Original Article

Descriptive and Geometric Morphometry of the Wings of *Phlebotomus sergenti* Populations in Central Morocco

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Abstract

Background: *Phlebotomus sergenti*, the proven vector of *Leishmania tropica*, the causative agent of anthroponotic cutaneous leishmaniasis, is widely distributed in Morocco. Previous works using molecular markers (Internal Transcribed Spacer 2 rDNA and Cytochrome B mtDNA) hypothesized the existence of multiple closely related populations of sand fly species (cryptic species) that would exhibit distinct vectorial capacities. This work studies morphotypic diversity using traditional and geometric morphometry analyses carried out on *Ph. sergenti*'s wings from central Morocco, where active *L. tropica* transmission occurs for 30 years.

Methods: Descriptive characteristics (size and shape) of the right wings were measured in *Ph. sergenti*'s specimens collected from fourteen stations in central Morocco. Both traditional and geometric morphometry methods were used to analyse geographic variations in *Ph. sergenti* wing's size and shape.

Results: These analyses support the existence of distinct *Ph. sergenti* populations, enlightening significant phenotypic variations of *Ph. sergenti*'s wings, regarding their size and shape, depending on geographic origin. In addition, traditional and geometric morphometric analyses of the wing's length, centroid size, β, ɵ, and γ distances allowed clear discrimination of *Ph. sergenti* sub-populations.

Conclusion: These data pinpoint the adaptative ability of *Ph. sergenti* to local environmental conditions. Additional studies are now required to further shed light on the genetic structure of *Ph. sergenti* populations in Morocco.

Keywords: *Phlebotomus sergenti*; Wing traditional morphometry; Wing geometric morphomery; Morocco

Introduction

Sand flies (Diptera, Psychodidae) are vectors of human pathogenic micro-organisms, including parasites of the genus *Leishmania*, bacteria (*Bartonella bacilliformis*), and various arboviruses belonging to the genera *Phlebovirus* and *Vesiculovirus* (1–3). They represent, therefore, a public health scourge. In Morocco, the phlebotomine fauna encompasses 24 species. Six species are proven, or suspected vectors involved in visceral or cutaneous leishmaniases (4).

Variability in genetic, biochemical, morphometric, or reproductive features between allopatric populations of the subfamily Phlebotominae are frequent and probably favored by their large

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geographic distribution and the limited dispersal capacity of populations with reproductive activity near their breeding site (5). These would lead to speciation processes not detectable with dichotomic keys (6). *Phlebotomus sergenti*, Parrot, 1917, is a proven *Leishmania tropica*, vector, the causative agent of anthroponotic cutaneous leishmaniasis (ACL) (1, 7–9). *Phlebotomus sergenti* is distributed from the southern to the western Mediterranean basins. It is replaced in the northeast of this region by *Ph. similis* Perfiliev, 1963 (10). Its distribution extends eastward into Iran, the former USSR, Pakistan, and India. Such geographic distribution vastly exceeds that of *L. tropica* (10). The genetic diversity of *Ph. sergenti* investigated with Internal Transcribed Spacer 2 (ITS2) rDNA and/or Cytochrome B (Cyt b) mtDNA lightened the possibility of closely related sand fly species (cryptic species) that may have distinct vectorial capacities (11-15). *Phlebotomus sergenti* is widely distributed throughout Morocco, with a preference for semi-arid habitats (16, 17). In ACL foci, *Ph. sergenti* is closely associated with the human habitat and involves a domestic and peridomestic transmission of *L. tropica* (17, 18). The analysis of the population's genetic structure of *Ph. sergenti* from Moroccan foci of Taza, Azilal, and Essaouira, using Cyt b mtDNA, disclosed the presence of three mitochondrial lineages with a striking genetic diversity in the Azilal focus (12).

Geometric morphometry (GM) has been applied to analyze morphological variations of insect wings (19). It has a taxonomic value for specimens belonging to puzzling morphological taxa (20) and highlights Spatio-temporal dynamics of some *Phlebotomus* populations; *Ph. tobbi* Adler and Theodor 1930, *Ph. papatasi* (Scopoli, 1786), *Ph. ariasi* Tonnoir 1921 and *Ph. sergenti* (21–25). In this work, traditional and geometric morphometry analyses were carried out on *Ph. sergenti*'s wings from central Morocco, where active *L. tropica* transmission occurs for 30 years (26).

Materials and Methods

Study area

The studied area locates in central Morocco (Fig. 1), where cutaneous leishmaniases (CL) are widespread (26); the first CL case due to *L. tropica* was reported in 1986 in Azilal (27). The environment is diverse, with altitudes varying from 491m to 1650m above sea level. It encompasses the Tadla plain, the phosphate plateau, and the High and Middle Atlas Mountains (Fig. 1, Table 1).

The climate is continental, with a wet season (November to March) and a dry season (April to October). The annual average temperature is 18 °C but ranges from 2 °C to 40 °C. The temperatures recorded during the capture period are given in Table 1. Precipitation varies from 100mm to 1200mm. Béni Mellal-Khénifra region endow the natural forest of holm oak (*Quercus rotundifolia*), junipers (*Juniperus phoenicea*), thuja (*Tetraclinis articulata*), pines (*Pinus halepensis*, *Pinus pinaster*), cedar (*Cedrus atlantica*), poplar (*Populus nigra*), and carob tree (*Ceratomia siliqua*). This region also has agricultural activity; cereals, olives, citrus fruits, sugar beets, grenadines, almonds, apples, carobs, sesame, and vegetables (28).

