

Geographical variation in populations of *Phlebotomus* (*Paraphlebotomus*) *caucasicus* (*Diptera: Psychodidae*) in Iran.

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Résumé : Variation géographique des populations de *Phlebotomus* (*Paraphlebotomus*) *caucasicus* (*Diptera : Psychodidae*) en Iran.

Une étude comparative morphologique et moléculaire a été menée sur 11 populations de *Phlebotomus* (*Paraphlebotomus*) *caucasicus* Marzinovsky 1917, capturées dans 7 provinces d'Iran entre 2004 et 2006. Le niveau d'implantation des épines distales du style, le nombre de soies du lobe basal de la coxite et la longueur du troisième segment antennaire mettent en évidence la présence de deux morphotypes chez *P. (Pa.) caucasicus*, une espèce à l'histoire confuse, en raison d'une mise en synonymie peu claire avec *Phlebotomus* (*Paraphlebotomus*) *grimmi* Porchinsky, 1874. Le séquençage d'une partie de l'ADN mitochondrial (cytochrome *b*, gène de la sérine et une partie du gène de la NADH1) et l'analyse en Neighbour-Joining des dites séquences a montré une corrélation partielle entre les morphotypes et les haplotypes, ainsi qu'avec l'origine géographique des exemplaires étudiés. Ces résultats nécessitent d'être pris en compte dans des études futures afin d'apprécier le rôle de chacun de ces morphotypes et/ou haplotypes dans la transmission de *Leishmania major*.

Summary:

A comparative morphological and molecular study was carried out on 11 different populations of *Phlebotomus* (*Paraphlebotomus*) *caucasicus* Marzinovsky 1917 caught in 7 provinces in Iran (2004-2005). Differences in the implantation level of the two distal spines of the style, the number of setae of the basal lobe of coxite, and the length of the third antennal segment, revealed the existence of two morphotypes within *P. (Pa.) caucasicus*, a species having a confused history if we take into account an unclear synonymisation with *Phlebotomus* (*Paraphlebotomus*) *grimmi* Porchinsky, 1874. Sequencing of mtDNA (a fragment of cytochrome *b* gene, tRNA for serine gene and a fragment of NADH1 gene) and Neighbour-Joining analysis showed a partial correlation between morphotypes and haplotypes. We also found a correlation between the latter and the geographical origin of the specimens. These results need further studies in order to appreciate the role of each morphotype/haplotype in the transmission of *Leishmania major*.

Introduction

The story of the binomen *Phlebotomus* (*Paraphlebotomus*) *caucasicus* Marzinovsky, 1917, a proven vector of *Leishmania major* in Iran (11, 17), is rather confused. Indeed, in his initial description, the author did not take into account an earlier description of a very close species: *Phlebotomus* (*Paraphlebotomus*) *grimmi* Porchinsky, 1872 that most researchers considered afterwards as an old synonym of *P. (Pa.) caucasicus*, some of them using the name *P. (Pa.) grimmi* arguing for the principle of priority while some others preferred *P. (Pa.) cau-*

Phlebotomus (*Paraphlebotomus*) *caucasicus*
Phlebotomus (*Paraphlebotomus*) *grimmi*
Leishmania major
morphologie
ADN mitochondrial
vecteur
Iran
Moyen-Orient

Phlebotomus (*Paraphlebotomus*) *caucasicus*
Phlebotomus (*Paraphlebotomus*) *grimmi*
Leishmania major
morphology
mtDNA
vector
Iran
Middle South Asia

casicus, the description of *P. (Pa.) grimmi* being incomplete. From a nomenclatural point of view, the case seemed to be settled with LEWIS proposition (1982) asking the International Commission of Zoological Nomenclature (ICZN) to statute on *P. (Pa.) grimmi* as *nomen nudum* (9). However, the ICZN didn't publish any comment related to *P. (Pa.) grimmi* or *P. (Pa.) caucasicus* since 1915 to 2000 (1, 2), and has not replied yet to our query. Without access to the types (the place of deposit of the type specimens of *P. (Pa.) grimmi* is unknown and the type specimens of *P. (Pa.) caucasicus* which could be located in a research Institute in Moscow seem to be

Figure 1.

Geographical distribution of *Phlebotomus (Paraphlebotomus) caucasicus*, the two morphotypes (C and G) of the species and foci of zoonotic cutaneous leishmaniasis, in Iran, 2006.

