

RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) ANALYSIS OF *Lutzomyia longipalpis* LABORATORY POPULATIONS

Edelberto S. DIAS (1), Consuelo L. FORTES-DIAS (2), John M. STITELER (3), Peter V. PERKINS(3) & Phillip G. LAWYER (3)

SUMMARY

The phlebotomine sand fly *Lutzomyia longipalpis* has been incriminated as a vector of American visceral leishmaniasis, caused by *Leishmania chagasi*. However, some evidence has been accumulated suggesting that it may exist in nature not as a single but as a species complex. Our goal was to compare four laboratory reference populations of *L. longipalpis* from distinct geographic regions at the molecular level by RAPD-PCR. We screened genomic DNA for polymorphic sites by PCR amplification with decamer single primers of arbitrary nucleotide sequences. One primer distinguished one population (Marajó Island, Pará State, Brazil) from the other three (Lapinha Cave, Minas Gerais State, Brazil; Melgar, Tolima Department, Colombia and Liberia, Guanacaste Province, Costa Rica). The population-specific and the conserved RAPD-PCR amplified fragments were cloned and shown to differ only in number of internal repeats.

KEYWORDS: RAPD-PCR; Fingerprintings; Sand fly; *Lutzomyia*; Genotyping

INTRODUCTION

Classical or alpha taxonomy of most organisms relies on detectable differences in morphologic characters between species. Among some vectors of disease, many "species" indistinguishable by currently used morphological characters are now recognized to contain reproductively isolated populations which may have very different biological characteristics.

The vectors of the leishmaniasis in humans and other mammalian hosts are the phlebotomine sand flies. As observed for other vectors, difficulties in the taxonomy of these insects are due mainly to similar morphology and the existence of sibling species²⁰. These difficulties in identification associated with a wide range of causative parasites, pose problems in deciding with certainty which sand fly is responsible for the transmission of a particular species of *Leishmania* in a given locality⁷. Differentiation and characterization of those populations are fundamental for epidemiological studies as some may act as important vectors of the *Leishmania* parasite, while others may be unable to transmit the parasite due to differences in their behavior or physiology.

In the last few years, several modern techniques have been introduced to complement the classical taxonomy data. The use of DNA probes is also becoming established as one important taxonomy tool, mainly for the identification of vector sibling

species^{3,13}, although requiring a prior knowledge of specific region in their genomic DNAs. More recently, another molecular technique, random-amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) was developed¹⁹. This approach is independent of previous DNA sequence information, involving the amplification via PCR of random segments of genomic DNA using a series of single primers of arbitrary nucleotide sequences. RAPD-PCR fingerprintings are generated by analysing the amplification products by electrophoresis on agarose gels. Polymorphic RAPD fragments can be used as diagnostic markers or, after cloning and sequencing, they can generate specific primers to be used in diagnostic PCR⁶.

The phlebotomine sand fly *Lutzomyia longipalpis* has been incriminated as the vector of American visceral leishmaniasis⁴ caused by *Leishmania chagasi*^{7,8,10}. The geographic distribution of *L. longipalpis*, extending from Mexico to Southern Brazil, coincides in general with the geographic distribution of the disease. *L. longipalpis* was found in all known foci of visceral leishmaniasis and is a proven vector in some of them⁵. There is strong evidence that this phlebotomine sand fly may exist in nature as a complex of species^{9,16}. The aim of our work was to study four geographic laboratory populations of *L. longipalpis* for the existence of DNA polymorphisms by RAPD-PCR.

(1) Laboratório de Leishmanioses, Centro de Pesquisas "René Rachou", FIOCRUZ, Belo Horizonte, MG, Brasil.

(2) Centro de Pesquisa e Desenvolvimento, Fundação Ezequiel Dias, Belo Horizonte, MG, Brasil.

(3) Department of Entomology, Walter Reed Army Institute of Research, Walter Reed Army Medical Center, Washington, D.C., USA.

Correspondence to: Dr. Edelberto Santos Dias, Laboratório de Leishmanioses, Centro de Pesquisas "René Rachou", Avenida Augusto de Lima 1715, 30190-002 Belo Horizonte, MG, Brazil.

