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Phenetic studies on randomly amplified polymorphic DNA-polymerase chain reaction-variability of four geographical populations of *Lutzomyia whitmani* (Diptera: Psychodidae) in Brazil

Estudos fenéticos de variabilidade de polimorfismos de DNA amplificados ao acaso pela reação em cadeia da polimerase em quatro populações geográficas de *Lutzomyia whitmani* (Diptera: Psychodidade) no Brasil

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ABSTRACT

Previous evaluation of the genetic variability of four biogeographical populations of Lutzomyia whitmani *from known foci of cutaneous leishmaniasis in Brazil demonstrated two main spatial clusters: Corte de Pedra-BA, Ilhéus-BA and Serra de Baturité-CE in the first cluster, and Martinho Campos-MG in the second. Further analysis showed a high degree of homogeneity in Corte de Pedra population but not in the others, which presented a significant percentage of specimens displaced from their phenon of origin (discrepant individuals). In the present work we analyzed the frequencies of association coefficients in the matrixes of similarity per population of* Lutzomyia whitmani *from both sexes and the general phenograms obtained, in a more detailed study of those discrepant specimens. Populational stability was observed for Corte de Pedra population, whereas the three remaining populations showed varying degrees of heterogeneity and different displacements according to sex. Our results strongly suggested the existence of a genetic flow between the lineages North-South/North-East and Ilhéus/ Serra do Baturité of* Lutzomyia whitmani.

Key-words: Lutzomyia whitmani. *Sand fly. Genetic variability. RAPD-PCR.*

RESUMO

Uma avaliação prévia da variabilidade genética de quatro populações biogeográficas de Lutzomyia whitmani *oriundas de focus conhecidos de leishmaniose cutânea no Brasil, evidenciou 2 agrupamentos espaciais principais: Corte de Pedra (BA), Ilhéus (BA) e Serra de Baturité (CE) no primeiro grupo, e Martinho Campos (MG) em um segundo. O aprofundamento da análise acusou um alto grau de homogeneidade na população de Corte de Pedra mas não nas outras, nas quais uma porcentagem significativa de espécimens deslocou-se do seu feno de origem (indivíduos discrepantes). Neste trabalho analisamos as freqüências dos coeficientes de associação nas matrizes de similaridade por população de* Lutzomyia whitmani, *de ambos os sexos, e o fenograma geral obtido, em um estudo mais detalhado daqueles espécimens discrepantes. Para Corte de Pedra foi observada estabilidade populacional, enquanto as outras três populações restantes mostraram graus de heterogeneidade variáveis e deslocamentos distintos, de acordo com o sexo dos indivíduos. Nossos resultados sugerem fortemente a existência de um fluxo genético entre as linhagens Norte-Sul/Norte-Leste e Ilhéus/Serra do Baturité de* Lutzomyia whitmani.

Palavras-chaves: Lutzomyia whitmani. *Flebotomíneo. Variabilidade genética. Reação em cadeia da polimerase.*

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Lutzomyia whitmani (Antunes & Coutinho, 1939) is a phlebotomine sand fly in the subgenus *Nyssomyia* that has been prevalent at Brazilian foci of cutaneous leishmaniasis in the state of Minas Gerais[13 15], Bahia[4 14] and Ceará[3 17 18]. It has been incriminated as a vector of human American cutaneous leishmaniasis, following the finding of specimens naturally infected with *Leishmania* parasites from the *braziliensis* complex[4 10], associated with human cases of the disease. Furthermore, a high anthropophilic behavior has been described for this sand fly species[3 8 9 16].

Several studies have suggested that *L. whitmani* is, in fact, a complex of species. Phylogenetic analysis of morphometric, biochemical and molecular characters of *L. whitmani* indicated that there are, at least, three to four distinct mitochondrial lineages in Brazil[11 19 20]. The first lineage is an Amazonian form, strictly silvatic, described from the northeast and southeast regions of the Amazon river, state of Pará. A second form was found in the northeastern Atlantic Forest zone, including the species type locality, distinguishable from the third group of haplotypes, which is located in the drier interior of Brazil (north-south group). A fourth lineage has been more recently described in the state of Rondônia, based on phylogenetic studies of mitochondrial DNA. An overall analysis showed that those lineages from forested regions (Rondônia, Amazonian and Northeastern) form a clade totally separated from the north-south lineage.

In a previous work, we analyzed the genetic variability of four geographical populations of *L. whitmani* in Brazil by randomly amplified polymorphic DNA-polymerase chain reaction[12]. The great majority of individuals fitted into the interpopulational phenograms according to their region of origin. However, a small discrepant group displaced from their region of origin was noted. The objective of the current work was to focus on those specimens unlinked to the phenon of origin.