Distribution géographique de *Phlebotomus (Paraphlebotomus) caucasicus*, des deux morphotypes de l'espèce (C et G) et du foyer de leishmaniose cutanée d'origine zoonotique, Iran, 2006.



inaccessible), we cannot state on the taxonomic status of these two taxons. Following the last authors reviewing the *Paraphlebotomus* subgenus (3, 4, 5), and waiting for new data, we will be using the name *P. (Pa.) caucasicus*, including for us *P. (Pa.) caucasicus* s. str. and *P. (Pa.) grimmi*. *Phlebotomus (Pa.) caucasicus* has a wide distribution area over Iran (figure 1).

In some regions such as Tehran and Isfahan in the central part, south of Elburz mountain chains in the north, Mashhad in the north-east and Geremi, Pars-Abad and Meshkin-Shahr in the north-west, the species is found as a domestic fly, but, in the other regions it is present in rodent and reptile burrows. It is found as a domestic fly in the northern part of Isfahan, in central Iran, but is active as wild in the eastern part of Iran (10, 16, 17).

These facts suggest that closely related sand flies species (cryptic species) within *P. (Pa.) caucasicus* with different ecological characteristics can be encountered in Iran. We had at our disposal an important collection of *P. (Pa.) caucasicus* caught in various parts of the country. Consequently, we undertook a comparative morphological, morphometrical, and molecular study of these different populations.

Materials and methods

Sand flies collection

Sand flies were collected from different provinces using sticky traps and aspirators. They were stored in 96% ethanol at 4°C. The specimens caught on sticky papers were washed in a bath of acetone before being stored. Since more reliable morphological characters were available on male specimens, only these were selected for studying their morphological and molecular variability.

Morphological analysis

The head and genitalia of individual male sand flies were cut off in a drop of ethanol, cleared in boiling Marc-André solution, and mounted between slide and cover slide for species

identification using the keys of THEODOR & MESGHALI, 1964 (15). The body related to the specimen was stored dried in a vial at -20°C before DNA extraction.

Specimens were observed using a BX 50 microscope (Olympus, Japan). Measurements were obtained from the Perfect Image software (Aries, Chatillon, France) by using a video camera connected to the microscope.

Statistical analysis

All statistical procedures were carried out on a microcomputer using Systat (version 10, Systat Software Inc., Richmond, CA, USA) and GraphPad Prism for Windows (version 4.03, GraphPad Software, San Diego, CA, USA). The distribution of all parameters was checked for deviations from Gaussian curve by means of Shapiro-Wilk and D'Agostino-Pearson omnibus normality tests. When appropriate, statistical comparisons between morphotypes were performed using the non-parametric Kruskal-Wallis test with a priori level of significance set at $p < 0.05$. Non-parametric tests were used because the number of samples was small, and there was no certainty that the data were normally distributed.

Molecular analysis

Based on ecological conditions where samples were collected and morphological differences observed by morphometric analysis, samples from each morphotype within a given ecological condition were chosen for molecular investigation. Genomic DNA was extracted from the thorax, wings, legs and abdomen of individual sand flies using the QIAmp DNA Mini Kit (Qiagen, Germany) following the protocol used by DEPAQUIT *et al.* (6, 7). PCR and sequencing were performed according to ESSEGHIR *et al.* (1997) and DEPAQUIT *et al.* (2005) (8, 7). Sequences were aligned using CLUSTALW software package (www.ebi.ac.uk/clustalw) and examined using Neighbour-Joining (NJ) (12).

Results

Morphological approach

As shown on table I, 11 populations of *P. (Pa.) caucasicus* from 7 provinces of Iran were collected. Morphological observations revealed two distinct morphotypes called C and G in the present study. Regarding morphotype G, the two terminal spines on the style were not implanted at the same level, which seemed to be in agreement with the description of *P. (Pa.) grimmi* whereas morphotype C had two terminal spines implanted at the same level, as described for *P. (Pa.) caucasicus* (figure 2).

Comparison of morphological parameters showed that the number of setae on the basal lobe of the coxite was slightly lower in morphotype G than in morphotype C. However, in the light of the small number of samples collected for morphotype G, this difference did not reach statistical significance ($P = 0.0671$). We also found that the length of the third antennal segment was significantly smaller in morphotype G as compared to morphotype C ($P < 0.01$). With the exception of these two parameters, no other statistically significant morphometric difference could be noted (see figures 3 and 4 and table II).

Except for three samples (one morphotype C in Urmia and two G in Isfahan), these morphotypes have completely different geographical distribution in the country (figure 1). As such morphotype C is common in all ZCL foci, whereas morphotype G is mainly found in the north-west of Iran.