MATERIAL AND METHODS

Source of genomic DNA – Genomic DNA was isolated by standard procedures¹¹ from four well characterized sand fly reference populations of *L. longipalpis* from established colonies maintained at the Walter Reed Army Institute of Research (WRAIR). Two populations originated from distinct geographic regions in Brazil, Lapinha Cave, Minas Gerais State (approximate coordinates 20°0'S, 44°0'W) and Marajó Island, Pará State (approximate coordinates 1°0'S, 49°0'W), one from Colombia, near Melgar, Tolima Department (4°11'N, 74°18'W) and one from Costa Rica, near Liberia, Guanacaste Province (10°37'N, 85°26'W). Briefly, male adult sand flies in groups of ten were disrupted together with a mortar and pestil and lysed in 50 mM Tris-HCl, 100 mM NaCl, 50 mM EDTA, 0.5% SDS pH 8.0. The extracts were digested with 1 mg/ml of RNase A for 30 min at 37° C, followed by 50 µg/ml proteinase K for 3 hr at 55° C. The DNAs were phenol/chloroform extracted and concentrated by ethanol precipitation. After resuspension in 50 µl TE buffer pH 8.0, the DNA concentrations were estimated by comparison with known standards by agarose-ethidium bromide method (data not shown).

Amplification conditions – PCR amplifications were performed under conditions previously established in a 100 µl reaction volume of 10 mM TRIS-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.001% gelatin containing 10 ng of sand fly genomic DNA, 2.5 units of AmpliTaq DNA polymerase (Perkin-Elmer/Cetus), 0.2 µM of a single RAPD-PCR primer and 200 µM each of dATP, dCTP, dGTP and dTTP. Control reactions were run omitting genomic DNA. Decamer primers were arbitrarily selected for this study (Kit#G, Operon Technologies, Alameda, CA). Reactions were overlaid with 50 µl of mineral oil and amplified by cycling in a Perkin Elmer Cetus DNA Thermal Cycler 480 for 35 cycles of 30 sec at 94° C, 1 min at 34° C, and 2 min at 72° C, prior to soaking at 4° C.

RAPD-PCR fingerprintings – 40 µl of each of the RAPD-PCR products were loaded on a 1.5% low-melting agarose gel in TAE buffer (0.04 M TRIS-acetate and 0.001 M EDTA) and electrophoresis was run for 3 hr at 80 V. Hae III restriction fragments of ϕ X174RFI DNA were used as molecular weight markers. DNA bands were stained with ethidium bromide and visualized using a U.V. transilluminator.

Cloning of RAPD-PCR bands – RAPD-PCR bands of interest were extracted from a 1.5% low-melting agarose gel using the Magic PCR™ kit (Promega, Madison, WI) and reamplified by PCR with the same random primer used for the primary amplification. The secondary PCR products were cloned with the TA Cloning™ kit version 1.0 (Invitrogen, San Diego, CA). Ligation to pCR1000™ vector and transformation of *E. coli*-competent cells were performed according to the manufacturer's instructions.

Screening of recombinants and plasmid DNA isolation

– The presence and size of inserts in the recombinants were checked by direct PCR amplification of the DNA from bacterial

colonies using M13 reverse (-48) 24-mer (5'-AGCGGATAACAATTTTCACACAGGA-3') and M13 forward (-40) 17-mer (5'-GTTTCCCAGTCACGAC-3') primers (New England Biolabs, Beverly, MA). The amplification products were analysed by electrophoresis in a 1.0 % agarose gel in TAE buffer. Selected recombinants were propagated by growing individual clones in LB media containing kanamycin (50 µg/ml) until log phase. Plasmid DNA was isolated by cesium chloride gradient or by the Wizard DNA purification kit (Promega, Madison, WI).

Sequencing of recombinant DNA – DNA was sequenced according to the dideoxy-chain termination method¹⁴ using sequenase® T7 DNA polymerase version 2.0 (United States Biochemical Co.) and appropriate internal and external primers. DNA sequencing runs were analyzed using the software MacVector from IBI, Kodak Co. (New Haven, CT). Homology comparison was performed in the database at NCBI (National Center for Biotechnology Information, NIH, Bethesda, MD) using the basic local alignment search tool (BLASTN) developed by ALTSCHUL et al.².

RESULTS

Among all the primers tested for the generation of RAPD-PCR fingerprintings from the genomic sand fly DNA, OPG-01 (5'-CTACGGAGGA-3') was the only one to generate a polymorphic fragment (about 620bp) unique for population B, besides a non-polymorphic band for all four populations of about 560bp. All the other decamer tested produced only non-polymorphic bands. Figure 1 shows the typical RAPD-PCR fingerprinting obtained with male DNA amplified with the primer OPG-01. Two different samples from each of the four populations of *L. longipalpis* were loaded in a single run to exemplify the profiles obtained. The same profiles were obtained on several independent occasions. No detectable band was seen in control reactions missing genomic DNA (data not shown).

