

# Limits of a rapid identification of common Mediterranean sandflies using polymerase chain reaction-restriction fragment length polymorphism

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*A total of 131 phlebotomine Algerian sandflies have been processed in the present study. They belong to the species Phlebotomus bergeroti, Phlebotomus alexandri, Phlebotomus sergenti, Phlebotomus chabaudi, Phlebotomus riouxi, Phlebotomus perniciosus, Phlebotomus longicuspis, Phlebotomus perfiliewi, Phlebotomus ariasi, Phlebotomus chadlii, Sergentomyia fallax, Sergentomyia minuta, Sergentomyia antennata, Sergentomyia schwetzi, Sergentomyia clydei, Sergentomyia christophersi and Grassomyia dreyfussi. They have been characterised by sequencing of a part of the cytochrome b (cyt b), tRNA serine and NADH1 on the one hand and of the cytochrome C oxidase I of the mitochondrial DNA (mtDNA) on the other hand. Our study highlights two sympatric populations within P. sergenti in the area of its type-locality and new haplotypes of P. perniciosus and P. longicuspis without recording the specimens called lcx previously found in North Africa. We tried to use a polymerase chain reaction-restriction fragment length polymorphism method based on a combined double digestion of each marker. These method is not interesting to identify sandflies all over the Mediterranean Basin.*

Key words: Algeria - mtDNA - PCR-RFLP - *Phlebotomus sergenti*

Algeria is a country where four leishmaniasis are endemic. The leishmaniasis due to *Leishmania infantum* is transmitted by phlebotomine sandflies belonging to the subgenus *Larrousius*: *Phlebotomus perniciosus*, *Phlebotomus perfiliewi* [proven vectors according to Killick-Kendrick (1990)] and possibly *Phlebotomus longicuspis* (suspected vector) (Izri et al. 1990, Izri & Belazzoug 1993, Harrat et al. 1996, Berdjane-Brouk et al. 2012). *Leishmania major* is transmitted by the proven vector *Phlebotomus papatasi* (Izri et al. 1992). *Leishmania tropica* and *Leishmania killicki* are transmitted by the proven vectors *Phlebotomus sergenti* (Guilvard et al. 1991, Boubidi et al. 2011, Jaouadi et al. 2012).

The phlebotomine sandfly fauna of Algeria has been studied in the past (Parrot 1917, 1935, 1942, Rioux et al. 1970a, b, Dedet et al. 1973, 1984, Dedet & Addadi 1977, Belazzoug & Mahzoul 1980, 1986, Belazzoug et al. 1986, Berchi et al. 1986, Belazzoug 1991, Russo et al. 1991). Recently, two molecular studies characterised two closely related species (*Phlebotomus chabaudi* and *Phlebotomus riouxi*) having undistinguishable or very difficultly distinguishable females from North Africa (Bounamous et al. 2008, Boudabous et al. 2009) and a new species for the country (and for Africa) has been recorded: *Phlebotomus mascittii* Grassi (Berdjane-Brouk et al. 2011).

In a recent paper, Latrofa et al. (2012) suggested to use mitochondrial DNA (mtDNA) cytochrome b (*cyt b*) polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) as a rapid molecular identification of the common phlebotomine sandflies in the Mediterranean Region. The goal of this paper is to check the proposed method on a sampling of 17 Algerian species (131 specimens) belonging to three genera: *Phlebotomus*, *Sergentomyia* and *Grassomyia*. We coupled *cyt b* and cytochrome C oxidase I (*COI*) of the mtDNA. The first marker is considered as the "gold standard" for phlebotomine sandflies systematic. The second one serves as a DNA barcode for the identification of animal species (Hebert et al. 2003). However, it was used in a few studies carried out on phlebotomine sandflies to study taxa from the Americas (Arrivillaga et al. 2002, Azpurua et al. 2010), from North Africa (Boudabous et al. 2009) and in India (Kumar et al. 2012).

## MATERIALS AND METHODS

**Sandflies collection** - Sandflies were collected from different provinces of Algeria coupling three methods in order to increase the diversity (Rioux et al. 2013): sticky traps, CDC and ultraviolet miniature light traps and aspirators (Fig. 1). They were stored in 96% ethanol. One hundred thirty-one males and females selected for this study are indicated in the Supplementary data.