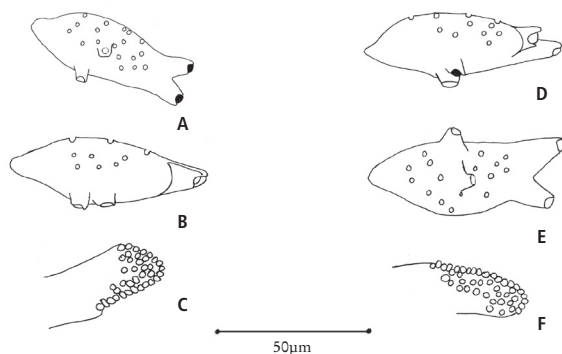
Tableau I.

Details of collected samples used in the study of intraspecific variation within *Phlebotomus* (*Paraphlebotomus*) *caucasicus* populations in Iran (2004-2005).
 Échantillons collectés et utilisés lors de l'étude de variation intraspécifique des populations de *Phlebotomus* (*Paraphlebotomus*) *caucasicus* en Iran (2004-2005).

province	city	latitude	longitude	altitude (meter)	bioclimate	village/district	collection method	collection sites	area	date of collection
Khorassan-e-Razavi	Neishabur	36°12N	58°48E	970	moderate, cold in mountainous area	Dehnoo, Ghalenoo	sticky trap	outdoor	plain	June 2005
	Sabzevar	36°52N	57°47E	985	moderate, very hot and dry in desert	Mahal-e-Mazar Jovin	sticky trap sticky trap	outdoor indoor	plain mountain	June 2005 June 2005
Ardebil	Meshkin-shahr	38°30N	47°60E	1335	moderate, cold in mountainous areas	Ghoort-Tapeh	sticky trap	indoor	plain	Aug. 2004
Eastern-Azerbaijan	Tabriz	38°07N	46°14E	1366	cold in mountainous areas, moderate in plain	Emamie	hand-catch	indoor	plain	June 2005
Western-Azerbaijan	Urmia	37°33N	45°06E	1340	mediterranean, cold in mountainous areas, moderate and rather warm near the lake	Sheikh-Tapeh	hand catch	outdoor	plain	June 2005
Kerman	Kerman	30°21N	57°05E	1757	arid climate, hot in summer, cold in winter	Mahaleh Shahr	sticky trap	indoor	plain	June 2005
Yazd	Yazd	31°54N	54°24E	1240	desert climate, hot and arid in summer, cold and arid in winter		hand-catch	indoor	plain	Sep. 2005
Isfahan	Isfahan	32°34N	51°44E	1590	desert climate, hot in summer, cold in winter	Parvaneh	sticky trap	outdoor	rodent burrow	June 2005
						Komshecheh Joibareh	sticky trap sticky trap	outdoor indoor	plain plain	June 2005 June 2005

Figure 2.

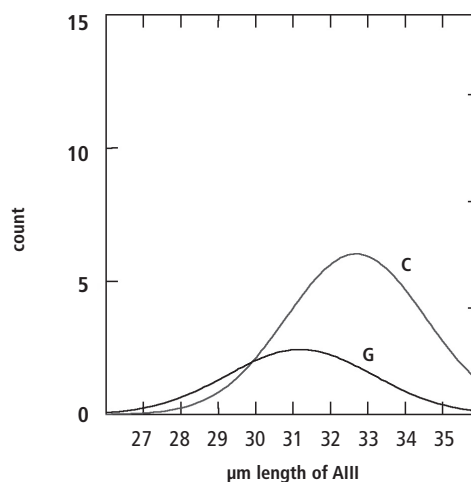
Morphological variation between different morphotypes of *Phlebotomus* (*Paraphlebotomus*) *caucasicus*, from Iran.
 Variations morphologiques entre les différents morphotypes de *Phlebotomus* (*Paraphlebotomus*) *caucasicus*, Iran, 2006.



A, B and C: morphotype C
 D, E, and F: morphotype G
 A, B, D, E: styles.
 C and F: basal lobes of coxites

Figure 3.

Distribution of the length of third antennal segment (AIII) in morphotypes C and G of Iranian *Phlebotomus* (*Paraphlebotomus*) *caucasicus* populations.
 Distribution de la longueur du 3^e segment antennaire (AIII) des morphotypes C et G de *Phlebotomus* (*Paraphlebotomus*) *caucasicus* en Iran.



Molecular approach

PCR amplification with the primer-paired CB3-PDR and N1N-PDR was performed for all samples from different geographical areas and for both morphotypes. All samples uniformly showed 549bp length PCR product, including 336 bp of 3' end of cytochrome B gene, 67 bp of complete tRNA for serine, 22 bp interval sequences (including stop codons), and 124 bp of 3' end of NADH1 gene. The results of all 23 selected samples are deposited in Genebank (accession numbers EF017349-EF017371).

