Article

Wings' descriptive and geometric morphometry analyses support distinct *Phlebotomus sergenti* Parrot populations in central Morocco.

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Simple Summary: In Morocco, anthroponotic cutaneous leishmaniases (ACL) are of public health concern. They are caused by *L. tropica,* whose proven vector, *Phlebotomus* (*Paraphlebotomus*) *sergenti*, has an extensive distribution in Morocco. Here, applied descriptive and geometric morphometry to investigate potential population diversity of *P. (Paraphlebotomus) sergenti* and pinpoint clear discrimination of *P. sergenti* sub-populations. Furthermore, the vectorial competence and the genetic structure of the *P. sergenti* population have to be probed to address transmission risk and the emergence of ACL.

Abstract: *Phlebotomus* (*Paraphlebotomus*) *sergenti* Parrot, 1917, the proven vector of *Leishmania tropica* Wright, 1903, the causative agent of anthroponotic cutaneous leishmaniasis, is widely distributed in Morocco. Previous works using molecular markers (ITS2 rDNA and Cyt b mtDNA) have hypothesized the existence of multiple closely related populations of sandfly species (cryptic species) that would exhibit distinct vectorial capacities. In this work, descriptive characteristics of wings (size and shape of the right and left wings) were measured in samples collected from fourteen stations in central Morocco. These analyses support the existence of distinct *P. sergenti* populations, enlightening significant phenotypic variations of *P. sergenti*'s wings, regarding their size and shape, depending on geographic origin. In addition, geomorphometric analyses of wing's length, centroid size, alpha, and beta distances allowed clear discrimination of *P. sergenti* sub-populations. These data pinpoint the adaptative ability of *P. sergenti* to local environmental conditions. Additional studies are now required to further shed light on the genetic structure of *P. sergenti* populations in Morocco.

Keywords: *Phlebotomus sergenti*; Wing Morphometry, Wing Geomorphomery, Morocco

1. Introduction

Sandflies (Diptera, Psychodidae) are vectors of human pathogenic micro-organisms, including parasites of the genus *Leishmania*, bacteria (*Bartonella bacilliformis*), and of various arboviruses belonging to the genera *Phlebovirus*, *Vesiculovirus,* and *Arbovirus* [1-3]. They represent, therefore, a public health scourge.

Variability in genetic, chemical, morphometric, or reproductive features between allopatric populations of the Phlebotominae subfamily are frequent and probably favored by their large geographic distribution and the limited dispersal capacity of populations that have reproductive activity near their breeding site [4]. These would lead to speciation processes not detectable with dichotomic keys [5]. *Phlebotomus* (*Paraphlebotomus*) *sergenti*, Parrot, 1917, is a proven *Leishmania tropica* vector (Wright, 1903), the causative agent of anthroponotic cutaneous leishmaniasis [1,6,7]. *P. sergenti* is distributed from the southern to the western Mediterranean basins. It is replaced in the northeast of this region by *P. (Paraphlebotomus) similis* [8]. Its distribution extends eastward into Iran, the former USSR, Pakistan, and India. Such geographic distribution vastly exceeds that of *L. tropica* [8]. Using *ITS2* rDNA and/*or Cyt b* mtDNA, previous works lightened a high genetic diversity and the possibility of closely related sandfly species (cryptic species) with distinct vectorial capacities [9-11]. In Morocco, *P. sergenti* is widely distributed throughout the country with a marked preference for semi-arid habitats but presents high adaptability to diverse bioclimatic conditions [12,13]. In ACL foci, *P. sergenti* is closely associated with the human habitat and involves a domestic and peridomestic transmission of *L. tropica* [13,14]. The analysis of the population's genetic structure of *P. 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 [10].

Geomorphometry (GM) that uses morphometric geometry principles to analyze morphological variations of an organism can be applied to an insect's wings [15]. It is a helpful taxonomic tool for identifying specimens belonging to puzzling morphological taxa [16]; it also has an interest in analyzing Spatio-temporal variations of various *Phlebotomus* species; *P. tobbi, P. papatasi,* and *P. ariasi* [17,18].

In this work, morphometric and geo-morphometric analyses were carried out on *P. sergenti's* wings to compare populations from central Morocco, where *L. tropica* transmission took place actively for over 30 years [19].

References should be numbered in order of appearance and indicated by a numeral or numerals in square brackets—e.g., or [2,3], or [4–6]. See the end of the document for further details on references.

2. Materials and Methods

2.1. Study area

The studied area locates in central Morocco (Figure. 1), where cutaneous leishmaniases (CL) are widespread [19]; the first CL case due to *L. tropica* was reported in 1986 in Azilal [20]. The environment is diverse, with altitudes varying from 491 m to 1650 m elevations. It encompasses the Tadla plain, the phosphate plateau, and the High and Middle Atlas, with altitudes ranging from 491 m to 1650 m elevation (Figure 1, Table 1).

Table 1. *P. sergenti* sampling stations in central Morocco.