MATERIAL AND METHODS

Phlebotomine. Sand flies were captured at night by Shannon[24] and CDC[26] traps at the Brazilian municipalities of Corte de Pedra, Bahia (13°26'23"S, 39°39'36"W); Ilhéus, Bahia (14°47'2"S, 39°02'58"W); Martinho Campos, Minas Gerais (19°19'54"S, 45°14'13"W) and Serra de Baturité, Ceará (04°19'43"S, 38°53'05"W). The traps were mounted at 5:00 p.m. and taken down at 7:00 a.m. the next day, for 5-7 consecutive days per region. In the laboratory, female sand flies were fed on non-infected hamsters (*Mesocricetus auratus*). After 24h they were isolated into individual containers until oviposition[14] after which they were mounted on microscope slides in Berlese medium[27] and identified according to Young and Duncan[30]. Eggs from 500 females identified as *L. whitmani* were combined, transferred to new containers and incubated in a humid chamber at 27ºC until eclosion. We obtained aproximately 200 specimens of *L. whitmani* per population in F1, from which 50 males and 50 females were frozen and stored in liquid nitrogen for later use.

DNA extraction. Fifteen specimens of *L. whitmani* of each sex and population were randomly selected from the frozen stock. Each specimen was macerated in a microtube using a plastic pestle, followed by addition of 50µl of lysis buffer (100 mM TRIS-HCl, 100mM NaCl, 25mM EDTA, 0.5% SDS, pH 8.0) and macerated again. Genomic DNA extraction was performed as described by Ausubel et al[2]. The final pellet was ressuspended in 20µl of TE 1X. Ten microliters were used for estimation of DNA concentration and purity by optical readings at 280 and 260nm in a spectrophotometer and then discarded. The remaining 10µl were stored at 4ºC until use.

DNA amplification. DNA was amplified by RAPD-PCR[28 29] in a thermocycler (Perkin-Elmer 2400) in a total reaction volume of 60µl. The reaction mixture was prepared as follows: 12µl of PCR buffer (100mM TRIS-HCl, 500mM KCl, 25mM $MgCl_2$, pH 8.3), 9.6µl dNTPs (10 mM each), 8µl of a random primer (70ng) and 18.4µl of ultrapure water. Six microliters of DNA (2ng) were added per reaction and the microtubes incubated at 94ºC for 1 min. After addition of 6µl (1.5U) of diluted Taq polymerase (Perkin Elmer), the amplification reaction was performed for 45 cycles of 30 s at 94ºC, 30 s at 34ºC and 1 min at 72ºC. The random primers were: OPG09 (CTGACGTCAC), OPG12 (CAGCTCACGA), 3302 (CTGATGCTAC) or 3303 (TCACGATGCA). A negative (no DNA) and a positive (lambda DNA) control were used in all amplifications.

DNA PAGE. Twelve microliters of the amplification products were analyzed by 4% PAGE (10 x 8.5 x 1.5cm) in TBE buffer for 10 min at 50V followed by 90 min at 80V, and developed by silver staining[5 23]. ϕX174RF DNA/*Hae*III fragments and 1Kb ladder (Gibco-BRL) were used as size markers. Gels were documented by a Polaroid Camera (Kodak) or an Eagle Eye System (Stratagene, La Jolla, US).

Data analysis. The PAGE fragments selected for analysis were the most consistently visible in all gels. The presence or absence of a given gel band was codified as 1 or 0, respectively, and used in the construction of a series of matrixes taxon/character. Association coefficients were determined following Dice[7] and the phenograms were constructed by unweighted pair-group method analysis (UPGMA)[25] through the NTSYS - pc version 2.0 software[22].

RESULTS

Absolute frequencies of the association coefficients derived from the matrixes of similarity including both sexes were analyzed per region of origin showing association coefficients in the range 0.47-0.60, 0.53-0.86, 0.39-0.77 and 0.53-0.84 for Corte de Pedra (BA), Ilhéus (BA), Martinho Campos (MG) and Serra de Baturité (CE), respectively (Figure 1). In general, a high variability in frequency and a persistent modal discontinuity were observed in all cases. It is worth noting the oscillation in the profile of frequencies for Martinho Campos population (0.50, 0.53, 0.57, 0.59, 0.61 and 0.69) at the highest frequency modes (0.52, 0.56, 0.58 and 0.60).

