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Full Length Research Paper

Identification of mitochondrial cytochrome B haplotypes by single strand conformation polymorphism in *Phlebotomus chabaudi* Croset, Abonnenc and Rioux, 1970 (Diptera, Psychodidae)

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Single strand conformation polymorphism (SSCP) was used to recognize the presence of *P. (Paraphlebotomus) chabaudi* Croset, Abonnenc and Rioux, 1970 haplotypes of Tunisian specimens; these were verified subsequently by sequencing the polymerase chain reaction products. The designed primers amplified a 545 pb fragment including the 3' end of mitochondrial cytochrome b (cyt b). The sensitivity of SSCP was demonstrated by (1) the detection of single nucleotide differences in SSCP variants, and (2) no sequence variation in specimens with the same SSCP mobility. The application of SSCP technique provided a valuable addition to available population genetic tools: It increased the efficiency of detection of variability in the cyt b gene and decreased the time required for screening large numbers of specimens to detect nucleotide variation.

Key words: Phlebotominae, Central east Tunisia, *Paraphlebotomus chabaudi,* identification, haplotypes SSCP.

INTRODUCTION

Molecular techniques have been developed and used for at least three decades to provide data for the determination of vector population structure. For phlebotomus, populations, genetic variation has been evaluated by isoenzyme analysis (Ward et al., 1981; Rogo et al., 1988; Perroti et al., 1991; Ben et al.,1991; Pesson et al., 1991), restriction fragment-length polymorphism (Aransay et al., 1999), random amplified polymorphic DNA (RAPD) (Mukhopadhyay et al., 2000), and DNA sequencing (Esseghir et al., 2000). These techniques differ in terms of sensitivity, cost, execution time and amount of DNA required. In Tunisia, *Leishmania killicki* is the agent of chronic cutaneous leishmaniasis form (CLK) (Rioux et al., 1986a, 1986b). The reservoir and the vector of this species are unknown. The vector could belong to the subgenera *Paraphlebotomus* as described elsewhere for *Leishmania tropica* Wright, 1913, a closely related species to *L. killicki* (Rioux et al., 1986a; Lawyer et al., 1991; Rioux et al., 1990; Jacobson, 2003; Gebre-Michael et al., 2004). Three species belonging to the *Paraphlebotomus* subgenus (*Phlebotomus sergenti*, *Phlebotomus alexandri* and *Phlebotomus chabaudi*) were suspected to transmit this cutaneous leishmaniasis form in Tunisia. *P. chabaudi* Croset, Abonnenc and Rioux, 1970 was the major species suspected to transmit *L. killicki* in south and recent central foci of *L. killick*i in Tunisia.

P. chabaudi population studies are uncommon and have been based on the comparison of mitochondrial

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cytochrome B sequences (Esseghir et al., 2000). An advantage of mitochondrial DNA (mDNA) as a population genetic marker is that it has a relatively rapid rate of mutation, providing readily identified haplotypes (DNA sequence variants). Within a single organism, the mitochondrial genome has multiple haploid copies (usually identical) of DNA that are maternally inherited and do not participate in recombination events. Maternal inheritance produces an effective population size (Ne) that is one-fourth that of nuclear genes. The smaller Ne reflects an increase in the effects of genetic drift among populations resulting in greater detection of genetic differentiation. The lack of recombination results in nucleotide sequences that will more accurately reflect the evolutionary history of the molecule if mDNA is assumed to be neutral with respect selection (Avise, 1994). Mitochondrial cytochrome b (cyt b) in sand flies exhibits sufficient variation for evaluation of the genetic structure of populations (Esseghir et al*.,* 1997, Ishikawa et al., 1999). A mutation detection technique, single strand conformation polymorphism (SSCP), was first described by Orita et al. (1989). The method is based on the observation that single stranded DNA is unstable, reannealing to itself to form conformations that vary depending on the sequence pattern. These differences are reflected in the migration rate during polyacrylamide gel electrophoresis. SSCP is a sensitive, inexpensive and easily performed method for haplotype detection. Population genetic studies in general (Sunnucks et al., 2000) and more specifically evaluations of human vector insect populations have employed SSCP only recently (Black and DuTeau, 1997; Stothard et al., 1998; De Merida et al., 1999; Wohlford et al., 1999; Gorrochotegui-Escalante et al., 2000; Birungi and Munstermann, 2002).