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

* Lack of data.
**[19]
CL: Cutaneous leishmaniasis
R: Right; L: Left

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. Precipitation varies from 100 mm to 1200 mm. Béni Mellal-Khénifra region is endowed by natural forests 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 an agricultural activity; cereals, olives, citrus fruits, sugar beets, grenadines, almonds, apples, carobs, sesame, and vegetables [21].
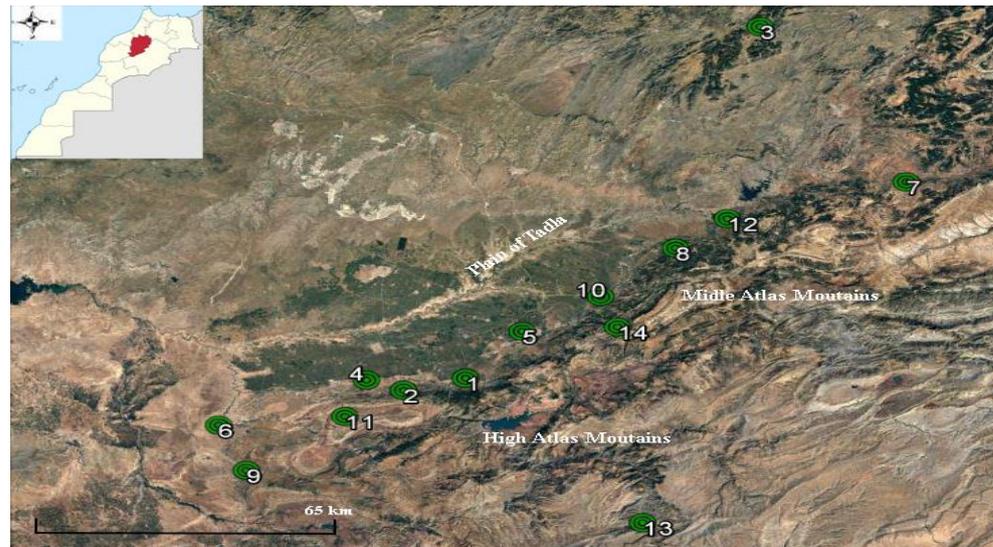
Figure 1. Location of the sampling area for *P. sergenti* populations in central Morocco (1: Afourer ; 2: Ait Imloul ; 3: Aguelmouss ; 4: Béni Ayyat ; 5: Béni Mellal ; 6: Bzou ; 7: El Kbab ; 8: El Ksiba ; 9: Foum Jamaa ; 10: Ragzirt ; 11: Tiski ; 12: Zaouiat Cheikh ; 13: Zaouiat Ahansal ; 14: Benssarou) (Source Google Earth).

2.2. Sandflies sampling and identification

Sampling is carried out using sticky traps placed in domestic, peridomestic, and wild biotopes in fourteen stations between May and October 2017. Traps were left in the field a single night to avoid deteriorations of wings. In the laboratory, each specimen is dissected under a binocular. First, the wings are separated, then the head and genitalia of males or the spermathecae of females are dissected and mounted in Canada balsam. Males are identified by examining the morphology of the external genitalia. The examination concerned the spermathecae and the cibarial and pharyngeal teeth for females. Identification is performed using published keys and descriptions [22,8].

2.3. Wings preparation

Well-preserved wings of specimens identified as *P. sergenti* were prepared following the described protocol [23], with modifications for better visualization of veins and ribs. After treatment and coloring, 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.

2.4. Morphometry and Geomorphometry

The measurements were carried out using the Gryphax 12.0 software (Jenoptik). Pictures were processed with tps-UTIL 32 version 1.74 and tps-Dig 2 version 2.30 software [24]. In addition, the 17 landmarks already used in studies concerning other sandfly species were considered [17,18]. These landmarks are located at the intersections of the wing veins with their margin and the crossroads of the transverse veins with the prominent veins, as depicted in Figure 2. The size of *P. sergenti* wings is determined via the measure of the length (5-17), the width (1-9), and the centroid size (CS) (Figure 2). The latter is defined by the square root of the sum of the squared distances of a set of reference points to their centroid. The analysis of the wing shape is based on seven distances α, β, δ, ε, θ, π and γ and on the coordinates of the 17 landmarks [5,25].
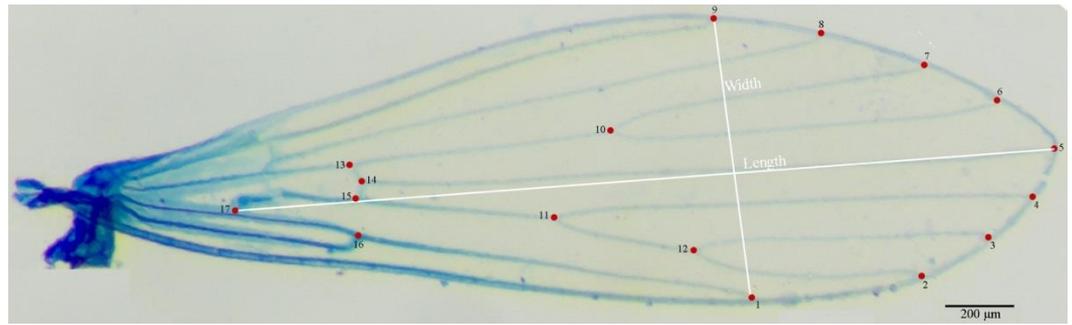
Figure 2. Location of the 17 landmarks used in this study on the right-wing of *P. sergenti*.