Sandflies sampling and identification

Sampling was carried out using sticky paper traps placed in domestic, peridomestic, and wild biotopes in fourteen stations between May and October 2017. Traps were left in the field for a single night to avoid the deterioration of wings. In the laboratory, each specimen was dissected under a binocular. We first separated the wing for each male, and then the head and genitalia were dissected and mounted in Canada balsam. Next, we identified specimens by examining the morphology of the external genitalia. Identification was performed using published keys and descriptions (10, 29).

Wings preparation

Well-preserved wings of specimens identified as *Ph. sergenti* were prepared following the described protocol (30), with modifications by reducing the incubation time to 10min in 5% KOH, in Methylene Blue to 10min. This is sufficient to visualize veins and ribs without damaging the wings. After treatment, wings are mounted in Canada balsam on marked slides and photographed with a digital camera (Leica microsystem CH) connected to a microscope (Leica) in the Museum of Natural History of Marrakech Cadi Ayyad University, Morocco.

Measurements

Measures were carried out using the Gryphax 12.0 software (Jenoptik) (31). In addition, pictures were processed with tps-UTIL 32 version 1.74 and tps-Dig 2 version 2.30 software (32). We considered the 17 landmarks already used in previous studies of other sand fly species (21, 22). These landmarks are located at the wing veins' intersections with their margin and the crossroads of the transverse veins with the prominent veins, as depicted in Fig. 2. The size of *Ph. sergenti* wings is given by measures of length (5–17), width (1–9), and centroid size (CS) (Fig. 2). The CS is defined by the square root of the sum of the squared distances between the centre of the configuration of landmarks and each landmark. It was extracted from each matrix using MorphoJ 1.07a version 1.8.0_251 software (33).

The analysis of the wing shape is based on the coordinates of the 17 landmarks and seven distances α (2-12), β (12-11), δ (1-11), ε (3-12), ɵ (4-11), π (10-11) and γ (11-14) (5, 25).

Data analysis

Traditional Morphometry

Traditional morphometry aims to describe the wing's morphology and delineate quantitative morphological characteristics that allow discrimination of *Ph. sergenti* subpopulations. Firstly, we performed a descriptive analysis of the length, width, CS, and seven distances. Then, the mean, standard deviation, median, mode, and variation ratio were calculated. Next, the normality and variance homogeneity assumptions were analysed for each variable using normalized plots. Secondly, for each character, the Student t-test was used to compute the significance of the differences at a significance level of 0.05. Finally, the link between each measure (size, shape) (quantitative variable) and station (qualitative variable) was examined by estimating the correlation ratio (r) that is used for allometric analysis to test the link between the wing's shape and size (CS, length, width). Univariate and bivariate statistical analyses were performed using Microsoft Excel 10.

In addition, a typology of wing morphs (size, shape) according to the geographical origin was made using canonical analysis, carried out with the STATISTICA software (34).

Geometric morphometry

The geometric morphometric analysis allows the visualization and comparison of the geometric configurations of *Ph. sergenti* wings. First, Ward's method (35) is used to construct the hierarchical classification dendrograms for wing size and shape, using Past 3.23 software (36). Then, the *Ph. sergenti* wing geometric configurations were investigated using a Generalized Procrustes Analysis (GPA) (37). After GPA, shape variables were measured and analyzed by the principal component of the "relative warps" scores calculated using the Past 3.23 software (36).

Results

This study used descriptive and geometric morphometry analyses of *Ph. sergenti* wings. It investigates the size and shape of a set of stations from central Morocco differing in their ecological characteristics. Ninety-two wings of *Ph. sergenti* were collected in 14 stations in central Morocco (Table 1).

Wing size and shape

As described in the Material and methods section, the wing's size measured is presented in Supplementary data Table 3. Overall, in males, the length of the right wings is 2137.01±124.05µm, the width is 738.17±69.78 µm, and the CS is 3236.79±160.66µm.

The shape of *Ph. sergenti* wings is characterized by seven distances: α, β, δ, ε, ɵ, π, and γ (Supplementary data Table 4). The wing shape varies mainly in the β (12-11), ɵ (4-11), and γ (11-14) distances. All measurements are log-normally distributed.

Allometric analysis was performed to assess the link between wing size and shape by estimating the correlation coefficient. As a result, a positive correlation is recorded particularly between, on the one hand, the 3 distances ɵ, α, and β, and on the other hand, the size parameters, mainly the CS and the width, as shown in Table 2.

Wings variability analysis

To investigate the spatial variations of *Ph. sergenti*'s wing parameters from stations where the *Ph. sergenti* density is low, we gathered samples from stations 2, 3, 4, 5, 7, 10, 12, 13, and 14 into the label "other stations" as shown in (Figs. 3 and 4).

The size of the right wing shows significant differences linked to their geographic origin (Fig. 3), as demonstrated by the correlation ratios computed between the origin and the length, width, or CS (0.68 and 0.56 and 0.74, respectively).

Similarly, the seven distances characterizing the wing shape showed differences according to the stations (Fig. 4), with β, ɵ, and γ as the most variable distances. The correlation ratios between the collection site and the distances α, β, δ, ε, ɵ, π and γ are 0.39, 0.49, 0.37, 0.39, 0.55, 0.31 and 0.46, respectively.