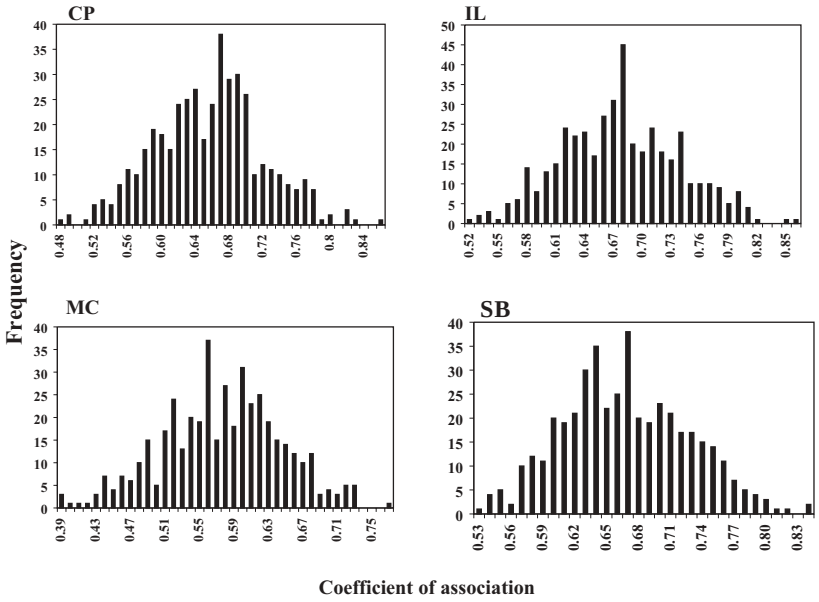

Figure 1 - Frequencies of association coefficients in the matrixes of similarity per population of Lutzomyia whitmani *from both sexes. Origin of populations- CP: Corte de Pedra/MG, IL: Ilhéus/BA, MC: Martinho Campos/MG and SB: Serra de Baturité/CE.*

Despite the high variability of association coefficients in the populations, the great majority of specimens was grouped according to their region of origin, in the interpopulational analysis per sex (Figures 2 and 3). However, a discrepant group was clearly formed by a limited number of specimens. For the females, it was composed of specimens numbered 301, 322 and 325 from Serra de Baturité and 18, 19, 20, 22, and 23 from Martinho Campos (Figure 2). Another discrepant group was observed for the males, comprising specimens 358 from Serra de Baturité, 275 and 280 from Ilhéus (BA) and 52 and 81 from Martinho Campos (Figure 3). Analysis of the displacement of similarities for the discrepant groups is summarized in Table 1.

DISCUSSION

The histogram profiles pointed to a variable range of association coefficients for the populations under study (Figure 1). Modal discontinuity was a common point, indicating the existence of a high level of intrapopulational genetic variability as previously described[12]. Intrapopulational variability associated with a low value of the highest frequency coefficient (0.56) distinguished Martinho Campos population from the others. The persistent discontinuity in the association coefficients (0.65) in the histograms of Corte de Pedra, Ilhéus, and Serra de Baturité might represent an aspect of similarity between these three populations, if one considers the positioning of this discontinuity in the same mode.

The phenon lines (equivalent to the mean of similarities among all the specimens analyzed) in the interpopulational phenograms of males and females (Figures 2 and 3) denote the greater homogeneity of Corte de Pedra and Serra de Baturité populations. All the individuals in these populations were grouped according to their region of origin. Females from Ilhéus formed a phenon including the discrepant group (Figure 2)