First, we performed a descriptive analysis of the length, width, CS, and each of the seven distances. Then, the mean, standard deviation, median, mode, and variation ratio were calculated. Next, the normality and variance homogeneity assumptions were analyzed for each variable, using normalized plots. Finally, the correlation ratio was calculated to examine the link between a qualitative variable (wing side, station) and a quantitative one (size, shape). For each character, the Student t-test was used to verify the significance of the differences with a significance level of 0.05. All statistical analyses were performed with Microsoft Excel 10. Then, the variations in the wing's shape and size according to the geographical origin are characterized using canonical analysis, carried out with the STATISTICA software [26]. Finally, stations with low specimen numbers were grouped and considered an out-group.

Unique link hierarchical classification dendrograms were performed for wing size and shape, according to the method of Ward's, using the Past 3.23 software [27,28]. Visualization and comparison of the geometric configurations of *P. sergenti* wings are performed by the Procrustes superposition method or a Generalized Procrustes Analysis (GPA) [29], using the MorphoJ 1.07a software version 1.8.0_251 [30].

3. Results

This is the first study using descriptive and geomorphometric analyses of *P. sergenti* wings that investigates the size and shape of a set of stations from central Morocco differing in their ecological characteristics. Ninety-six wings of *P. sergenti* were collected in 14 stations of central Morocco (Table 1), 92 male wings (77 right and 15 left), and 4 right wings in females.

3.1. Wing size and shape measures

The wing's size measured as described in the Material and methods section is presented in Supplementary data `Table S1. Overall, in males, the length of the right wings is 2137.01 ± 124.05 µm, the width is 738.17 ± 69.78 µm, and the CS is 3236.79 ± 160.66 µm. In the left wings, the length, width, and CS are 2293.27 ± 65.79 µm, 790.00 ± 40.17 µm, and 3484.06 ± 95.03 µm, respectively. The length of the right wings of females is 1897.50 ± 457.04 µm, the width is 687.56 ± 142.96 µm, and the CS is 3033.50 ± 342.53 µm. In males, the left-wing are bigger in size; significantly longer (student t-test = -11.36, p = 0.000), wider (t = -4.79, p = 0.000) and larger CS (t = -12.41, p = 0.000) than the right one (Figure 3). The correlation ratios between wing's side and length, width, and CS are 0.73 and 0.46, and 0.84, respectively. Female wings are, in general, smaller than males. The normality test and the homogeneity distribution for the three characters' right and left male wings showed polymodal distributions, particularly of the length and CS responsible for sub-populations structuring.
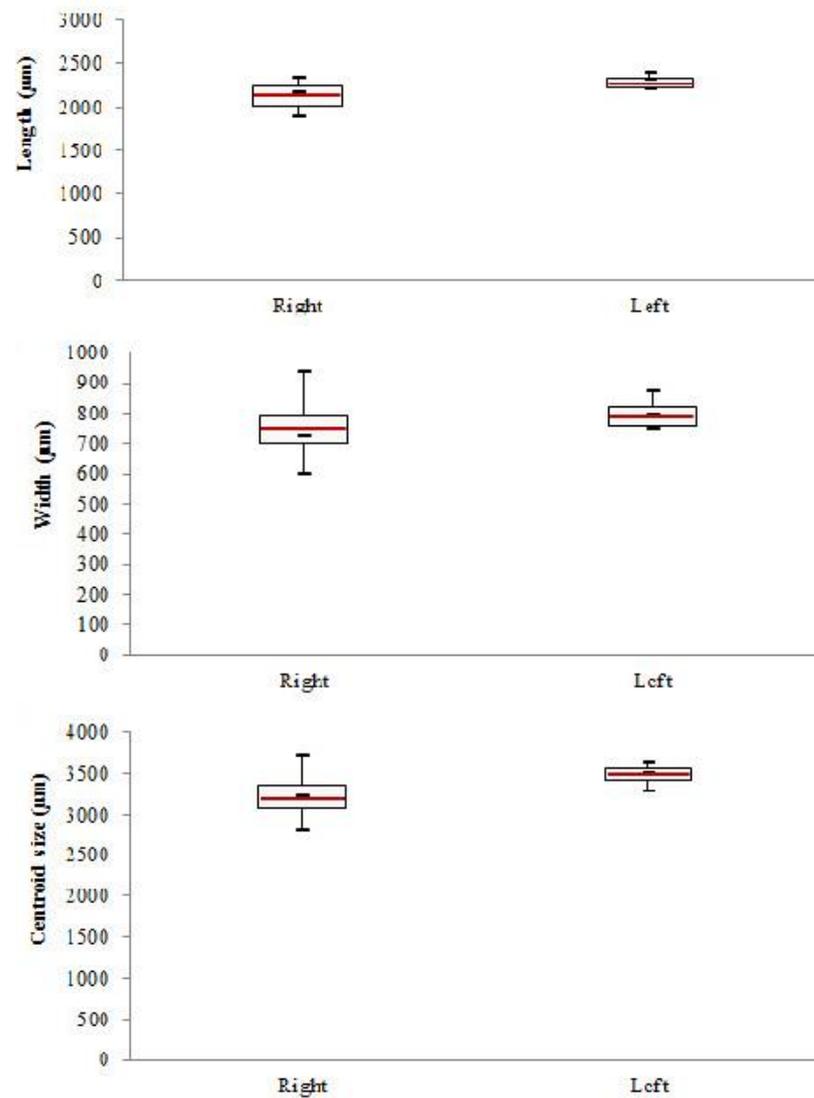
Figure 3. Boxplots representation of length, width, and centroid size of *P. sergenti*'s right and left male wings. The correlation ratio between the wing side and the length, width, or size of CS are 0.73, 0.46, and 0.84, respectively.