Wings of *Ph. sergenti* of Afourer exhibit peculiar characteristics, as shown in Figs. 3 and 4. Differences were noticed, especially for the CS, β, and ɵ distances. Furthermore, the CS in Afourer is significantly different from all other stations, even the neighbouring ones. The student t-test are; Bzou (t= -10,626), Foum Jamaa (t= -23,531), Tiski (t= -4,281), Ksiba (t= -10,747).

As for results from the descriptive analysis, canonical analyses of the size and shape of *Ph. sergenti* wings, illustrated in figure 5, point to substantial variation according to the wings' geographical origin (sampling site). Canonical analysis of the size (Fig. 5A) discloses that the plane (1-2) explains 92.57% of the total inertia (canonical variate 1: 76.43% and canonical variate 2: 16.14%). These results indicate that the wing size differs from station to station. First, specimens from Afourer and Tiski are grouped on the right of the canonical analysis, followed by those from Bzou, Foum Jamaa, and the other stations (stations 2, 3, 4, 5, 7, 10, 12, 13, and 14). For the wing shape, the projection in the plane (1-2) explains 63.06% of the total inertia (canonical variate 1: 43.23% and canonical variate 2: 19.83%) (Fig. 5B). As for the parameter of wing size, the canonical analysis reveals gradual variations according to the stations.

The hierarchical single-link classification trees for the measured parameters of size (Fig. 6A) and wing shape (Fig. 6B) demonstrate a clustering according to the geographic origin, which again reinforces the results of the canonical analysis. But, again, and particularly for wing size, two groups stand out; the populations of Afourer and Tiski with smaller wings according to measured parameters vary from those of Foum Jamaa, Bzou, or the other stations.

Likewise, the mean configurations of the wing shapes vary according to the geographical origin, with marked differences in the position of landmarks 2, 9, 10, and 11, as presented in Fig. 7.

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Table 1. Stations sampled for *Phlebotomus sergenti* in central Morocco during the period May–October 2017

Stations	N°	Longitude (W)	Latitude (N)	Altitude (m)	Temperature (°C) Mean (Min-Max)	Urbanization	CL cases** (2009 to 2015)	Specimens number
Afourer	1	06°30´00´´	32°13´00´´	491	26 (18-34)	Urban	433	13
Ait Imloul	2	06°38´18´´	32°11´00´´	1118	*	Rural	*	6
Aguelmouss	3	05°50´35´´	33°09´30´´	1217	*	Rural	*	2
Béni Ayyat	4	06°34´01´´	32°12´31´	535	*	Rural	37	1
Béni Mellal	5	06°22´32´´	32°20´32´´	507	27 (23-31)	Urban	90	2
Bzou	6	07°03´18´´	32°05´09´´	429	26.5 (19-34)	Rural	489	11
El Kbab	7	05°31´01´´	32°44´27´´	1198	*	Rural	*	1
El Ksiba	8	06°01´58´´	32°33’54´´	1003	*	Urban	5	8
Foum Jamaa	9	06°59´26´´	31°37´54´´	813	24.5 (17-32)	Rural	386	21
Tagzirt	10	06°12´01’’	32°26´08´´	594	*	Rural	33	3
Tiski	11	06°46´12´´	32°06´44´´	677	25.5 (18-33)	Rural	178	16
Zaouiat Cheikh	12	05°55´01´´	32°38’38´´	798	*	Urban	202	1
Zaouiat Ahansal	13	06°06´15´´	31°49´59´´	1629	*	Rural	2	3
Benssarou	14	06° 09´42´´	32°21´16´´	1650	*	Rural	*	4

*Lack of data.
**(26)
CL: Cutaneous leishmaniasis

Table 2. Correlation coefficient (r) between *Phlebotomus sergenti* wing's parameters (shape and size) during the period May–October 2017

Shape	Size		
	CS	Length	Width
θ	0.69	0.54	0.70
α	0.55	0.32	0.66
β	0.47	0.30	0.48
ε	0.34	0.26	0.28
π	0.32	0.14	0.27
δ	0.26	0.08	0.29
γ	0.08	0.19	0.0007

Table 3. Morphometry of *Phlebotomus sergenti* male's right-wing collected in the study region in central Morocco during the period May–October 2017

Numerical parameters	Length	Width	Centroid size
Mean±SD (µm)	2137.01±124.05	738.17±69.78	3236.79±160.66
(Min-Max) (µm)	(1880-2340)	(598-935)	(2855-3668)
Mode (µm)	1990.00	700.00;725.00; 740.00	3136.00;3149.00; 3181.00
Median (µm)	2170.00	725.50	3205.00
Variation ratio (%)	5.80	9.45	4.96

Table 4. Numerical characteristics of the distances α, β, δ, ε, ɵ, π and γ of *Phlebotomus sergenti* male's right-wing collected in central Morocco during the period May–October 2017

Numerical parameters	α	β	δ	ε	ɵ	π	γ
Mean±SD (µm) **(Min-Max) (µm)**	540.19±70.91 (399-782)	375.61±86.86 (230-835)	173.84±54.86 (108-502)	367.30±60.60 (255-787)	604.79±44.55 (510-690)	260.5±37.65 (200-488)	621.92±104.47 (385-984)
Mode (µm)	510.00; 525.00	420.00	118.0;144.0; 161.0;171.0; 182.0; 203.0	320.00; 350.00	560.00; 600.00	243.00	515.00; 560.00; 610.00
Median (µm)	530.00	359.00	168.0	360.00	605.00	253.00	610.00
Variation ratio (%)	13.13	23.12	31.55	16.50	7.36	14.45	16.80