**Sandflies mounting and identification** - The head and genitalia of individual male sandflies were cut off within a drop of ethanol, cleared in boiling Marc-André solution and mounted between slide and cover slide for spe-

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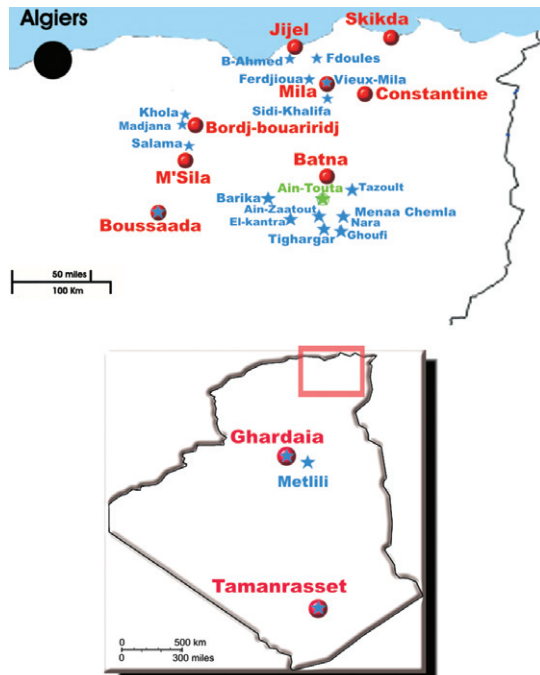


Fig. 1: sampling realised for this study in different areas of Algeria. Ain-Touta is the type locality of *Phlebotomus sergenti*. Circles: main cities; stars: locations where sandflies have been caught.

cies identification. The body related to the specimen was stored dried in a vial at  $-20^{\circ}\text{C}$  before DNA extraction.

The specimens have been identified by observation of the head and genitalia under a BX50 microscope. The identification keys and characters used for the identification of specimens are those of Abonnenc (1972), Dedet et al. (1984), Depaquit et al. (1998a), Pesson et al. (2004) and Bounamous et al. (2008). Measures and photos have been performed using the Perfect Image software (Aries Company, Chatillon, France) and a video camera connected to the microscope.

**DNA extraction** - Genomic DNA was extracted from the thorax, wings, legs and abdomen of individual sandflies using the QIAmp DNA Mini Kit (Qiagen, Germany) following the manufacturer's instructions, modified by crushing the sandfly tissues with a piston pellet (Treff, Switzerland) and using an elution volume of 200  $\mu\text{L}$ , as detailed in Depaquit et al. (2004).

**PCR amplification and sequencing** - All the mtDNA amplifications were performed in a 50  $\mu\text{L}$  volume using 5  $\mu\text{L}$  of extracted DNA solution and 50 pmol of each of the primers. The PCR mix contained (final concentrations) 10 mM Tris HCl (pH 8.3), 1.5 mM  $\text{MgCl}_2$ , 50 mM KCl, 0.01% Triton X 100, 200  $\mu\text{M}$  deoxynucleotide triphosphate each base and 1.25 units of 5 prime *Taq* polymerase (Eppendorf, Germany).

The cycle profiles were marker dependent. Each PCR begins by an initial denaturation step at  $94^{\circ}\text{C}$  for 3 min and finishes by a final extension at  $68^{\circ}\text{C}$  for 1 min. Amplification of a fragment of *cyt b* gene has been done by

using the primers N1N-PDR and C3B-PDR, following the method previously published by Essegir et al. (1997): five cycles (denaturation at  $94^{\circ}\text{C}$  for 30 s, annealing at  $40^{\circ}\text{C}$  for 60 s and extension at  $68^{\circ}\text{C}$  for 60 s) followed by 35 cycles (denaturation at  $94^{\circ}\text{C}$  for 60 s, annealing at  $44^{\circ}\text{C}$  for 60 s and extension at  $68^{\circ}\text{C}$  for 60 s). Their *COI* domain was amplified using the primers used by Hajibabaei et al. (2006): LepF and LepR, under the following thermal profile (Costa et al. 2007): five cycles (denaturation at  $94^{\circ}\text{C}$  for 30 s, annealing at  $45^{\circ}\text{C}$  for 90 s and extension at  $68^{\circ}\text{C}$  for 60 s), then 35 cycles (denaturation at  $94^{\circ}\text{C}$  for 30 s, annealing at  $51^{\circ}\text{C}$  for 90 s and extension at  $68^{\circ}\text{C}$  for 60 s).