The shape of *P. sergenti* wings is characterized by seven distances; α, β, δ, ε, θ, π, and γ (Supplementary data Table S2). The left wings have longer distances α, β, δ, θ, ε and π than the right ones (Fig. 4). The differences are statistically significant for the distances α and β (t = -2.65, p = 0.019; t = -2.63, p = 0.020, respectively). The distance γ is the only one that appears to be smallest in left wings (t = -0.342, p = 0.013) (Figure 4). The correlation ratios between wing's side and distances α, β, δ, ε, θ, π and γ are 0.59, 0.55, 0.27, 0.26, 0.16, 0.14 and 0.15, respectively. The normality test and the homogeneity distribution show variations for each parameter. However, the wing shape varies particularly in the α and β distances, with correlation ratios exceeding 50%.
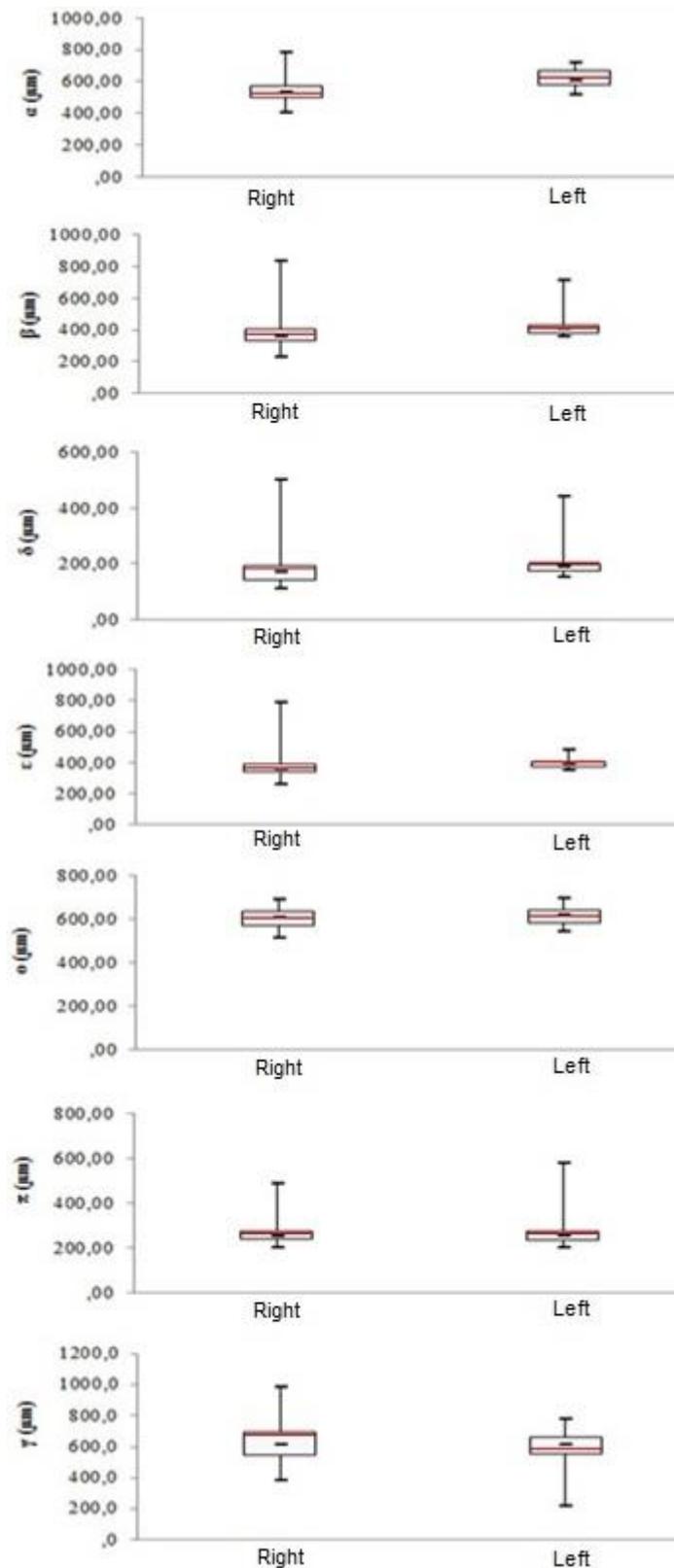
Figure 4. Boxplots representation of the α, β, δ, ε, ɵ, π and γ distances of *P. sergenti* male's right and left wings. (The ratios of correlation between the wing side and the distances α, β, δ, ε, ɵ, π and γ are 0.59, 0.55, 0.27, 0.26, 0.16, 0.14 and 0.15, respectively).