In the current report, sequence analysis of the 3' end of the cyt b gene, combined with SSCP, supports the reliability of the SSCP technique for characterizing *P. chabaudi* genetic variation.

MATERIALS AND METHODS

Collection, storage and identification of adult sand flies

The collections were carried out from September to October 2005 in central region of Tunisia (from two localities), during the main season of activity of adult sand flies in Tunisia. Sand flies were collected on sticky papers (A4 sheets of white paper soaked in castor oil) placed at least 3 nights in ruined outhouse and centers for disease control (CDC) miniature light traps were set overnight to sample sand flies in domestic and per domestic animal shelters

Sandflies captured in light traps were narcotized with freezing and those caught on sticky papers were removed with needles or fine brushes dipped in 70% ethanol. All specimens were then stored in analytical grade 80% ethanol at -20°C.

All sand flies were identified based on external and internal morphological characters of the head and abdominal terminalia described by Croset et al. (1978), which were slide-mounted in Berlese fluid (Lewis, 1982) following dissection with sterilized forceps and microneedles (Testa et al., 2002). This was carried out in a room away from the molecular biology laboratory, to reduce the risk of polymerase chain reaction (PCR) carryover, that is, the contamination of a sample with a DNA fragment that had already been amplified by PCR.

DNA isolation, amplification of DNA

Total genomic DNA was extracted from frozen single sand flies by the methods of Ready et al. (1997). Usually with these methods, 50 to 250 ng of DNA is extracted from each sand fly and, following ethanol precipitation, the genomic DNA was dissolved in 50 ml of 1XTE (10 mM Tris-HCl, 1 mM EDTA pH 8.0). 1 to 1.5 µl of this solution was added to each 50 µl of polymerase chain reaction (PCR) mix, which contained Invitrogen buffer with-out 2 mM $MgCl_2$, 100 µM of each dNTP, and 40 pmol of each primer. The forward primer CB3FC (5'-3' CA(C/T)ATT CAACC(A/T)GAATGATA) and the reverse primer NINFA (5'-3' GGTA(C/T)(A/T) TTGCCTCGA(A/T)TTCG(A/T) TATGA) (Esseghir et al., 1997) were used to amplify a 545 base-paire (bp) fragment of mtDNA, which contains the 3'end of Cyt b gene (Ready et al., 1997). 1.5 units of Taq DNA polymerase (Invitrogen) were always used. The primer sequences are homologous to nucleotides 11214 to 11233 and 11759 to 11734, respectively (GeneBank accession number L20934), of the mitochondrial genome of *Anopheles gambiae* (Beard et al., 1993). PCR consisted of 5 cycles of denaturation at 94°C for 30 s, annealing at 40°C for 30s. and extension at 72°C for 1 min, followed by 35 similar cycles but with annealing at 44°, and a final extension at 72°C for 10 min, all on an PTC-100™ Programmable thermal controller cycler (MJ Research INC).

The size of the amplified DNA fragments were determined by fractionation in 1.5% agarose gels along with DNA standards (Promega PCR markers 100 bp DNA Ladder 0.5 µg/lane). PCR products were purified before analysis using the (Promega Wizard®SV Gel and PCR Clean-Up System, according to the supplier's specifications. This step was necessary; it removed bands that interfered with interpretation of results. Samples were stored at -20°C.

Single strand conformation polymorphism analysis (SSCP)