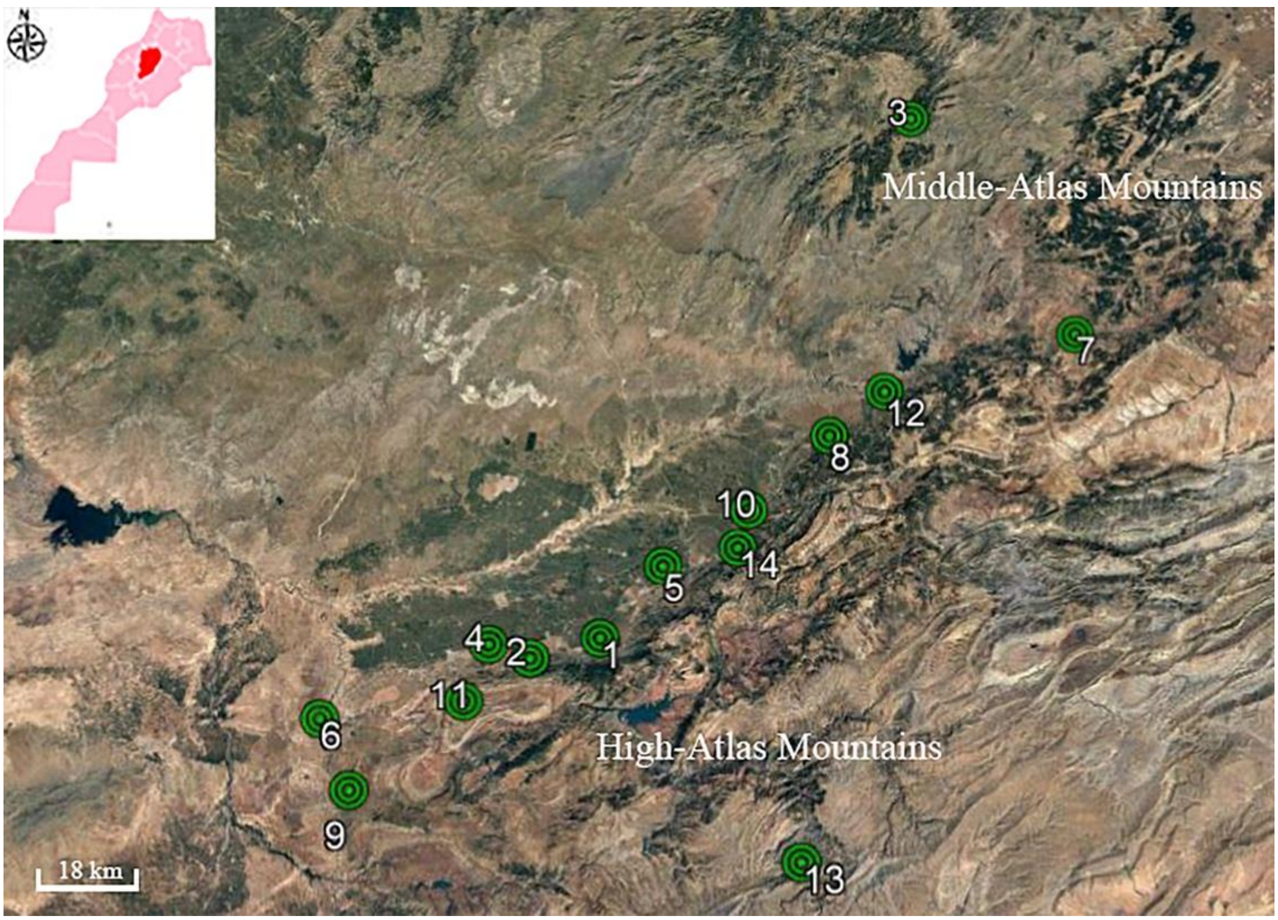

Fig. 1. Location of the sampling area for *Phlebotomus sergenti* populations in central Morocco (Source Google Earth)

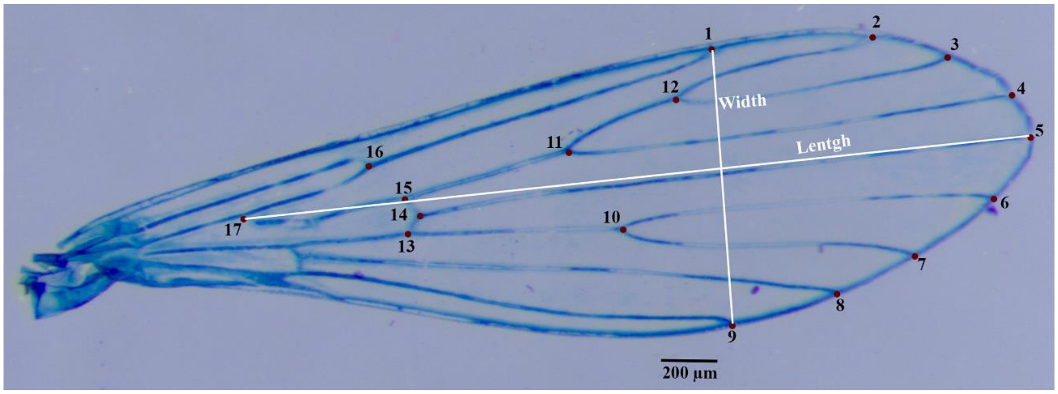

Fig. 2. Location of the 17 landmarks used in this study on the right-wing of *Phlebotomus sergenti*