3.1. Spacial variations analyses

The size of the right-wing shows significant differences linked to their geographic origin (Figure 5), 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).
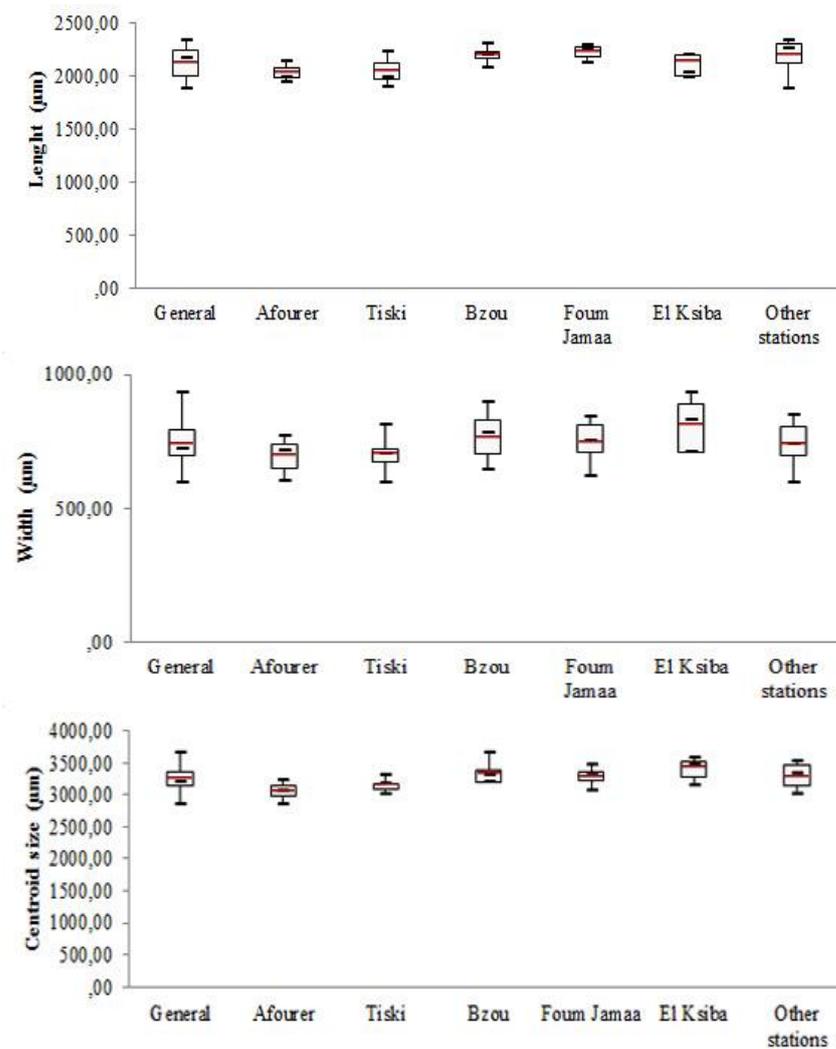
Figure 5. Boxplots of the lengths, widths, and centroid sizes of the right wings of *P. sergenti* in the various station of central Morocco. (The correlation ratios between stations and centroid size, width, and length are 0.68, 0.56, and 0.74, respectively).