The 620bp fragment was isolated and cloned into a suitable vector as described. After PCR screening for the presence of the insert, four recombinant clones were obtained (Fig. 2). Their sizes were estimated as 760bp for clones 1 and 3, and 820bp for clones 2 and 4, respectively. These sizes roughly correspond to the sum of the lengths of the non-polymorphic (560bp) and polymorphic (620bp) bands plus the internal vector sequence flanking the insert site (194bp).

The inserts in clones 1 and 4 were subjected to DNA sequencing. Figure 3 presents both nucleotide sequences for comparison. The non-polymorphic band, with estimated size of 560bp, is composed of 565 nucleotide pairs. It displays a 35bp repeat at the beginning of its sequence that starts (nucleotides 1-10) and ends (nucleotides 26-35) with the OPG-01 sequence. The 35bp repeat is present in inverted position at the end of the DNA sequence. The polymorphic band is composed of 626 nucleotides and contains additional 26bp plus another 35bp inverted repeat when compared to the non-polymorphic fragment.

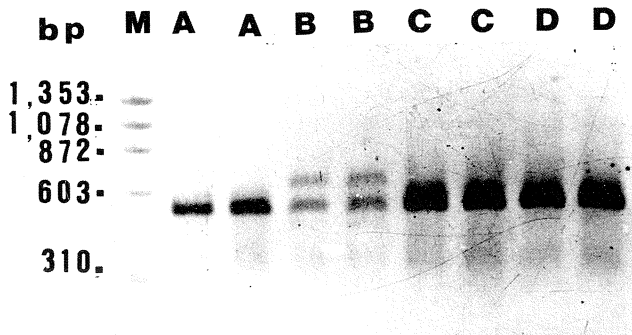


Fig. 1 - RAPD-PCR fingerprintings on 1.5% agarose gel after amplification of genomic DNA. Two different samples from the four populations of *L. longipalpis* were loaded onto the same gel to exemplify the profile generated by amplification of *L. longipalpis* DNA with OPG-01. Origin of populations: A - Lapinha Cave, Minas Gerais State - Brazil; B - Marajó Island, Pará State- Brazil; C - Melgar, Tolima Department - Colombia; D - Liberia, Guanacaste Province- Costa Rica. Lane M represents the molecular weight markers.

DISCUSSION

RAPD-PCR analysis has been previously used for genetic mapping and population studies^{6,17}. Recently, it was successfully applied in the distinction of two sympatric species of sand fly in Venezuela¹, as well as in the identification of unknown specimens of *Anopheles (Nyssorhynchus) albitarsis*, an important vector of malaria parasites constituted by a complex of cryptic species¹⁸.

The use of RAPD-PCR analysis presents the advantages of a simple, fast, effective and powerful technology. However, recent reports have shown that the amplification conditions have to be optimized and very well defined to allow for reproducible fingerprintings^{12,15,21}. In our study, the RAPD-PCR fingerprintings were consistently reproducible for several pools of male DNA from the four populations.

Although resulting in a simple band pattern, amplification of *L. longipalpis* DNA by OPG-01 was able to discriminate population B (Marajó Island, Brazil) from A (Lapinha Cave, Brazil), C (Tolima, Colombia) and D (Guanacaste, Costa Rica) as the 626bp fragment is amplified only in specimens from population B.

The screening of recombinants demonstrated the insertion in the vector of not only the 620bp band, but also of the lowest 540bp band, common to all four *Lutzomyia* populations. The cloning technique used here comprises an initial PCR step in the presence of OPG-01, using the 620bp as template. As shown by the nucleotide sequence determined for the 620bp and 560bp fragments (Fig. 3), the latter is contained in the 620bp band. This would explain the presence of both bands inserted into the vector later (Fig. 2).

The real sizes of the 620bp and 560bp fragments, based on their nucleotide sequence, are 626bp and 565bp, respectively.