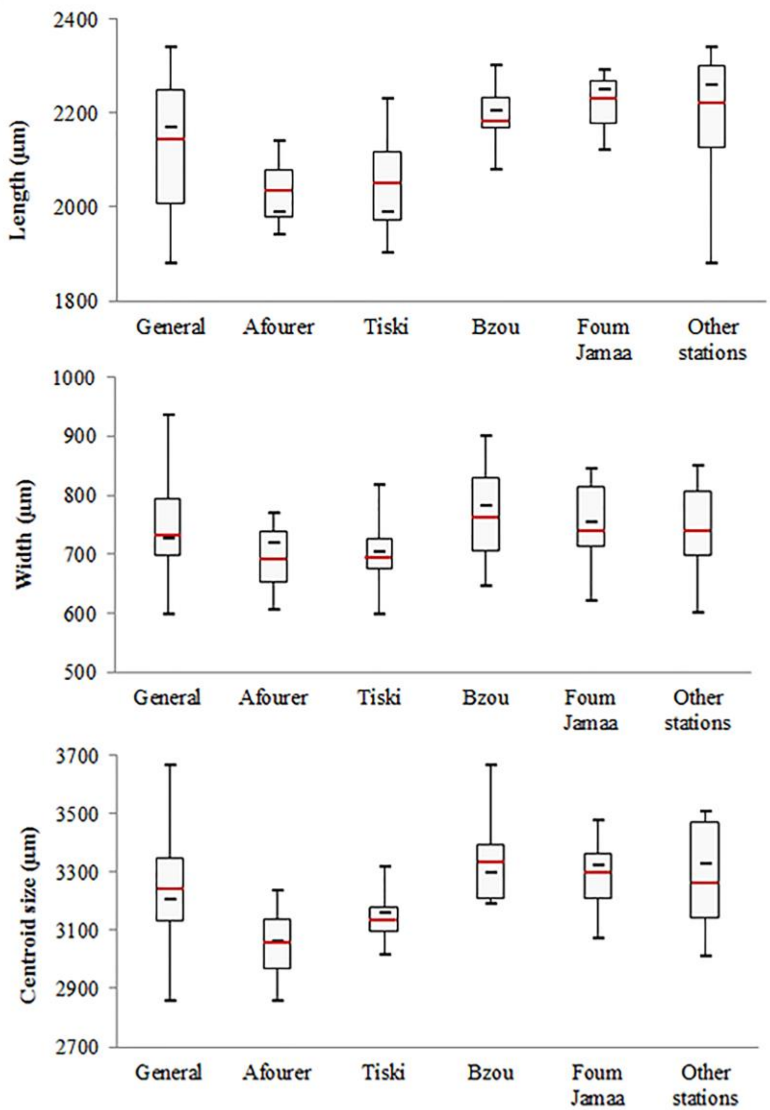

Fig. 3. Boxplots of the lengths, widths, and centroid sizes of *Phlebotomus sergenti* male's right wings in the various station of central Morocco during the period May–October 2017