Similarly, the seven distances characterizing the wing shape (Figure 6) showed differences according to the stations (correlation ratio and Student's t-test), with β, ө, and γ as the most variable distances. Whether in size or shape, the wings of *P. sergenti* of Afourer were statistically significantly different from all other stations (Figures 5 and 6).
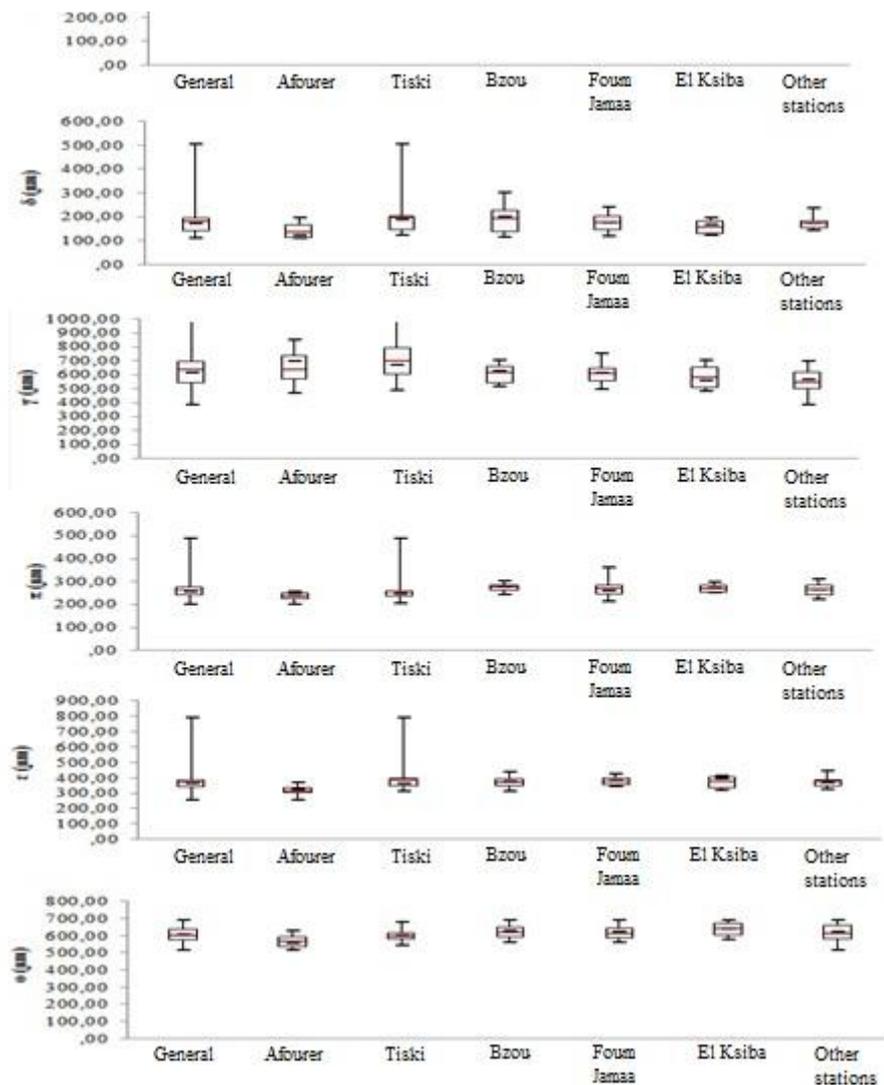
Figure 6. Boxplots representation the distances α, β, δ, ε, ϴ, π and γ of the right wings of *P. sergenti*'s males in each station. (The correlation ratios between the collection stations and the distances α, β, δ, ε, ϴ, π and γ are 0.39, 0.49, 0.37, 0.39, 0.55, 0.31 and 0.46, respectively).

As for results from the descriptive analysis, canonical analyses of the size and shape of *P. sergenti* wings, illustrated in figure 5, point to substantial variation according to the geographical origin of the wings. Factorial analysis of the size (Figure 7A) discloses that the factorial 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 according to its geographical origin. First, specimens from Afourer were located on the right of the canonical analysis, followed by those from Tiski, then 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 a factorial plane (1-2) explains 63.08% of the total inertia (canonical variate 1: 43.23% and canonical variate 2: 19.85%) (Figure 7B). As for the parameter of wing size, the canonical analysis reveals gradual variations according to the stations.
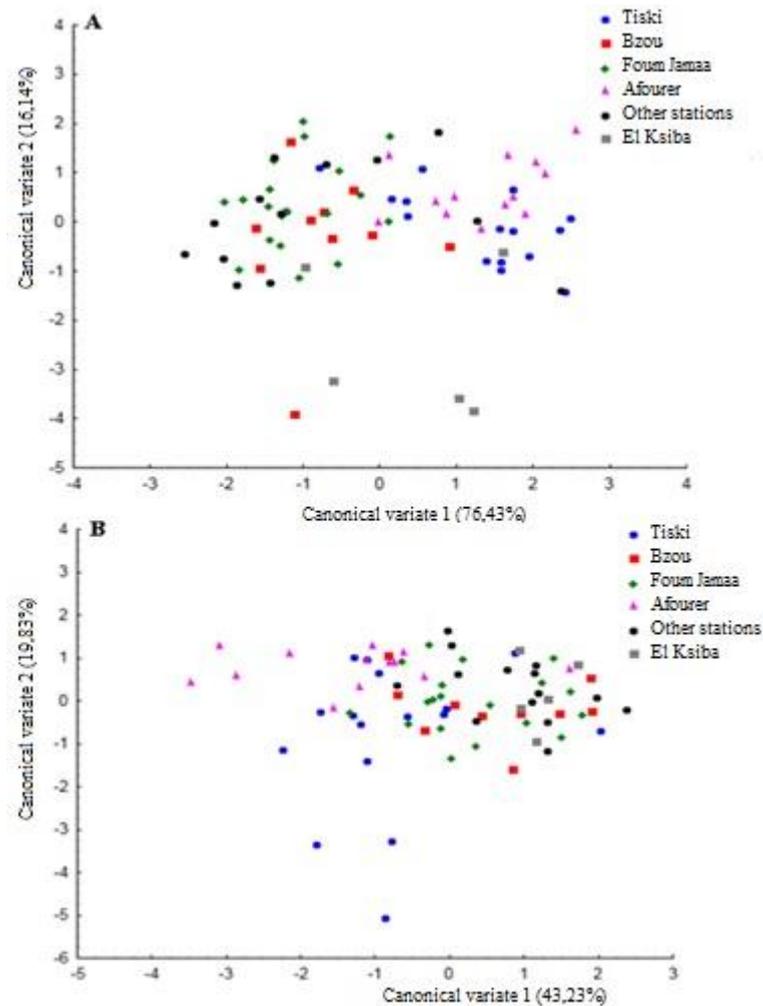
Figure 7. Canonical analysis of the size variations **(A)** (92.57% of the total inertia) and the shape **(B)** (63.08% of the total inertia) of *P. sergenti*'s wings depending on the geographical origin.