Amplicons were analysed by electrophoresis in 1.5% agarose gel containing ethidium bromide. Direct sequencing in both directions was performed by Sanger's method using the primers used for DNA amplification. Reagents for PCR cleanup were Agencourt® AMPure XP-PCR Purification and those for sequencing reaction were Big Dye Sequencing Buffer (Applied Biosystems Foster City, California, USA); Agencourt® CleanSEQ kit: CleanSeq® beads. The instruments used for the sequencing were Biomek NXp BiomekFXP (Bekman Coulter); Sequencer 3730 XLT (Applied Biosystems). The correction of sequences was done using Pregap and Gap softwares included in the Staden Package (Bonfield & Staden 1996).

**Molecular analyses** - They are based on the two datasets of sequences. Analyses were performed using haplotypes obtained from this study and sequences of *P. chabaudi* and *P. riouxi* available in GenBank (Supplementary data). Sequence alignment was performed using the CLUSTALW routine included in the MEGA v.4 software (Tamura et al. 2007) and checked by eye. The objective of this study is to provide a database for sandflies identification, not to do any phylogenetical analysis. Consequently, a neighbour-joining (NJ) analysis was performed using MEGA 4 software, with the Kimura-2 parameters model and using uniform rates among sites. Gaps were treated as missing data.

**RFLP** - The diagnostic endonuclease restriction sites on *cyt b* and *COI* mtDNA sequences were predicted for each specimen using CLC workbench 5.2 software (clc-genomics-workbench.com-about.com). A panel of restriction enzymes was tested including *AseI* enzyme proposed by Latrofa et al. (2012) for *cyt b* digestion. Because one restriction enzyme cannot provide an original digestion pattern per species, we selected double digestion for both molecular markers.

PCR-RFLP assays were performed in a 50- $\mu\text{L}$  total volume reaction mix, containing 15  $\mu\text{L}$  of PCR product (from PCR vials), 0.1  $\mu\text{L}$  of *AseI*, 0.4  $\mu\text{L}$  of *MnII* for *cytb* and 0.05  $\mu\text{L}$  of *MspI*, 0.05  $\mu\text{L}$  of *TaqI* for *COI*, 5  $\mu\text{L}$  of NEB buffer 3 for *cyt b* and 5  $\mu\text{L}$  of NEB buffer 4 for *COI* containing bovine serum albumin (New England Biolabs, France).

For *cyt b* PCR-RFLP, we selected a double digestion coupling *AseI* with *MnII*. PCR products were digested during 2 h at  $37^{\circ}\text{C}$ .

For *COI*PCR-RFLP, we selected a double digestion coupling *MspI* with *TaqI*. According to their different temperature of activity ( $37^{\circ}\text{C}$  and  $65^{\circ}\text{C}$ , respectively), PCR products were digested during 1 h at  $37^{\circ}\text{C}$  then 1 h at  $65^{\circ}\text{C}$ .

The digested samples were separated by electrophoresis in a 3% agarose gel to produce DNA fragments and sized by comparison with markers 50 bp ladder, 100 bp ladder and 20 bp ladder (Cliniscience, France).

According to the sequencing of all the specimens processed in the present study, we did not use any positive control, but we checked that each restriction enzyme had functioned properly in each reaction by comparison to the predicted digestion.

## RESULTS

PCR amplification was successful for all the specimens processed. The GenBank accessions for *COI* and *cyt b* are indicated in the Supplementary data. The length of the analysed markers is of 680 bp for *COI*. It varies from 510-525 bp for *cyt b*. Each gene from each haplotype had an open reading frame (ORF). The sequences labelled *COI* include exclusively this marker. The sequences labelled "cytB" included in fact an ORF of cytB (positions 1-321), the tRNA serine (positions 321-378), then the ORF for NADH subunit 1 (from 379 to the last position). These ORFs are translated in proteins, explaining the low probability they could be pseudogenes (Rogers & Griffiths-Jones 2012).

Global trees based on *cyt b* and *COI* sequences are presented in Figs 2, 3, respectively. According to Depaquit et al. (1998b), *Phlebotomus bergeroti* has been selected to root the tree. All the species morphologically recognised are well individualised. We note that *P. sergenti* included two populations without any morphological difference. The identification of *P. perniciosus* and *P. longicuspis* is not doubtful. We did not record any atypical specimen.