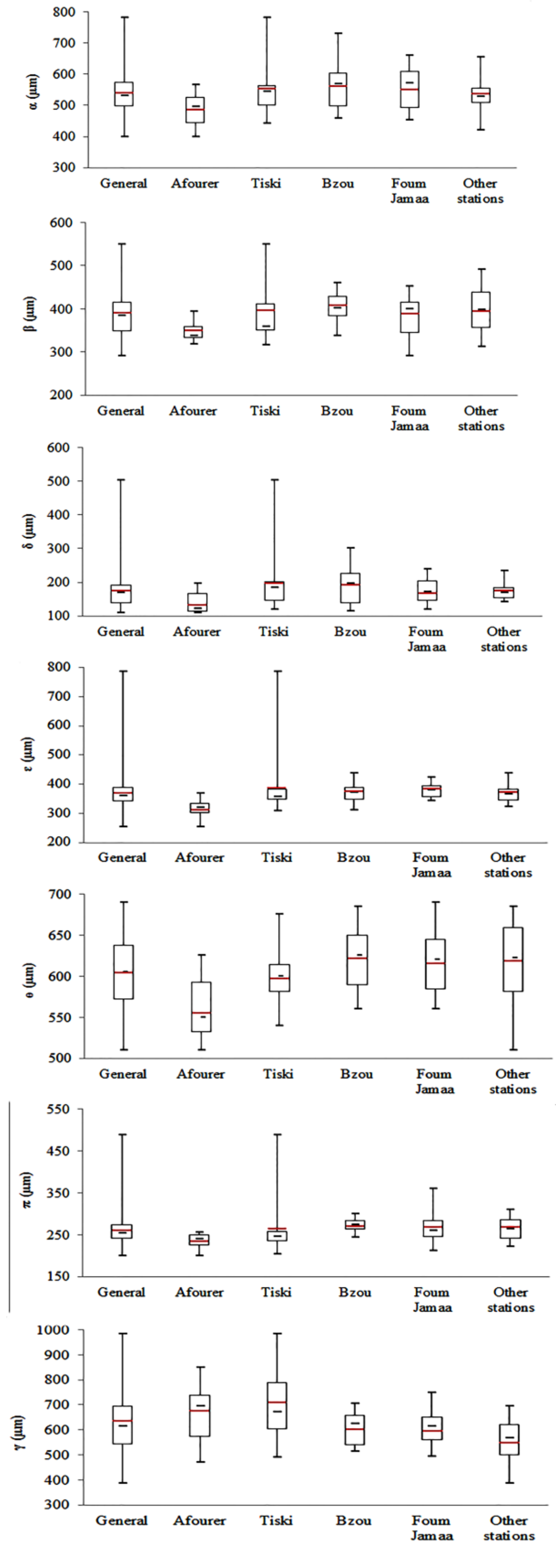

Fig. 4. Boxplots representation of the distances α, β, δ, ε, ɵ, π, and γ of the right wings of *Phlebotomus sergenti*'s males in each station of central Morocco during the period May–October 2017

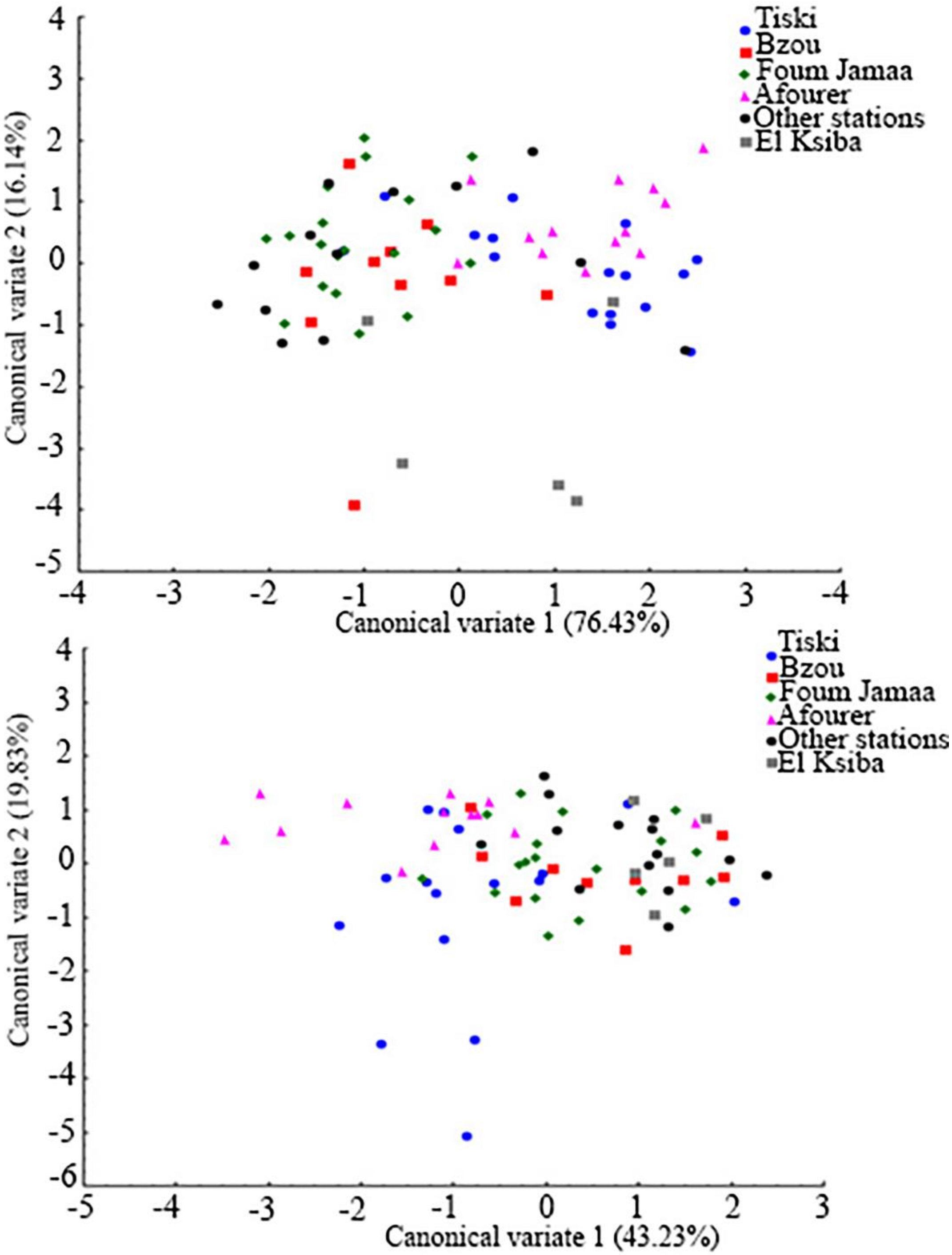

Fig. 5. Canonical analysis of the size variations **(A)** (92.57% of the total inertia) and the shape **(B)** (63.06% of the total inertia) of *Phlebotomus sergenti*'s male right wings depending on the geographical origin of central Morocco during the period May–October 2017