The hierarchical single-link classification trees for the measured parameters of size (Figure 8A) and wing shape (Figure 8B) demonstrate a clustering according to the geographic origin, which again reinforces the results of the canonical analysis. But, again, 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 other stations.
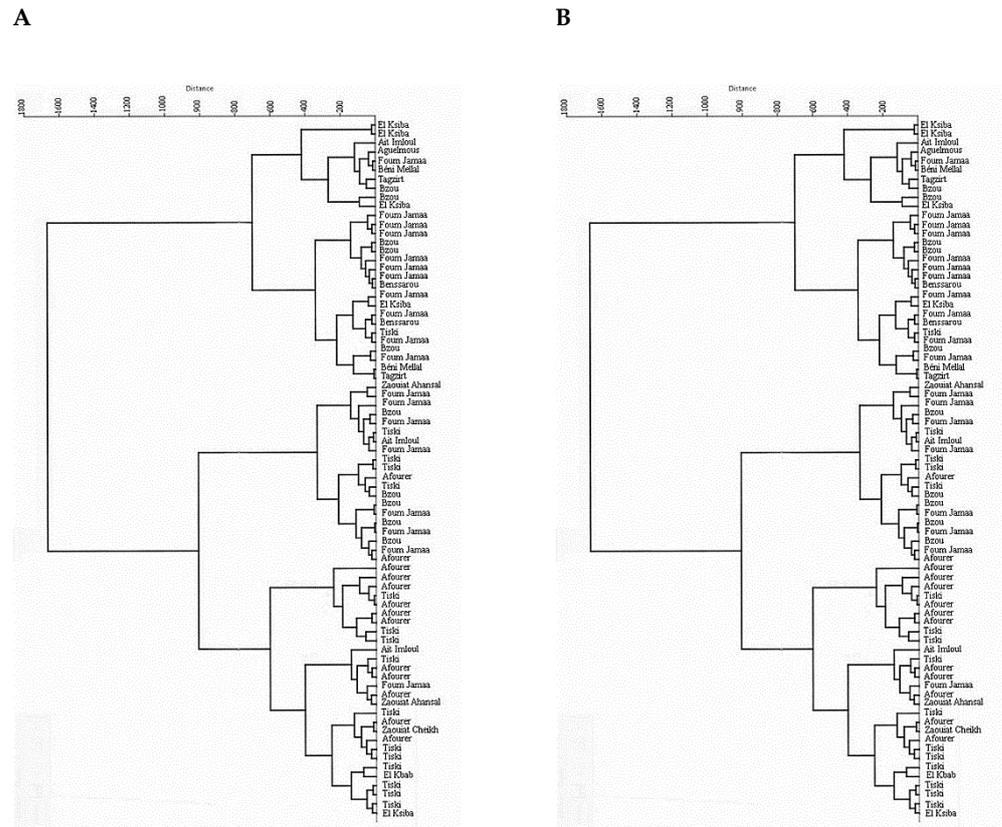
Figure 8. Single link hierarchical classifications tree of *P. sergenti* wings size (A) and (B) left wings.