Gel plates were cleaned with 95% EtOH and ddH_2O. Acrylamide was prepared before use, filtered and stored at 4°C. The acrymlamide solution consisted of the following: 3 ml, 5XTBE (0.45 M Tris base, 0.45 M boric acid, 10 mM EDTA, pH 8); 3 ml, 50% acrylamide-N,N'-methylene bis-acrylamide solution (99:1); 3 ml, 50% glycerol; 20 ml ddH_2O. Before pouring into the gel plate assembly, 0.75 ml, 1.6% ammonium persulfate and 30 µl TEMED were added. Samples of purified PCR product were diluted, 1:1 in loading buffer (20 µM EDTA, 0.05% bromophenol blue, 95% formamide and 0.05% xylene cyanol), heated at 95°C for 5 min and immediately placed on wet ice. Aliquots were loaded on a prerun gel (1 mm thick, 16 by 18 cm) and subjected to electrophoresis for 3.5 h at 200 V, 19°C, using a vertical slab gel unit and 0.5 X TBE buffer. The gel was silver stained (Sanguinetti et al., 1994) to visualize the reannealed single-stranded PCR products. Initial fixation of the gel was for 10 min (10% EtOH, 0.5% acetic acid), followed by staining in silver nitrate (0.2% in fixative) for 5 and 1 min wash in deionized water.

The gel was then placed in chilled developer (3% NaOH, 0.1% formaldehyde) until bands became visible. Following a final fixation for 5 and 10 min wash in deionized water, the plate was air dried at room temperature and photographed. All solutions were prepared in deionized water to reduce background staining. The genetic molecular human laboratory (LGMH), Faculté de Medecine de Sfax-Tunisia, performed automated sequencing of variants. Sequence data were aligned using the program Sequencher 4.6 Demo.