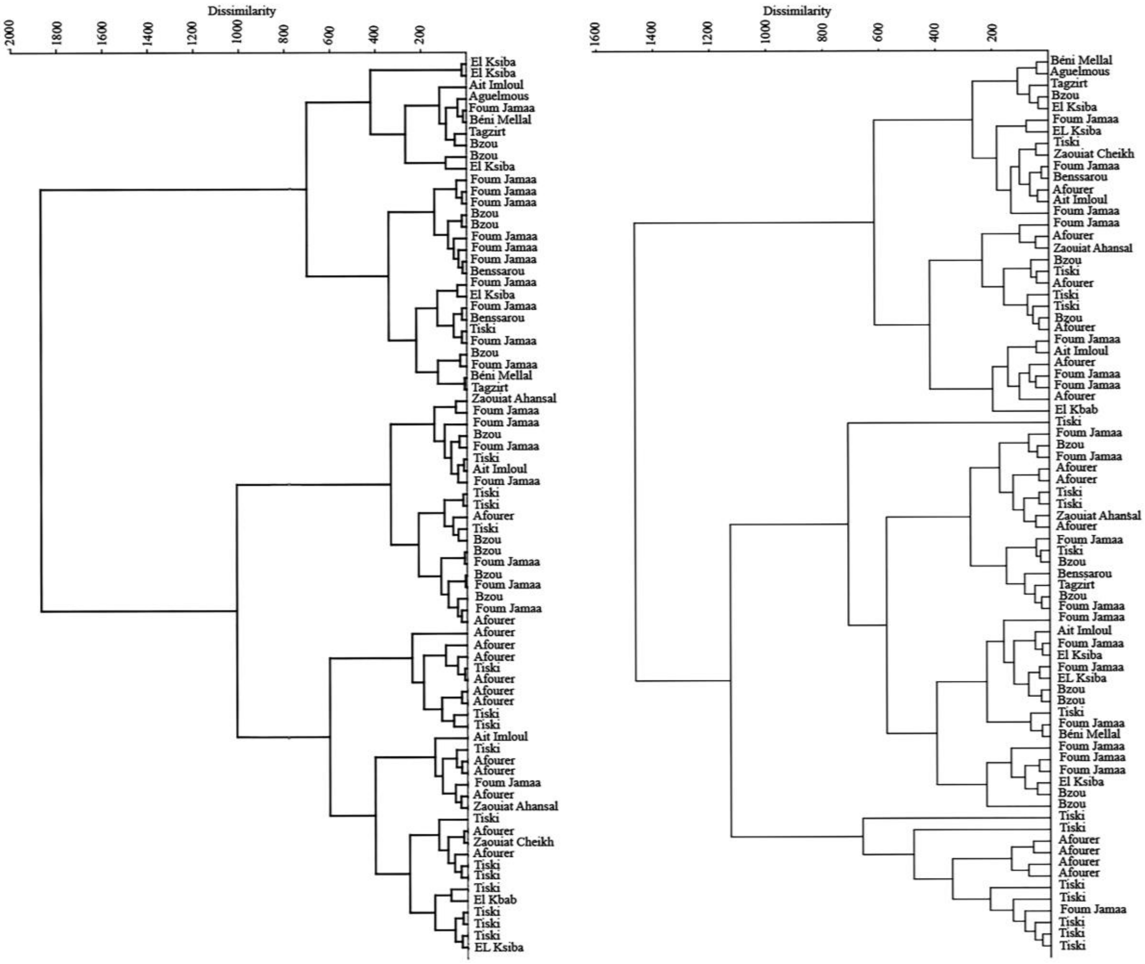

Fig. 6. Hierarchical classifications tree of *Phlebotomus sergenti* right-wings size (A) and shape (B) of central Morocco

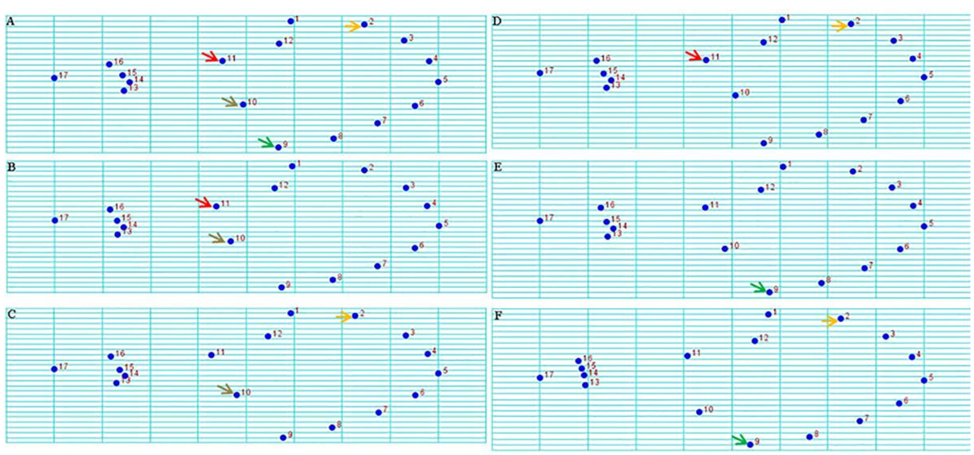

Fig. 7. Superposition of the right-wing landmarks (mean measures) of *Phlebotomus sergenti* males from Afourer **(A)**, Tiski **(B),** El Ksiba **(C),** Bzou **(D),** Foum Jamaa **(E),** and other stations **(F)**. (Arrows of a given color show variations in the position of a given landmark) of central Morocco during the period May–October 2017