Both sequences contain a 35bp repeat at the 5' end that is inversely repeated at the 3' end of the 565bp clone. As this 35bp repeat starts and ends with motifs that are complementary to the OPG-01, one would expect hybridization of OPG-01 at both sites. However, this event did not happen during the experiments carried out so far. No fragments have been obtained corresponding to priming at the end of the 35bp repeat. The 626bp clone corresponds to the 565bp sequence followed by

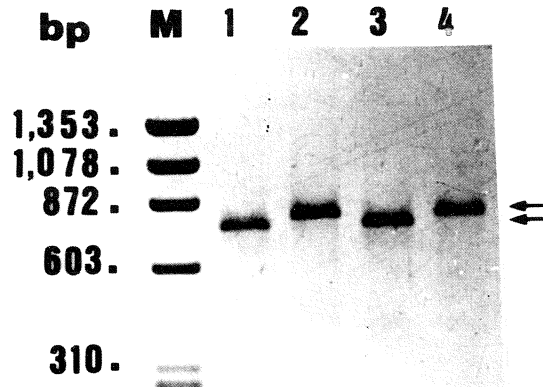


Fig. 2 - Agarose gel analysis of the amplification products obtained by PCR of selected recombinant colonies with M13 reverse (-48) and forward (-40) primers. Lanes M and 1-4 represent molecular markers and clones 1 to 4, respectively.

	10	20	30	40	50	60	70
<u>CTACGGAGGA</u>	<u>CAACACCGGG</u>	<u>TACGCTCTAGC</u>	<u>GAGGA</u> GAAGA	TGGCAGAAAA	AAATAAACTT	TCACTCACGC	
<u>CTACGGAGGA</u>	<u>CAACACCGGG</u>	<u>TACGCTCTAGC</u>	<u>GAGGA</u> GAAAGA	TGGCAGAAAA	AAATAAACTT	TCACTCACGC	
	80	90	100	110	120	130	140
AATTTGCTTT	CGACGTGAA	TTTCCGCGAC	TGATTTGCTA	TTTTCTTCAG	ACAATGAATG	ATAATTTGGT	
AATTTGCTTT	CGACGTGAA	TTTCCGCGAC	TGATTTGCTA	TTTTCTTCAG	ACAATGAATG	ATAATTTGGT	
	150	160	170	180	190	200	210
AGTATATAGA	ATTGAAAAGT	GCAAAGAGTA	GCGAACGGGA	TCATGTACA	CTGTGGAAG	GTACAAAATA	
AATTTGCTTT	CGACGTGAA	TTTCCGCGAC	TGATTTGCTA	TTTTCTTCAG	ACAATGAATG	ATAATTTGGT	
	220	230	240	250	260	270	280
AAAGTTAATG	TGGATGATGT	AGGTATATAT	AAAAACGGTC	GAGGCTGTTT	TAAATTGGGA	TAGGCTCTTT	
AAAGTTAATG	TGGATGATGT	AGGTATATAT	AAAAACGGTC	GAGGCTGTTT	TAAATTGGGA	TAGGCTCTTT	
	290	300	310	320	330	340	350
CTCACGTGCT	GTGATTTTTT	TTTTTGTAGT	TTGAATCATT	TGAATAGAAA	AAGGGAATAA	GGGGTCATCG	
CTCACGTGCT	GTGATTTTTT	TTTTT-GAGT	TTGAATCATT	TGAATAGAAA	AAGGGAATAA	GGGGTCATCG	
	360	370	380	390	400	410	420
TCATCTTTTT	GGATGGATAG	TTACTTTTCC	GATAGATTCC	TTTGGCCATT	CCCCACAATG	TCCGGCAAC	
TCATCTTTTT	GGATGGATAG	TTACTTTTCC	GATAGATTCC	TTTGGCCATT	CCCCACAATG	TCCGGCAAC	
	430	440	450	460	470	480	490
AAGGTGGCTT	TTACCACTTA	ATGTGTGGCA	TCATATCATA	CTTTGGTTTG	CTGACACCAG	TGCCATCATT	
AAGGTGGCTT	TTACCACTTA	ATGTGTGGCA	TCATATCATA	CTTTGGTTTG	CTGACACCAG	TGCCATCATT	
	500	510	520	530	540	550	560
GAATGCACTA	TTAATCCGGT	CCGTATCACT	TTTCACAATG	<u>TCCTCCGTAG</u>	<u>ACGTACCCGG</u>	<u>GGTTGTCCTC</u>	
GAATGCACTA	TTAATCCGGT	CCGTATCACT	TTTCACAATG	<u>TCCTCCGTAG</u>	<u>ACGTACCCGG</u>	<u>GGTTGTCCTC</u>	
	570	580	590	600	610	620	
<u>CGTAGACCGG</u>	<u>TCCGTATCAC</u>	<u>TTTTTCACAAT</u>	<u>CTCTCCGTAG</u>	<u>GACGTACCCG</u>	<u>GTGTTGTCCT</u>	<u>CCGTAG</u>	
<u>CGTAG</u>							

Fig. 3 - Nucleotide sequence of the polymorphic (626bp) and non-polymorphic (565bp) RAPD-PCR bands generated with *L. longipalpis* DNA and OPG-01. OPG-01 nucleotide sequence is shown in bold type and the 35bp repeat is underlined.