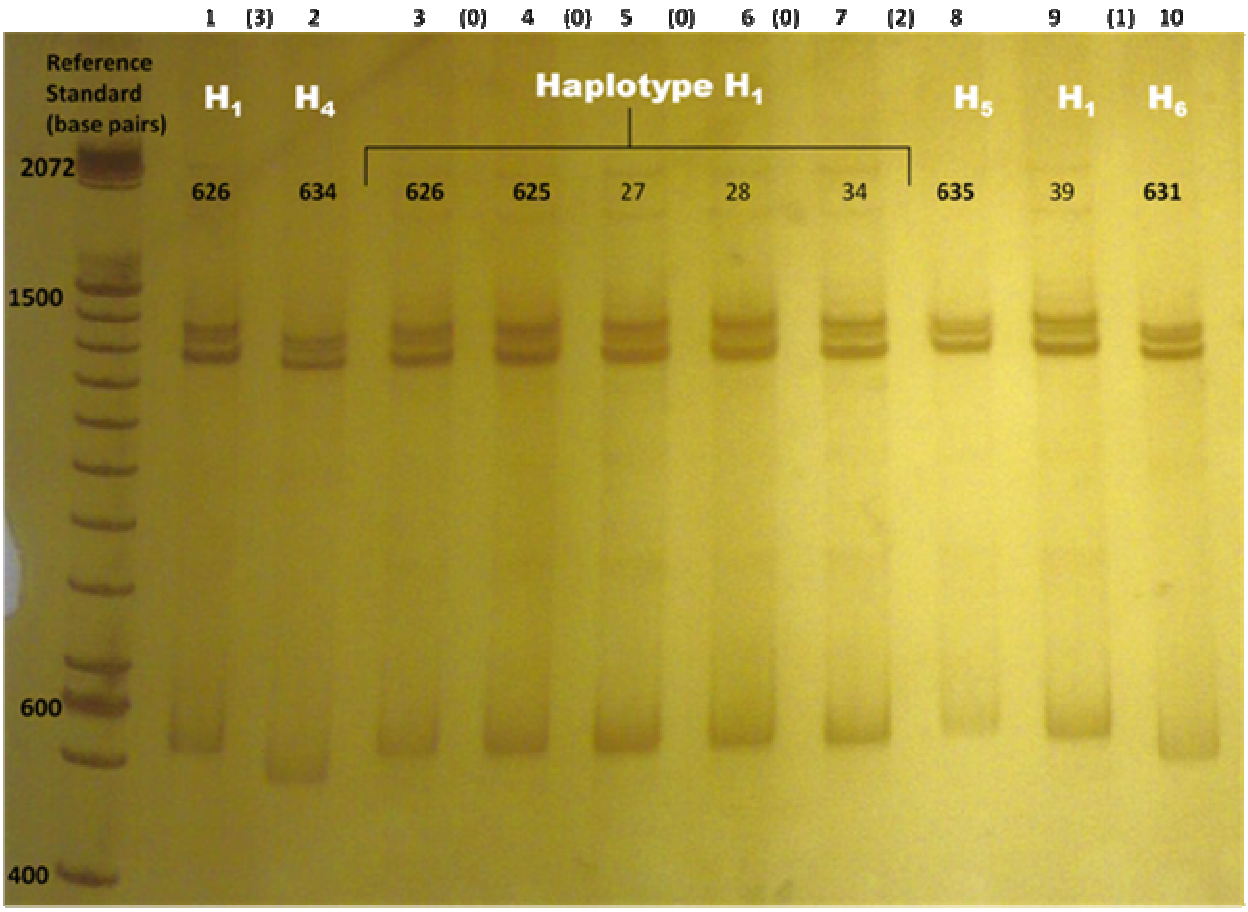

Figure 1. SSCP analysis of a 545 bp fragment from the 3' end of mitochondrial cytochrome b. DNA was isolated and amplified from field-caught individual *P. chabaudi* from two locations in Eastern Tunisia. Lanes 1 to 10 show haplotypes. Lanes 3, 4, 5, 6 and 7 were identical haplotypes from five specimens. The difference in band migration in adjacent lanes is associated with the number of nucleotide diffrences (in parentheses): lanes 1 to 2 (3), 7 to 8 (1) and 9 to 10 (1).

RESULTS AND DISCUSSION

Verification of the variants identified by SSCP was based on sequencing of PCR products. Comparison of the haplotypes identified by SSCP analysis (Figure 1) with the sequence (Figure 2) indicates that for amplified fragment from *P. chabaudi* specimens, haplotypes can be reliably distinguished. The sensitivity of the SSCP technique is illustrated (Figure 1) by its ability to distinguish between haplotypes that differ by a single nucleotide, e.g., lanes 9 and 10. Lanes 1, 3, 4, 5, 6, 7 and 9 contained samples exhibiting equal mobility, and were later verified by sequencing to be identical.

The current study identified no problems with the SSCP analysis of *P. chabaudi* mtDNA nucleotide variation. However, identical PCR products can migrate to different positions; likewise products with differing sequences can migrate to the same position (e.g. Wohlford et al. (1999) in *Glossina morsitans*). SSCP migration of the same haplotype to different positions may be due to variations in the amplified fragment near the primer site. Factors influencing the reliability of the SSCP technique have been reviewed (Yap and McGee, 1994; Hayashi et al.,. 1998). In the current analysis, standardization of the following procedures was necessary to produce reliable results: (1) Amplification regimes for the relatively small PCR fragment; (2) PCR product purification before electrophoresis; (3) Optimization of electrophoresis factors: Gel dimentions, acrylamide concentration, acrylamide/bis:acrylamide ratio, glycerol concentration, buffer pH, and temperature. Generally, SSCP resolution is better with fragments smaller than 500 bp, but a report by Kukita et al. (1997) presents' modifications permitting accurate evaluation of up to 800 bp.

In the current study, alignment of 490 bp from 20 specimens of *P. chabaudi* identified 9 segregating sites in a total of 6 haplotypes. Transversions exceeded transitions by 7 to 1 in 9 substitutions. We aligned the 3' end of cyt b sequences of *P. chabaudi* with reference to those of *P. chabaudi* (Esseghir et al., 1997). The dominant haplotype is H_1 (50%) (Figure 2). This paper reports the variation in mitochondrial cyt b from

