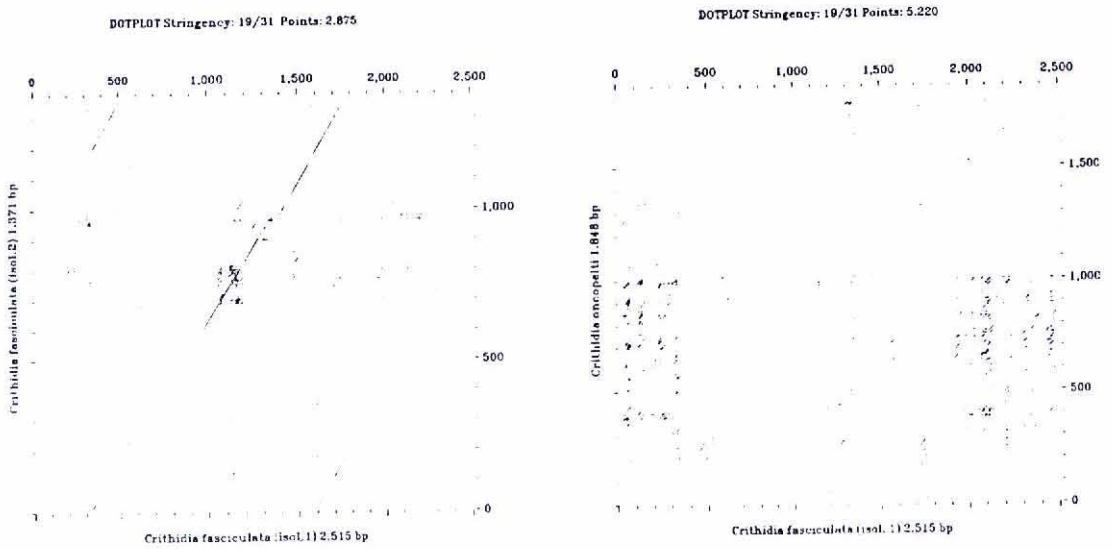

Fig.5 Compare-dotplot analysis using a window of 31 nt and a proportional match of 19/31. A) comparison between two minicircles from different C. fasciculata isolates (M19266 versus Maslov et al. 1988), B) comparison between a C. fasciculata minicircle (M19266) and a C. oncopelti minicircle sequence (X17109).

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                    1                                                  50
         Consensus  GGGBGGTTTC SGGGTGCCCG CGAAATATCA GAAATGGTCT CGGGTAGGGG
C. fasciculata 11   ...GT.G..T G......... .......... .......... ..........
C. fasciculata 12   ..CT.A.... C...GT.... .....A.... ....C..... ..........
C. fasciculata 21   ...T.....G G......... .......... ....C..... ..........
C. fasciculata 22   ..TC.A.... C.A.GT.... .......... .......... ..........
C. fasciculata 31   ...TC...CT G......... .......... ....C-.... .-........
C. fasciculata 32   ..TC...... C...GT.... .......... .......... ..........
C. fasciculata 41   .A.C.AGC.. C....T.... .......... ....C..... ..........
C. fasciculata 42   T.AG..G..T G.....-T.. .......... .......--. .CT...-...
C. oncopelti        T..G...... T...G.T..C AA...GT.TG .GGT.TTGG. TTT.......

                    51                                                 100
         Consensus  CGTTCTGCGA AAATCGACTT TTGATACAGG AAATCCCGTT CAAAAATGGS
C. fasciculata 11   .......... .......... .......... .......... .......G.C
C. fasciculata 12   .......... .......... .......... .......... .......-CG
C. fasciculata 21   .......... ......GG.. .......... .......... .......G.C
C. fasciculata 22   .......... .......... .......... .......... .......-CG
C. fasciculata 31   .......... .......... .......... .......... .......G.C
C. fasciculata 32   .......... .......... .......... .......... .......-CA
C. fasciculata 41   .......... ......GG.. .......... .......... .......CCG
C. fasciculata 42   T......... .......... .........-. .......... .......T.C
C. oncopelti        .....AA..T TTT...GGGG .CT......A CTT....C.. A.C.TT.T.G

                    101                                                150
         Consensus  WGATTTTTTC AATTTTGGAG GCAAACTGGG GATTTCCGGG GTTGGTGTAG
C. fasciculata 11   A.-....C.. G......... ..TCGGCT.. .......... ..........
C. fasciculata 12   T......... .......... .......... .......... ..........
C. fasciculata 21   A.-....C.. ......T... ....GT.... .......... ..........
C. fasciculata 22   T......... .......... .T.G...... ......A... ........G.
C. fasciculata 31   A.-....C.. ......T... ....GT.... ...C.G.... ..........
C. fasciculata 32   G.T.....CT ......T... ...-...... .......... ..........
C. fasciculata 41   T......... ....G..... ......C... .......... ..........
C. fasciculata 42   -......... G......... .......... .......... ..........
C. oncopelti        CC.....AG. GG...GC.GA CG....CT.. CG...GGA.. ........TA