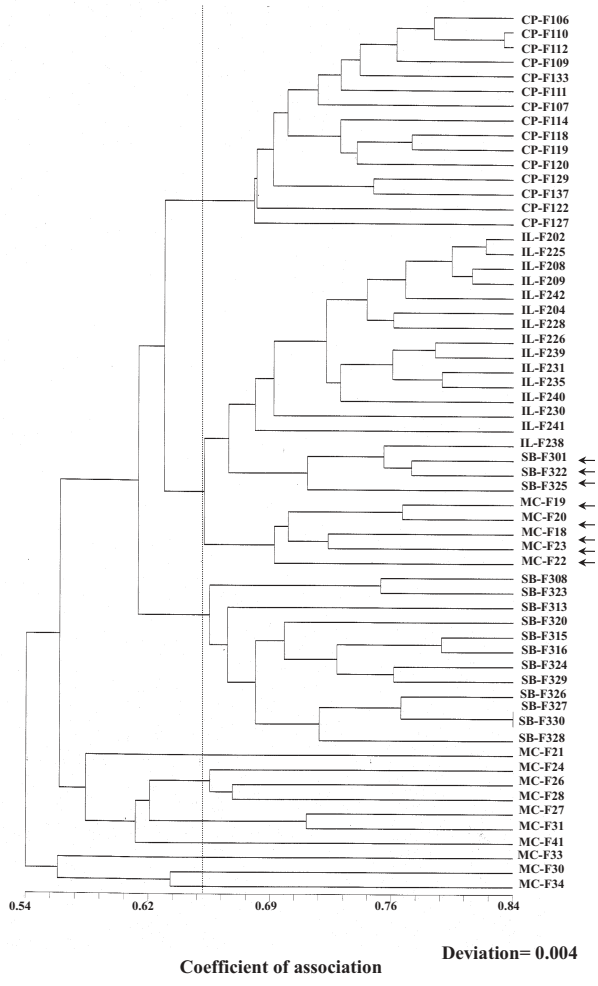

Figure 2 - General phenogram for females of Lutzomyia whitmani *generated by NT-SYS software. Each specimen was identified by the initials of the population (CP: Corte de Pedra/MG; IL: Ilhéus/BA; MC: Martinho Campos/MG; SB: Serra de Baturité/CE), sex (F for female) and a three digits number. The vertical line represents the average level of similarity between all the pairs of OTU's analyzed (phenon line).*

Table 1- Percentage of specimens of L. whitmani *linked in the same phenogram and displacement analysis of the discrepant group. CP: Corte de Pedra/ BA, MC: Martinho Campos/ MG, IL: Ilhéus/ BA and SB: Serra de Baturité/ CE, F- females, M- males.*

Software	Locality	Total number of individuals analyzed		Linked individuals				Individuals in the discrepant group				Locality of displacement of the discrepant group	
		F	M	F		M		F		M		F	M
				nº	%	nº	%	nº	%	nº	%		
	CP	15	15	15	100.0	15	100.0	0	0	0	0	-	-
NTSYS	IL	15	15	14	93.0	13	87.0	1	7.0	2	13.0	SB	SB
	MC	15	15	10	67.0	13	87.0	5	33.0	2	13.0	IL	SB
	SB	15	15	12	80.0	14	93.0	3	20.0	1	7.0	IL	SB

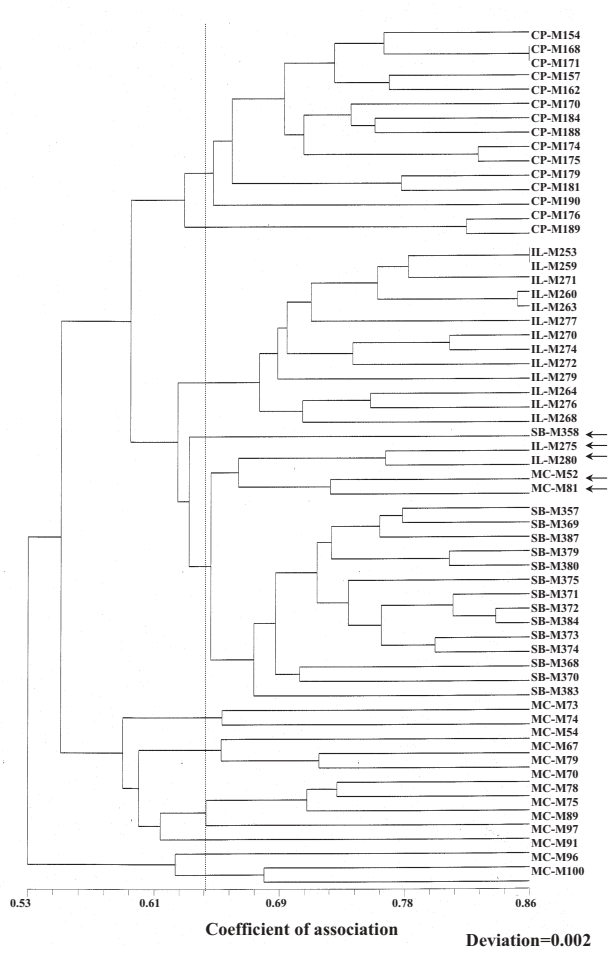

Figure 3 - General phenogram for males of Lutzomyia whitmani *generated by NT-SYS software. Each specimen was identified by the initials of the population (CP: Corte de Pedra/MG; IL: Ilhéus/BA; MC: Martinho Campos/ MG; SB: Serra de Baturité/CE), sex (M for male) and a three digits number. The vertical line represents the average level of similarity between all the pairs of OTU's analyzed (phenon line).*