The double digestion of each PCR product confirms the expected fragments for all haplotypes (Tables I, II). The resolution of DNA fragment size by the gel fractionation method used is about 10 bp. The double digestion of *COI* by restriction enzymes *TaqI* and *MspI* is very efficient, but cannot unequivocally distinguish *Phlebotomus ariasi* and *Sergentomyia schwetzi*.

The double digestion of *cyt b* by restriction enzymes *AseI* and *MnII* provide restriction profiles indicated in Table II. On the one hand, it cannot individualise one population of *P. sergenti*, *P. ariasi* and *P. riouxi*. On the other hand, it cannot distinguish *P. longicuspis* and *P. perfiliewi*.

No partial digests were recognised in the analysis.

## DISCUSSION

The specimens identified by morphology as belonging to a species are branched together regarding independently *cyt b* or *COI* sequences (Figs 2, 3).

*Cyt b* sequences provide a NJ tree in agreement with the traditional morphological taxonomy of the phlebotomine sandflies (Fig. 2). *Sergentomyia* are grouped together including *Grassomyia dreyfussi*. Concerning *Larroussius*, all of them are grouped together and two branches are individualised: one including *P. ariasi* and *Phlebotomus chadlii* and another containing *P. perniciosus*, *P. longicuspis* (including 2 lineages) and *P. perfiliewi*. These data are in accordance with those obtained by Esseghir et al. (2000) on *cyt b* and Di Muccio et al.

(2000) on rDNA internal transcribed spacer 2. Concerning the subgenus *Paraphlebotomus*, the species *P. sergenti*, *P. chabaudi* and *P. riouxi* are grouped together. The first one shows two lineages in its type-locality. Moreover, *Phlebotomus alexandri* is not included in this branch, as previously observed (Depaquit et al. 2000, Krüger et al. 2011).

The NJ tree based on *COI* sequences (Fig. 3) has a surprising topology: the species belonging to the subgenera *Sergentomyia*, *Larroussius* and *Paraphlebotomus* are not grouped together. Despite this curious branching, some results are congruent with *cyt b*: (i) the position of *P. alexandri*, (ii) the existence of two molecular lineages within *P. sergenti* topotypes, (iii) the individualisation of *P. chabaudi* and *P. riouxi*, (iv) the existence of two lineages within *P. longicuspis* and (v) a high variability within *Sergentomyia minuta* and *Sergentomyia antennata*.

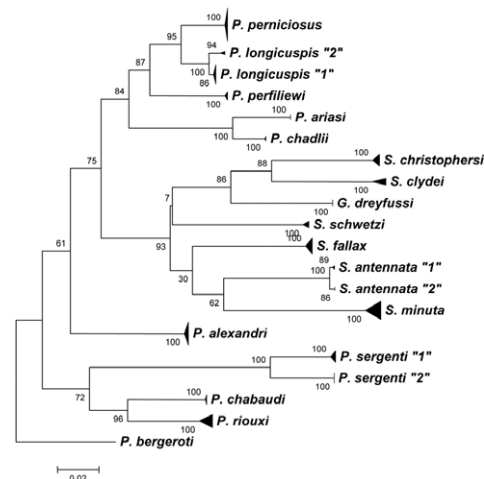


Fig. 2: neighbour-joining tree based on mitochondrial DNA cytochrome b sequences rooted on *Phlebotomus bergeroti*. Bootstrap values after 1,000 replicates are indicated on the branches.

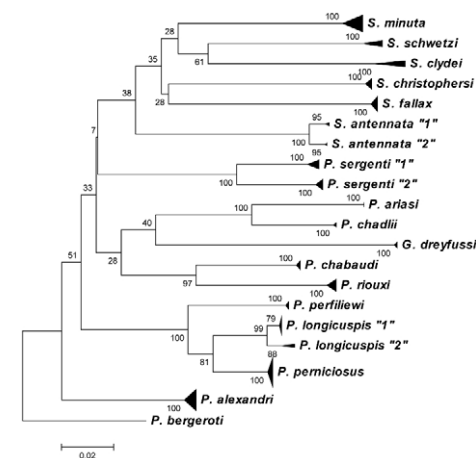


Fig. 3: neighbour-joining tree based on mitochondrial DNA cytochrome C oxidase I sequences rooted on *Phlebotomus bergeroti*. Bootstrap values after 1,000 replicates are indicated on the branches.