Likewise, the mean configurations of the right-wing shapes vary according to the geographical origin, with marked differences in the populations of Afourer and Tiski (Figure 9).
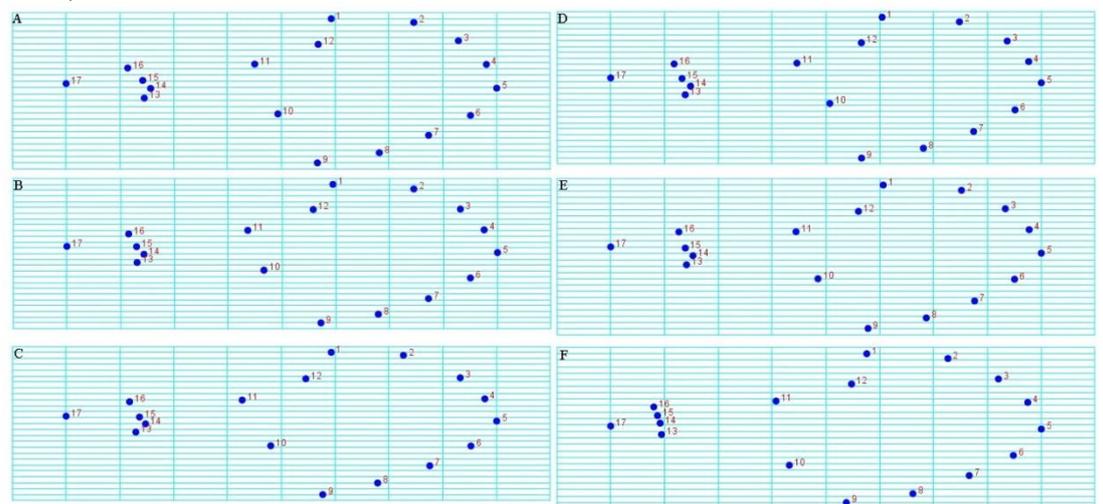

Figure 9. Superposition of the right-wing landmarks (mean measures) of *P. sergenti* males from Afourer **(A)**, Tiski **(B)**, El Kbab **(C)**, Bzou **(D)**, Foum Jamaa **(E)**, and other stations **(F)**.

4. Discussion

Phlebotomus sergenti is a proven vector of *L. tropica* [1] in Saudi Arabia [6], Israel [31], and Morocco [7]. Previous studies have led to consider *P. sergenti* as a species complex, regarding the tremendous genetic diversity recorded over its distribution [9-11,32]. Nevertheless, no morphotype indicator is currently available to probe *P. sergenti* population

diversity. Therefore, the wing's geo-morphometric analyses would help shed light on the population's diversity of *P. sergenti*. This has been used successfully on several sandfly species, like *P. tobbi*, *P. papatasi*, or *P. ariasi* [17,18,23,33,], and has provided evidence on the separation of *P. papatasi* populations between the southern and northern slopes of the High Atlas Mountains in Morocco [17]. This was further delineated via a genetic analysis of these populations [34]. Therefore, we applied GM analyses to probe *P. sergenti* population diversity in central Morocco for the first time.

Significant differences in *P. sergenti* wing's size and shape were recorded in populations we sampled in central Morocco. The size, length, width, and CS were significantly different depending on geographic origin, and the left wings were relatively bigger than the right ones. Such differences were absent between the right and left wings of *P. papatasi* originating from the northern or the southern slope of the Atlas Mountains, Morocco [17]. Despite the small number of *P. sergenti* females investigated in this study, we recorded that females presented smaller wings than males. Besides size variations, the shape of *P. sergenti* wings also showed significant variations, particularly at the two distances; α and β.

Furthermore, the right-wing shape was different from the left one. The size and shape of the *P. sergenti* wings showed spatial variations depending on geographic 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 being notably different.

Descriptive analyses point to a link between the size and shape and the geographical origin of the sample, which is further supported by multivariate analyses. Canonical studies reveal a gradual variation in size (92.57% of the total inertia) and shape (63.08% of the total inertia). The two populations of Afourer and Tiski are distinct with relatively smaller wings, following hierarchical classifications analyses. Superimpositions of the average configurations of the male right wings indicate significant variations in landmarks 2, 9, 10, and 14 positions. According to an altitudinal gradient, previous works have demonstrated a link between wing size and shape in *P. tobbi* and *P. papatasi* [17,23,35]. Even via an indirect effect, altitude can not be at the origin of the recorded gradual variation in *P. sergenti*. The individualized populations of Afourer and Tiski locate at the same altitudinal area as Bzou and Foum Jamaa, only about 60 km apart. At the same time, the slope effect described in *P. ariasi* female populations [33] cannot be involved in the variation we detected in *P. sergenti*. The stations compared are located on the same High Atlas Mountains slope. Therefore, it seems that the variations observed in *P. sergenti* in central Morocco should be correlated with the adaptation to a local environment. Moreover, ecologically, *P. sergenti* in Morocco is a constant species with an abundance and density directly related to the micro-habitat it occupies [13]. This adaptation phenomenon to a local environment is also reported in *P. ariasi* in the Oiselette massif in the south of France [33].

In conclusion, our results underline the diversity of *P. sergenti* in Morocco. It also points to *P. sergenti* to adapt to local environmental conditions. The correlation between the phenotypic differentiation and the genetic structure of *P. sergenti* in Morocco must be further investigated.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Table S1: Morphometry of *P. sergenti*'s wings collected in the study region in central Morocco, Table S2: Numerical characteristics of the distances α, β, δ, ε, θ, π and γ of the male (right and left) and right female wings of *P. sergenti* collected in central Morocco.

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