There were 14 (3.1%) polymorphism sites bearing 13 haplotypes among the 23 sequenced specimens. The region was AT rich, with a proportion of 79.7%. Pairwise genetic similarity was between 98 and 100%. Some samples with identical sequences originated from different localities or belonged to different morphotypes as, in some cases both morphotypes showed similar genotype.

Comparison among CytB, tRNA for serine and NADH1 loci of the sequence at the nucleotide level showed 3.8%, 1.5% and 2.4% variation, respectively. At amino acid (AA) level, NADH1 locus showed 100% similarity, but 1% variation was observed for CytB. Rate of informative substitution in CytB, NADH1 and tRNA for serine were 37.5%, 50%, and 100% in that order. Most substitutions were silent, whe-

reas 63.4% of them occurred in the 3rd position of codons. It should be noted that the third substitutions in the first position of codons were found in one sample (252-Cau C-Kerman) originating from Kerman province near central part of the country which were transversion. Other substitutions were transitions (78.6%).

Distance analysis of sequences indicated that there were two main branches. One of them includes exclusively haplotypes correlated to morphotype C whereas the other includes haplotypes correlated to both morphotypes (figure 5). The morphotype C displayed more variability than the morphotype G. Haplotypes of the morphotype G were similar except for one specimen collected in Urmia.

The morphotype G was found in three close provinces, including Western-Azerbaijan, Eastern-Azerbaijan and Ardebil (mostly mountainous areas, cold in winter and temperate in summer) located in the north-west of Iran. The morphotype C was found in the central, north-eastern and south-eastern part of Iran. It should be mentioned that the two specimens which were similar to morphotype G and found in Isfahan had sequences similar to those of other specimens (morphotype C) from this area. Moreover, one specimen was found as morphotype C in Urmia, North-West of the country (figure 1).

Figure 4.

Distribution of number of setae on the basal lobe of male coxites in morphotypes C and G of Iranian *Phlebotomus (Paraphlebotomus) caucasicus* populations.

Distribution du nombre de soies sur le lobe basal du coxite mâle chez les morphotypes C et G des population de *Phlebotomus (Paraphlebotomus) caucasicus* en Iran.

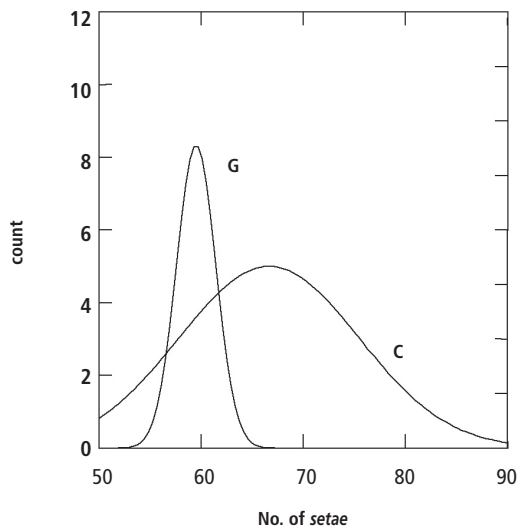


Figure 5.

Neighbour-Joining tree inferred from 459 bp of mtDNA sequences including 288 bp of 3' end CytB gene, 67 bp complete tRNA for serine and 82 bp of 3' end NADH1 gene of *Phlebotomus (Paraphlebotomus) caucasicus* populations from Iran.

Arbre phylogénique Neighbour-Joining tiré des 459 pb des séquences ADNmt comprenant 288 pb de l'extrémité 3' du gène *cyt B*, 67 pb de l'ARNt complet pour la serine et 82 pb de l'extrémité 3' du gène de la NADH1 des populations de *Phlebotomus (Paraphlebotomus) caucasicus* en Iran