26bp plus another 35bp at the 3' end. We expected the 626bp clone to contain a short specific region not present in the 565bp band, making it possible the development of a specific probe for population B. Unfortunately, the extra 26bp in the 620bp clone occurs also in the 506-530 region of both clones, thus excluding this possibility.

Structural data base comparison with the 626bp nucleotide sequence showed 68% identity with "gb[U28944]CELCL18A3" from *Caenorhabditis elegans* cosmid in a fragment 60bp long. The significance of this finding, if any, is unknown.

The existence of high level of genetic divergence among three out of the four laboratory populations of *L. longipalpis* used here (A, C and D), has been described elsewhere⁹. Population from Marajó Island (B) has not been used in their study. Those authors have found a number of diagnostic loci that discriminates populations A, C and D through isozyme analysis. Besides, sterility has been observed in male progeny from all intercolony crosses upon experimental hybridization. Those data provided strong evidence that *L. longipalpis* is not a single species, but exists in nature as a complex of at least three distinct species. Unfortunately, no polymorphisms were detected with the RAPD primers used in our study that would distinguish between populations A, C and D. However, there is a difference between the population from the Marajó Island (B) and the other three. This finding is not surprising considering the geographic isolation of Marajó Island (population B) from the American continental area (populations A, C and D). The epidemiological and/or vectorial significance of this finding remain to be determined when more data is available.

It should be emphasized that the populations used here were laboratory-reared. Further studies would be necessary using field specimens from the same four populations as well as testing of other series of random primers and use of female genomic DNA (not available at this time) to clarify the question of genetic divergence from the Marajó Island and continental populations of *L. longipalpis*.

RESUMO

Análise de DNA em populações de laboratório de *Lutzomyia longipalpis* através de amplificação ao acaso de polimorfismos (RAPD-PCR)

O flebotomíneo *Lutzomyia longipalpis* tem sido incriminado como vetor da leishmaniose visceral americana, causada pelo protozoário *Leishmania chagasi*. Entretanto, tem-se acumulado evidências que sugerem a existência de um complexo e não apenas uma espécie de *L. longipalpis* na natureza. Nosso trabalho teve como objetivo comparar, ao nível molecular, quatro populações de *L. longipalpis* de referência, utilizando espécimes criados em laboratório, provenientes de regiões geograficamente distintas, através de RAPD-PCR (reação de polimerase em cadeia com amplificação por iniciadores ao acaso). Para isso, o DNA genômico de grupos de flebotomíneos foi amplificado com iniciadores decaméricos únicos com sequência de nucleotídeos arbitrária, na

tentativa de se detectar sítios polimórficos. Apenas um dos iniciadores testados foi capaz de distinguir uma das populações (Ilha de Marajó, PA, Brasil) das outras três (Gruta da Lapinha, MG, Brasil; Melgar, Tolima, Colômbia e Libéria, Província de Guanacaste, Costa Rica). Os fragmentos população-específico e os conservados, amplificados por RAPD-PCR, foram clonados e sequenciados, mostrando que a diferença entre eles deve-se apenas à uma diferença no número de repetições internas.

ACKNOWLEDGMENTS

This work was partially supported by CNPq (PDE# 201125/90, PDE# 201124/90) and FAPEMIG (CBS 305/95), Brazil. We gratefully thank Dr. Yuan Lin from the Laboratory of Allergenic Products of the Food and Drug Administration (Bethesda, MD, USA) for the DNA sequencing facilities.