Many lineages have been identified in *P. sergenti* in the literature from populations from different parts of the species distribution area and no study emphasise a link between the molecular variability and the morphology (Depaquit et al. 2002, Yahia et al. 2004, Moin-Vaziri et al. 2007, Barón et al. 2008, Dvorak et al. 2011). The two mitochondrial lineages (*cyt b* as well as *COI*) within Algerian specimens of *P. sergenti* coming from different localities, all located just around the type locality area (Fig. 1) called Ain Touta, formerly Mac Mahon (Parrot 1917). This locality has not been precisely designated by Parrot (1917). The specimens processed in the present study can be considered as being topotypes, clearly labelled and stored in the collection of the laboratory of Parasitology of the Faculty of Pharmacy of Reims. These two populations are strongly separated and are characterised by many variable nucleotide positions: about 30 for *cyt b* and 40 for *COI* (Fig. 4). The mean pairwise distance between the two populations of *P. sergenti* (> 5%) is comparable to the pairwise distances individualising *P. perniciosus* from *P. longicuspis* or *P. perfiliewi* in the present study and question about the status of these populations.

The haplotypes obtained for *P. perniciosus* and *P. longicuspis* have been compared with those of *cyt b* available in the literature (Esseghir et al. 1997, Pesson et al. 2004, Perrotey et al. 2005) (Fig. 5). The main *P. perniciosus* Algerian haplotype is the same than the main Mediterranean haplotype (= pern01), but three new haplotypes are recorded from Algeria for this species.

Concerning *P. longicuspis*, the most common haplotype is the lcu01. However, we record four new haplotypes including a couple (LC2 and LC637) strongly individualised from the other ones. The *COI* sequences also individualise the latter specimens from all other *P. longicuspis* (Fig. 3).

Recently, the single digestion based on *cyt b* sequences by Latrofa et al. (2012) as a rapid molecular identification method for the common phlebotomine sandflies in the Mediterranean Region does not apply in Algeria. In fact, these authors focused on five species only, commonly caught in Italy: *P. papatasi*, *P. perniciosus*, *P. perfiliewi*, *Phlebotomus neglectus* and *S. minuta*. We tried this method on the Algerian sandflies, including *P. papatasi*. The method is not able to distinguish all the species and the combined double digestion of two different markers is needed for the specific identification. Due to the conservation of some parts of DNA sequences, the simple digestion of PCR products cannot separate some species belonging to different genera (like *P. ariasi* and *S. schwetzi*) or subgenera (like *P. sergenti* and *P. ariasi*). Consequently, this method is not enough discriminant to be used in routine all over the Mediterranean Basin. In fact, it is easier to firstly identify the species easy to recognise by a microscopical examination and secondly, to apply efficient PCR-RFLP methods to identify the species for which morphological identification is difficult, like the females of the Perniciosus complex, those of *P. chabaudi* and *P. riouxi* and for some *Sergentomyia* males.

TABLE I  
Species and populations examined in the present study - number and positions of cuts predicted for cytochrome C oxidase I mitochondrial DNA restriction fragment length polymorphism

Species	<i>TaqI</i> and <i>MspI</i> (expected)		Cut sites
	Cut (n)	Fragments (n)	
<i>Phlebotomus bergeroti</i>	4	5	85, 91, 101, 176, 254
<i>Phlebotomus alexandri</i>	5	6	22, 84, 85, 91, 156, 267
<i>Phlebotomus longicuspis</i>	4	5	22, 73, 91, 113, 408
<i>Phlebotomus perniciosus</i>	3	4	22, 91, 184, 408
<i>Phlebotomus chabaudi</i>	5	6	30, 85, 89, 91, 158, 256
<i>Phlebotomus sergenti</i> (group 1)	3	4	25, 62, 111, 507
<i>Phlebotomus sergenti</i> (group 2)	3	4	22, 85, 91, 507
<i>Phlebotomus chadlii</i>	6	7	14, 22, 85, 91, 101, 174, 224
<i>Phlebotomus riouxi</i>	6	7	21, 30, 85, 91, 141, 157, 186
<i>Phlebotomus ariasi</i>	2	3	85, 91, 527
<i>Phlebotomus perfiliewi</i>	2	3	11, 184, 408
<i>Sergentomyia fallax</i>	2	3	85, 265, 353
<i>Sergentomyia schwetzi</i>	2	3	85, 91, 527
<i>Sergentomyia clydei</i>	4	5	82, 91, 174, 176, 184
<i>Sergentomyia minuta</i>	3	4	88, 90, 176, 350
<i>Sergentomyia christophersi</i>	5	6	32, 81, 85, 91, 101, 319
<i>Sergentomyia dreyfussi</i>	3	4	70, 91, 184, 360
<i>Sergentomyia antennata</i>	3	4	82, 110, 157, 356