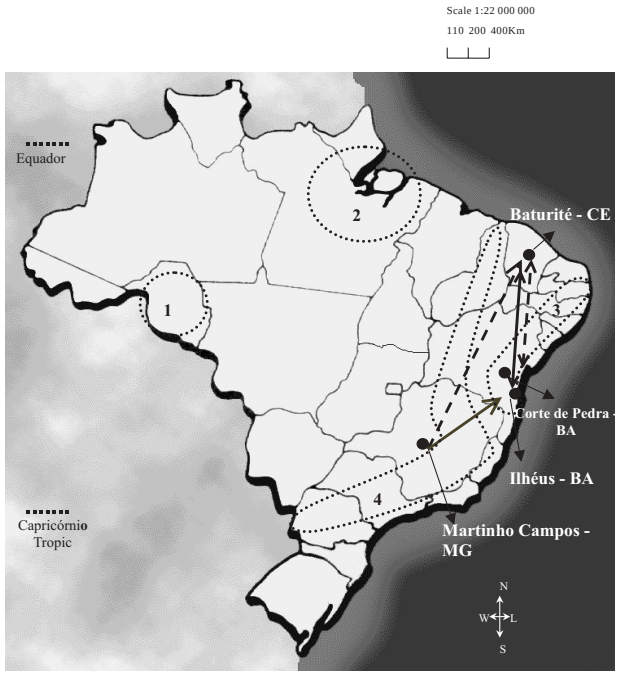

Figure 4 - Geographic distribution of the localities used for sand fly captures in Brazil, schematic representation of the Lutzomyia whitmani *lineages and displacement lines of similarities for the discrepant specimens from the four populations studied. Dotted lines delimit the lineages already described- 1: State of Rondônia; 2: Amazonian; 3: North-east; 4: North-south. Dotted and full arrows correspond to the displacement flow of similarity for males and females, respectively.*

evidencing the similarity between these insects. Incorporation of the discrepant group was not observed for the males from the same region (Figure 3). Spreading of discrepant individuals from Martinho Campos population in distinct groups suggested a non-uniformity in this population for both sexes.

Further analysis of the displacement of similarities for the discrepant groups confirmed that Corte de Pedra is the most uniform population with 100% of females and males grouped in a single phenon (Table 1). The high level of homogeneity in both sexes suggests a populational stability and perhaps a genetic isolation of this region. This hypothesis is reinforced by the overall analysis of discrepant individuals from the other regions with the Corte de Pedra phenon was never observed. The three populations remaining showed varying degree of heterogeneity and different displacements for females or males. In the first case, 93% of the specimens from Ilhéus formed a phenon while 7% suffered a displacement to the Serra de Baturité population. Martinho Campos displayed the highest heterogeneity with only 67% of specimens grouped in a characteristic phenon. Discrepant individuals (33%) were displaced consistently towards Ilhéus group. A similar displacement was observed for discrepant females from Serra de Baturité. Differently from previously reported[6,19]. Ilhéus and Serra de Baturité populations were not grouped into the same phenon but close to each other[12].

The discrepant females from Ilhéus population grouped consistently with Serra do Baturité (CE) phenon and vice-versa. The fact is indicative of a high degree of similarity between these two populations. The displacements observed (Martinho Campos and Serra de Baturité towards Ilhéus and Ilhéus towards Serra do Baturité) suggest the existence of genetic flows between the lineages north-south/north-east populations and Ilhéus/Serra do Baturité (Figure 4).

Analysis of male populations revealed aspects similar to those discussed for females. The great majority of specimens were grouped accordingly to their region of origin (Figure 3). However, differently from the females, 13% of males from Martinho Campos were displaced towards Serra de Baturité instead of Ilhéus. It is most probable that Martinho Campos population belongs to the north-south lineage, which is extensively distributed in Brazil from the States of Piauí (PI) to Paraná (PR)[20,21]. It is probable that the genetic non-uniformity of the lineage results from the existence of sympatric populations in those regions. As pointed out before, individuals with intermediate profiles could be explained by the occurrence of a gene flow.

In conclusion, analysis of the discrepant specimens of *L. whitmani* suggested some gene flow among the populations under study. We believe that further studies on the susceptibility of *L. whitmani* from different regions to *Leishmania* infection, associated with intra- and inter-lineages analyses, will help to clarify our understanding of sympatry, genetic flow and adaptability of these populations of leishmaniasis vectors.

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