REFERENCES

- ADAMSON, R.E.; WARD, R.D.; FELICIANGELI, M.D. & MAINGON, R. - The application of random amplified polymorphic DNA for sand fly species identification. *Med. vet. Entomol.*, 7: 203-207, 1993.
- ALTSCHUL, S.F.; GISH, W.; MILLER, W.; MYERS, E.W. & LIPMAN, D.J. - Basic local alignment search tool. *J. Molec. Biol.*, 215: 403-410, 1990.
- CRAMPTON, J.; KNAPP, T. & WARD, R. - DNA probes for vector taxonomy. In: HART, D.T. *Leishmaniasis: the current status and new strategies for control*. New York, Plenum Press, 1989. p. 957-964. (NATO ASI Series A, Life Sciences, v. 163).
- DEANE, L.M. - Leishmaniose visceral no Brasil. Estudos sobre reservatórios e transmissores realizados no Estado do Ceará. Rio de Janeiro. Serviço Nacional de Educação, 1956.
- GRIMALDI, G.; TESH, R.B. & MCMAHON-PRATT, D. - A review of geographic distribution and epidemiology of leishmaniasis in the World. *Amer. J. trop. Med. Hyg.*, 40: 687-725, 1989.
- HADRYN, H.; BALICK, M. & SCHIERWATER, B. - Applications of random amplified polymorphic DNA (RAPD) in molecular biology. *Molec. Ecol.*, 1: 55-63, 1992.
- KILLICK-KENDRICK, R. - Phlebotominae vectors of the leishmaniasis: a review. *Med. vet. Entomol.*, 4: 1-24, 1990.
- LAINSON, R. & SHAW, J.J. - Evolution, classification and geographical distribution. In: PETERS, W. & KILLICK-KENDRICK, R., ed. *The leishmaniasis in biology and medicine*. London, Academic Press, 1987. v.1, p. 1-120.
- LANZARO, G.C.; OSTROVSKA, K.; HERRERO, M.V.; LAWYER, P.G. & WARBURG, A. - *Lutzomyia longipalpis* is a species complex: genetic divergence and interspecific hybrid sterility among three populations. *Amer. J. trop. Med. Hyg.*, 48: 839-847, 1993.
- LEWIS, D.J. & WARD, R.D. - Transmission and vectors. In: PETERS, W. & KILLICK-KENDRICK, R., ed. *The leishmaniasis in biology and medicine*. London, Academic Press, 1987. v.1, p. 235-262.
- MANIATIS, T.; FRITSCH, E.F. & SAMBROOK, J. - *Molecular cloning: a laboratory manual*. New York, Cold Spring Harbor Laboratory, 1982.
- MURALIDHARAN, K. & WAKELAND, E.K. - Concentration of primer and template qualitatively affects products in random-amplified polymorphic DNA PCR. *Biotechniques*, 14: 362-363, 1993.
- POST, R.J. - DNA probes for vector identification. *Parasit. today*, 1: 89-90, 1985.

14. SANGER, F.; NICKLEN, F.S. & COULSON, A.R. - DNA sequencing with chain termination inhibition. **Proc. nat. Acad. Sci. (Wash.)**, 74: 5463-5467, 1977.
15. SCHIERWATER, B. & ENDER, A. - Different thermostable DNA polymerases may amplify different RAPD PCR products. **Nucleic Acids Res.**, 21, 4647-4649, 1993.
16. WARD, R.D.; PHILLIPS, A.; BURNET, B. & MARCONDES, C.B. - The *Lutzomyia longipalpis* complex: reproduction and distribution. In: SERVICE, M.W. **Biosystematics and haematophagous insects**. Oxford, Clarendon Press, 1988. p. 257-269. (Systematics Association Special, no. 37).
17. WELSH, J.; PETERSON, C. & MCCLELLAND, M. - Polymorphisms generated by arbitrarily primed PCR in the mouse: application to strain identification and genetic mapping. **Nucleic Acids Res.**, 19: 303-306, 1991.
18. WILKERSON, R.C.; GAFFIGAN, T.V. & BENTO LIMA, J. - Identification of species related to *Anopheles (Nyssorhynchus) albitarsis* by random amplified DNA polymerase chain reaction (Diptera: Culicidae). **Mem. Inst. Oswaldo Cruz**, 90: 721-732, 1995.
19. WILLIAMS, J.G.K.; KUBELIK, A.R.; LIVAK, K.J.; RAFALSKI, J.A. & TINGEY, S.V. - DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. **Nucleic Acids Res.**, 18: 6531-6535, 1990.
20. YOUNG, D.G. & LAWYER, P.G. - New world vectors of the leishmaniases. **Recent Top. Vector Res.**, 4: 29-71, 1987.
21. YU, K. & PAULS, K.P. - Optimization of the PCR program for RAPD analysis. **Nucleic Acids Res.**, 20: 2606, 1992.

Recebido para publicação em 01/09/1997

Aceito para publicação em 29/12/1997