TABLE II

Species and populations examined in the present study - number and positions of cuts predicted for cytochrome b mitochondrial DNA restriction fragment length polymorphism

Species	AseI and MnlI (expected)		Cut sites
	Cut (n)	Fragments (n)	
<i>Phlebotomus bergeroti</i>	3	4	14, 20, 74, 434
<i>Phlebotomus alexandri</i>	5	6	14, 20, 62, 92, 108, 248
<i>Phlebotomus longicuspis</i>	5	6	14, 20, 62, 92, 103, 263
<i>Phlebotomus perniciosus</i>	4	5	14, 20, 62, 103, 353
<i>Phlebotomus chabaudi</i>	4	5	14,20, 62, 198,248
<i>Phlebotomus sergenti</i> (group 1)	4	5	14, 20, 96, 208,209
<i>Phlebotomus sergenti</i> (group 2)	3	4	14, 20, 62, 449
<i>Phlebotomus chadlii</i>	3	4	20, 62, 107, 356
<i>Phlebotomus riouxi</i>	3	4	14,20, 62, 445
<i>Phlebotomus ariasi</i>	3	4	14, 20, 62, 453
<i>Phlebotomus perfiliewi</i>	5	6	14, 20, 62, 92, 103, 263
<i>Sergentomyia fallax</i>	3	4	20, 62, 103, 353
<i>Sergentomyia schwetzi</i>	4	5	20, 52, 52, 62, 356
<i>Sergentomyia clydei</i>	5	6	10, 20, 62, 103, 134,212
<i>Sergentomyia minuta</i>	6	7	14, 19,20, 42,74, 93,296
<i>Sergentomyia christophersi</i>	6	7	20, 34, 46, 58, 62, 134, 184
<i>Sergentomyia dreyfussi</i>	7	8	14, 15, 20, 28, 62, 103, 134,171
<i>Sergentomyia antennata</i>	1	2	20, 515

**Cytochrome B:**

[illegible]

## COI:

[illegible]

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1111111122222
12455777734466788901455
T995794824583578113714628
GAAATTTTCCTCTACATCGACAAGAT
.....A.....
pern01(France, Italy, Malta, Tunisia, Morocco) .....T.....T.....T.....
pern02(Italy) .....T.....T.....T.....
pern04(Spain) .....T.....T.....T.....
pern05(Spain) .....T.....T.....T.....
pern06(PNA: Tunisia, Morocco) .....G.....C.....T.....
pern07(PNA: Morocco) .....C.....T.....
pern09(France) .....T.....
PN-main-algerian-haplotype (n=16) .....T.....
PNFE1.2-CYT .....A.....
PN582-CYT .....A.....
PN642-PN643 .....A.....
lcus01 (Tunisia, Morocco) .....G.....TCG.....TC.....TCGA
lcus02 (Morocco) .....G.....TCG.....TC.....TCGA
LC3-549-601-BE1-545-644-509-548-553-331 .....G.....TCG.....TC.....TCGA
LC633 .....G.....TCG.....TC.....TCGA
LC67 .....G.....TCG.....TC.....TCGA
LC637 .....G.....TCG.....TC.....TCGA
LCVIM6 .....G.....TCG.....TC.....TCGA
lcx01 (Morocco) .....GCC.....CC.....CA.....T.A

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Fig. 5: alignment of cytochrome b haplotypes concerning *Phlebotomus perniciosus* and *Phlebotomus longicuspis* and showing exclusively the variable sites reported by Essegir et al. (1997), Pesson et al (2004) and Perrotey et al. (2005). The Algerian haplotypes are written in bold. The variations observed in Algerian specimens are underlined. The stars indicate the sites individualising the haplotypes LC2 and LC637.

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Fig. 4: variable positions observed within *Phlebotomus sergenti* for cytochrome b and cytochrome C oxidase I (*COI*) mitochondrial DNA. Stars indicate the sites characterising the two populations.

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