Discussion

Phlebotomus sergenti is a proven and primary vector of *L. tropica* in Saudi Arabia, Morocco, Iran and Israel (1, 7–9, 38). Previous studies have considered *Ph. sergenti* a species complex regarding the tremendous genetic diversity recorded over its distribution (11–13, 39). On the other hand**,** the cross-mating study showed that there is no reproductive barrier between *Ph*. *sergenti* from different geographical areas (24), and no statistically important differences were found in wing morphology for all local populations of *Ph. sergenti*, suggesting that the barriers are not sufficient to stop gene flow among local populations of sand flies (25). Despite this ambiguous context, no morphotype indicator is currently available to probe *Ph. sergenti* population diversity. Therefore, the wing's geo-morphometric analyses would help shed light on the population's diversity of *Ph. sergenti*. This has been used successfully on several sand fly species, like *Ph. tobbi*, *Ph. papatasi*, or *Ph. ariasi* (21–23, 30), and has provided evidence of the separation of *Ph. papatasi* populations between the southern and northern slopes of the High Atlas Mountains in Morocco (21). This was further delineated via a genetic analysis of these populations (40). Therefore, we applied descriptive and geometric morphometry analyses to probe *Ph. sergenti* populations' diversity in central Morocco for the first time.

The morphometric parameters of *Ph. sergenti*'s wings sampled in central Morocco show significant variability. The size is variable in length, width, and CS. The shape also showed substantial variations, particularly at the distances; β, ɵ, and γ. In addition, allometric analyses demonstrate a strong link between size and shape variability. A correlation is recorded between CS or wing width on one side and ɵ, α or β, on the other side.

Descriptive analyses point to a link between *Ph. sergenti* wing's size and shape and the geographical origin. Length, width, CS, and the seven distances characterizing the wing's shape differ significantly between stations, with correlation ratios higher than 50%. Population from Afourer locality is notably different, with relatively smaller wings. Multivariate analyses further supported these results. Canonical studies reveal, in addition to the variability, the existence of a gradual variation in size and shape (92.57% and 63.06% of the total inertia, respectively). This population's succession in the factorial plan could testify to a cline variation of the measured parameters.

The results of the geometric analyses corroborate those of the descriptive ones. Hierarchical classifications trace the gradual variation and define two types based on the geographic origin; the Foum Jamaa and Bzou types mainly differ from the Afourer and Tiski types. Relatively smaller wings characterize these last two populations. In addition, superimpositions of the average configurations of *Ph. sergenti* male right wings indicate significant variations in landmarks 2, 9, 10, and 11.

Previous works have focused on the relationship with altitude regarding the origin of the variability of sand fly wings in the natural environment. However, altitude is not an ecological factor, but it can express a variety of climatic factors. In this study, we conducted a bi-monthly sampling to integrate variations in connection with climatic factors in central Morocco. In areas where *Ph. sergenti* is abundant, it was active for only two months, July and August, during the dry period. Therefore, no significant temporal variation in wing parameters (size and shape) was recorded, probably due to the short active period.

Wings of *Ph. ariasi* (France), *Ph. papatasi* and *Ph. tobbi* (Turkey) showed variations in response to an altitudinal gradient (22, 23, 30). But, even via an indirect effect, altitude cannot explain the recorded gradual variation in *Ph. sergenti* from central Morocco. The individualized populations of Afourer and Tiski locate in the same altitudinal zone as Bzou and Foum Jamaa. Furthermore, the altitudinal structuring of sand flies observed in the High Atlas Mountains concerned *Ph. papatasi*, *Ph. ariasi*, and the species of the *Ph. perniciosus* complex (41). The density and distribution of *Ph. sergenti* did not follow any altitudinal gradient (41).

Altitude can also act indirectly through the slope effect. This has been observed in *Ph. papatasi* on the southern and northern slopes of the Moroccan High Atlas (21) and in *Ph. tobbi* (22) and *Ph. ariasi* (23). But this slope effect cannot be involved in the variation we detected in *Ph. sergenti*; the stations compared to Afourer, Bzou, Tiski, and Foum Jamaa are located on the same High Atlas Mountains slope. Moreover, the stations which show *Ph. sergenti* wing variations are located in the same zone of the High Atlas Mountains, at a maximum of 60km apart. Therefore, they are subject to a similar climate, and considering the low dispersal potential of male sand flies, this adds evidence for a microenvironmental adaptation. This phenomenon is also reported in *Ph. ariasi* in the Oiselette massif in the south of France (23).

Furthermore, local habitat adaptation of *Ph. sergenti* populations was observed in the western Moroccan High Atlas Mountains and other *L. tropica* foci. Although *Ph. sergenti* is a widely distributed species, its abundance is linked to a particular microhabitat where shelters are present (areas in basins) and where the dwellings (construction material) are not made of cement (17).

Conclusions

In conclusion, our results underline the diversity of *Ph. sergenti* in Morocco. Descriptive and geometric morphometry analyses are exciting tools for quantifying phenotypic variability in *Ph. sergenti* populations. They also point out the ability of *Ph. sergenti* to adapt to local environmental conditions. The correlation between the phenotypic differentiation and the genetic structure of *Ph. sergenti* in Morocco must be further investigated.

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Ethical considerations

No ethical issue.

Conflict of interest statement

The authors declare there is no conflict of interests.

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