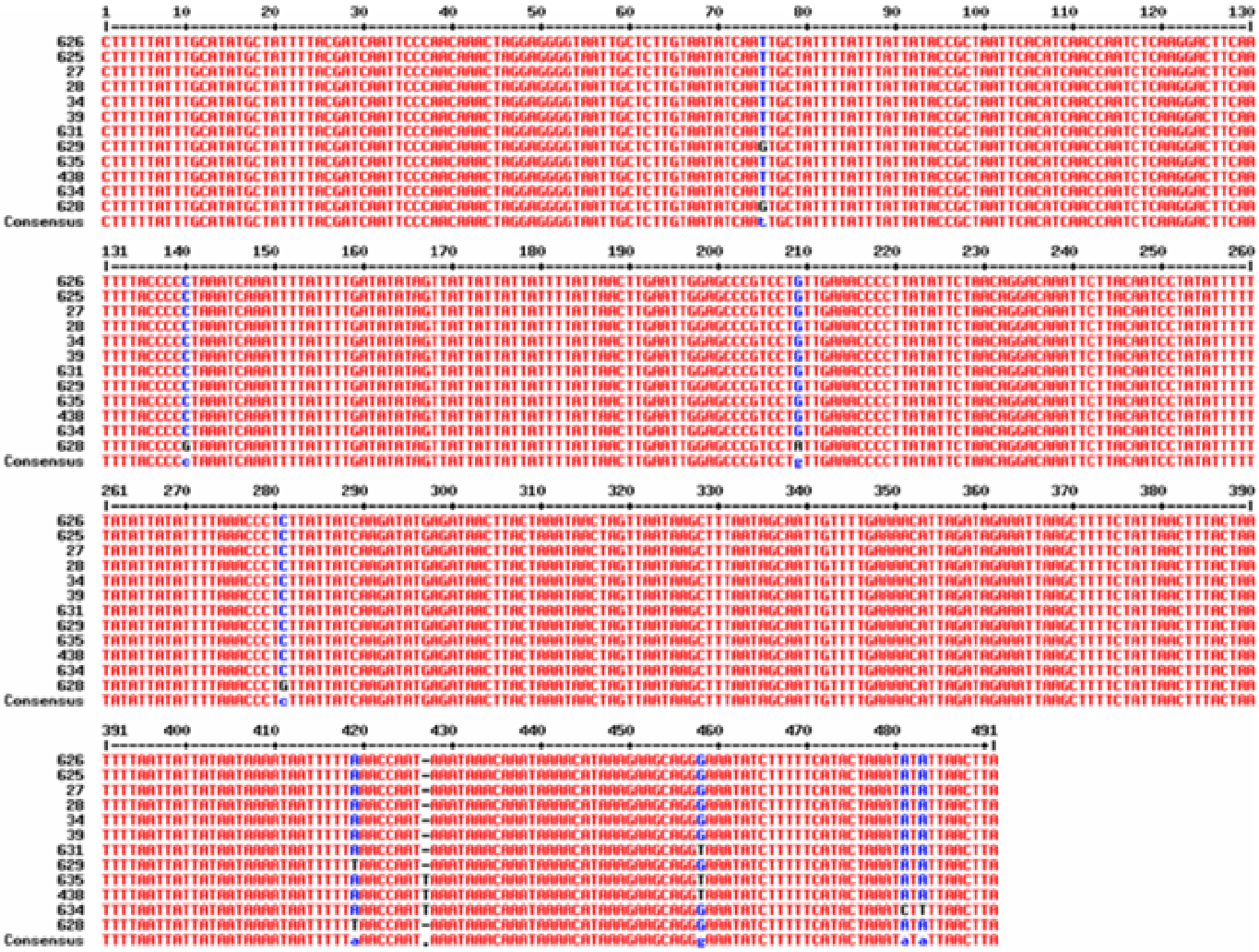

Figure 2. Distribution of haplotypes and segregating sites based on the sequencing and alignement of 490 bp located at the 3' end of cytochrome b gene of *P. chabaudi*.

P. chabaudi field populations and haplotype detection using SSCP. SSCP analysis of the 3' end of the cyt b gene provides a new genetic tool for population analysis of *P. chabaudi* and other related species especially *Phlebotomus* and *Larroussius* species proven vector of cutaneous and visceral leishmaniasis in Tunisia, respectively. Analysis of this cyt b fragment takes advantage of the variability that can be detected, along with the ease and reliability of the SSCP approach. Large samples can be rapidly screened for variation; sequencing is necessary only for the variant haplotypes when each is recognized on the SSCP gels.

Consequently, the proposed tool for identification of haplotypes has to be used during epidemiological field works, to study genetic structure of populations of sand flies. That allows epidemiologists making relationship between distributions of haplotypes to be associated with geological or climatic events and/or that there has been a relatively recent population occupying particular geographical range.

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