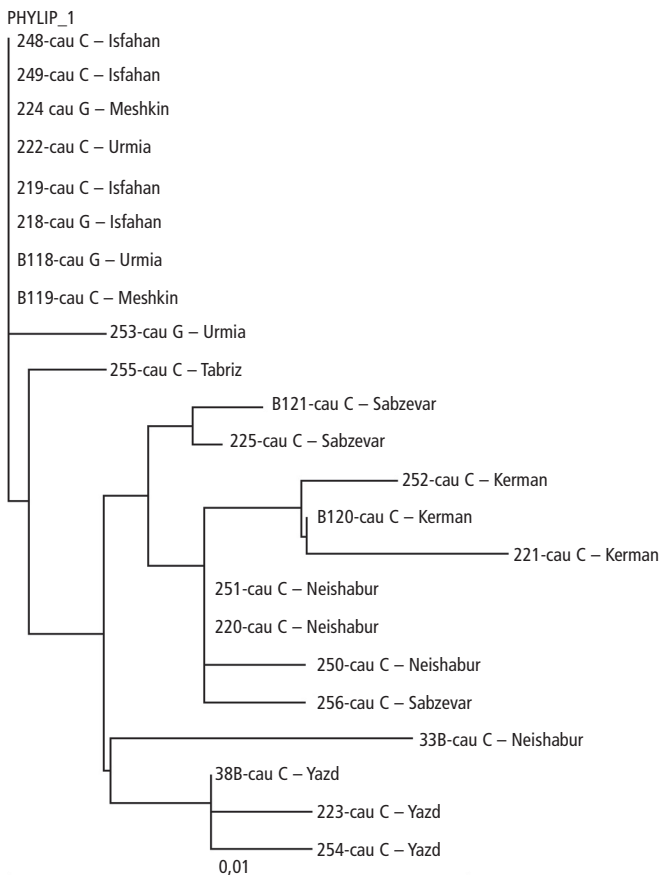


Tableau II.

Morphometric parameters of different Iranian morphotypes of *Phlebotomus (Paraphlebotomus) caucasicus*, 2006.

Paramètres morphométriques des différents morphotypes de *Phlebotomus (Paraphlebotomus) caucasicus*, Iran 2006.

characters	morphotype C (mean ± SD)	morphotype G (mean ± SD)
length of A-III	326.8 ± 18.5	311.9 ± 19.6
length of <i>labrum</i>	253.3 ± 13.4	248.5 ± 17.3
length of coxite	259.7 ± 23.2	256.1 ± 24.7
length of surstyle	327.7 ± 22.7	325.1 ± 19.1
length of genital filaments	231.2 ± 30.1	231.9 ± 29.2
length of genital pump	196.4 ± 9.8	194.3 ± 4.6
length of style	121.0 ± 5.8	118.6 ± 4.0
width of style	41.9 ± 4.3	40.7 ± 4.2
length of <i>aedeagus</i>	65.3 ± 5.0	64.8 ± 6.1
Number of setae	66.6 ± 8.8	59.5 ± 1.9
length of basal lobe	73.9 ± 9.0	74.1 ± 10.0
width of basal lobe	34.1 ± 5.0	33.7 ± 3.4

Discussion

Phlebotomus (Pa.) caucasicus includes two morphotypes (C and G). This morphological observation is strongly supported by statistical analysis. These two morphotypes showed significant variation in the length of AIII and number of setae on the basal lobe of coxite. The mtDNA sequence analysis coincides partly with the morphological data: most of morphotype C specimens were grouped together, although some of them (from Urmia and Isfahan) were associated with morphotype G specimens. Moreover, there were no consistency between the collection sites (rodent burrows, mountainous area, indoor and outdoor) and their sequences. The discrepancy between morphological and molecular characteristics could be explained as follows: *P. (Pa.) caucasicus* and *P. (Pa.) grimmi* are very close to each other from a morphological point of view and might have been diverged lately so that there is not enough accumulated genetic variation to perfectly resolve these two species. No mating barrier might exist yet if speciation is in course. Moreover, the region of mtDNA studied has been previously used for systematics of *Transphlebotomus* (7), *Phlebotomus (Paraphlebotomus) sergenti* (Institute of Public Health, unpublished data), but it might not be suitable for the species under investigation. SIMON *et al.* (2004) have shown that different genes have different genetic variation in organisms (13). We thus conclude that, in order to clarify the taxonomic state of these two species, further investigations on other parts of their genome (without maternal inheritance (14) like the rDNA-ITS2 region) should be done in order to better understanding of the recent evolution of these populations.

Regarding the results of the present study, it is interesting to compare the distribution of *P. (Pa.) caucasicus* morphotypes with the distribution of *Leishmania major*. Morphotype C was recorded in all foci of ZCL due to this parasite, whereas morphotype G was mainly recorded in three provinces (Eastern-Azerbaijan, Western-Azerbaijan and Ardebil) in the north-west of Iran which are considered free from ZCL. Further studies will be necessary to better understanding of the role of each morphotype/haplotype of *P. (Pa.) caucasicus* in the transmission of *Leishmania major*, including epidemiological studies.

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