                    151                                  185
         Consensus  TATTCYGGGG TYBBGGGSGG GTBTTBBGGG GTTTY
C. fasciculata 11   .CA.TCCT.. GTCC...C.. ..C.GGC... .G..C
C. fasciculata 12   ....TCT... .CCG.---.. ..CC.GA... ...CC
C. fasciculata 21   G....C.... GTCT...G.T CGT..CC... AC..T
C. fasciculata 22   .....T.... .CGG.-ACC. .GC.CCT... C..CC
C. fasciculata 31   .....T.... GTCT...G.T C.GGATCT.. ..C.T
C. fasciculata 32   .....T.... .CGGA..CT. ..T..CC... T..CC
C. fasciculata 41   .....CT... GGTC...T.. ..C.GGGA.. ....T
C. fasciculata 42   ...A.T.... .TTT...CC. .AG..TT... ....T
C. oncopelti        A..AGG.T.. .GTTTTAG.. ..TC.GG... T.CCG
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Fig.6 Multiple sequence alignment between the C. fasciculata and C. oncopelti minicircle conserved regions, numbered as in Material and Methods. A consensus sequence was drawn at the top using UPAC ambiguity codes where necessary. Dots indicate homology to the consensus sequence, dashes indicate deletion/insertions. The "universal 12-mer sequence" is underlined.

In this study we showed the genotypic diversity within the genus Crithidia using a number of different methods. Firstly, according to our hybridization experiments, kDNA minicircles (conserved regions) did not cross-hybridize between, respectively, C. oncopelti and C. deanei and the other Crithidia species analyzed. Some of this data were corroborated, by comparing previously published minicircle sequences, by multiple sequence alignment between the C. fasciculata and C. oncopelti minicircle conserved regions. To our knowledge eight C. fasciculata and one C. oncopelti minicircle complete or partial nucleotide sequence containing conserved sequences have been reported in the literature thus far (Rezepkina et al. 1984; Maslov et al. 1988; Pestov et al. 1990). The conserved region, which show about 80-100% intra-species homology are thought to contribute primary to the intra and inter-species kDNA hybridization signals, while variable regions probably only contribute where certain minicircle sequence classes are common to isolates from the same and closely related species. In C. fasciculata two conserved sequence regions have been identified in different kDNA minicircle classes, each containing a single copy of the 12 bp universal sequence. Molecules from the major class in isolate CF-C1 (Sugisaki and Ray 1987) are almost homogeneous in sequence, with a few point mutations distributed equally throughout the conserved and variable regions. In the multiple sequence alignment carried out in this study (see Fig.6), mini repeats from the minor sequence classes (regions 31, 32, 41 and 42) or from a different isolate (regions 21 and 22) seem to be more or less equally related. However, the minicircle molecule from C. oncopelti shows only one region corresponding to a mini repeat, based on the presence of a GGGGTTGGTGTA like sequence, lacking significant homology to the corresponding C. fasciculata sequence. We conclude that the C. oncopelti sequence seems only distantly related to the C. fasciculata sequences, an observation which would become even more apparent if we would consider only the central, most conserved part of the multiple alignment.

Secondly, cross-hybridization among kDNA from the species C. fasciculata, C. luciliae, C. guilhermei and C. ricardoi indicate a stronger sequence homology between minicircles molecules of these species. The stronger reaction obtained when C. luciliae was hybridized with probes from other species (Fig.2) is probably due to a greater quantity of kDNA spotted on the membrane. Although these species can be readily distinguished by the difference observed in their kDNA restriction patterns, thus supporting their status as separate taxa, they conserve overall sequence homology suggesting a close phylogenetic relationship. The genotypic heterogeneity in the genus Crithidia observed in this study parallels the study of Kolesnikov et al. (1990) who also found, through kDNA analyses, considerable heterogeneity in the genus Leptomonas. The picture that emerges from this diversity, also reinforced by Camargo et al. (1992), is that Crithidia as well as Leptomonas do not appear to be precise genera but rather groups of species complexes.

The cladistic analysis based on the results of isoenzyme electrophoresis showed similarity between C. deanei and C. ricardoi which formed a monophyletic group with C. luciliae and C. fasciculata. On the other hand, Camargo et al. (1990) based on biological and biochemical evidence suggest that C. deanei is a very atypical species and does not fit in either Crithidia or Herpetomonas genera. Some of its morphological and biochemical features are due to the presence of an endosymbiont in its cytoplasm (see Camargo et al. 1990).

Our results also corroborate the reports from Lima et al. (1982) and Motta et al. (1991), showing that C. fasciculata, C. luciliae and C. guilhermei are grouped, with C. oncopelti remaining outside the group. Although the presence of bacterium-like endosymbionts in the cytoplasm of both C. deanei (Mundim et al. 1974) and C. oncopelti (Newton 1957) can be considered a synapomorphy, as has been suggested by Motta et al. (1991), this feature could also be a result of a parallel evolution. It is not at all unexpected that the genus Crithidia comprises organisms of widely diverse nature, as thus far no fine taxonomic and phylogenetic studies with several different and sensitive tools have been undertaken. Analyses of endosymbiont-free lineages together with an enlarged number of isolates will

further contribute to a better assessment of the phylogenetic relationships within this and other genera of monogenetic trypanosomatids.

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