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Original Article

Discrimination of *Phlebotomus perfiliewi transcaucasicus*, *Ph. major* sensu lato and *Ph. tobbi* (Diptera: Psychodidae) Using Morphometric and DNA Barcoding Methods in the Endemic Foci of Visceral Leishmaniasis in Ardabil Province, North West of Iran

Alireza Barmaki[1, 2], *Yavar Rassi[1,3], Azad Absavaran[4], Amir Ahmad Akhavan[1], Eslam Moradi-Asl[3,5], Alireza Zahraei-Ramazani[1], Sayana Rafizadeh[6]

[1]Department of Vector Biology and Control of Diseases, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran
[2]Department of Public Health, School of Public Health, Qazvin University of Medical Sciences, Qazvin, Iran
[3]Arthropod-Borne Diseases Research Center, Ardabil University of Medical Sciences, Ardabil, Iran
[4]Department of Vector Biology and Control of Diseases, School of Medicine, Zanjan University of Medical Sciences, Zanjan, Iran
[5]Department of Public Health, School of Public Health, Ardabil University of Medical Sciences, Ardabil, Iran
[6]Ministry of Health and Medical Education, Tehran, Iran

***Corresponding author:** Dr Yavar Rassi, E-mail: rassiy@tums.ac.ir

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Abstract

Background: Visceral leishmaniasis, commonly known as kala-azar, and prevalent in more than 70 countries and several regions of Iran. It is one of the main diseases transmitted by sand flies.
In this work, geometric morphometrics and DNA barcoding were employed as novel techniques to enhance the diagnostic tools used in this study.

Methods: *Phlebotomus perfiliewi transcaucasicus*, *Phlebotomus major* s.l., and *Phlebotomus tobbi* caught from three districts in the Ardabil Province, northwest of Iran. The right wings of 286 female sand flies were analyzed using geometric morphometric (GM) tools. Additionally, the COI gene was isolated from each of the three species, amplified using universal primers, and sequenced through the DNA barcoding method for classification. This sequencing data was then formatted to generate morphometric analyses.

Results: The landmarks with the most variations were found in sets 10, 12, 13, and 14, whereas the first set's landmarks at 1 and 11, along with those from the second set at positions 2, 3, and 5 exhibited the greatest variations. Analysis of the size and shape variations in the wings indicates the presence of distinct populations ($P< 0.05$). Furthermore, the DNA barcoding results not only confirmed the findings from the geometric morphometric analysis but also revealed both interspecific and intraspecific distances.

Conclusion: This study was the first attempt to assess whether wing geometry morphometrics, combined with DNA barcode techniques, can effectively distinguish the three mentioned species in the studied areas. Furthermore, the identification of *Phlebotomus neglectus* in this area prompted recommendations for additional research.

Keywords: Visceral leishmaniasis; *Phlebotomus perfiliewi transcaucasicus*; DNA barcoding; Iran

Introduction

Visceral leishmaniasis is one of the most important parasitic diseases transmitted by phlebotomine sand flies, also known as kala-azar, and it affects Iran and Endemic in over 70 countries worldwide (1). In Iran, the disease is reported sporadically from all provinces, but focally from Ardabil, East Azerbaijan, Fars, and Bushehr provinces. The country has recently shown the introduction of new endemic foci in other regions (2). Meshkin-Shahr, Bileh Savar,

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and Germi districts in the Ardabil Province are the main endemic foci for visceral leishmaniasis. *Leishmania infantum* is the causative agent of kala-azar in Iran, including in the province of Ardabil, and is transmitted by *Phlebotomus kandelakii*, *Ph. perfiliewi transcaucasicus*, and *Ph. tobbi* as the proven or probable vectors of disease (3-7).

According to studies by Iranian researchers, 48 phlebotomine sand fly species have been confirmed in Iran, including 18 species of the genus *Sergentomyia* and 30 species of the genus *Phlebotomus* (8). The Ardabil Province has become a high-risk and endemic region for kala-azar due to canid reservoir infections, particularly domestic dogs, and their close contact with the Sand flies as a vector. Therefore, visceral leishmaniasis has an annual prevalence of 1–2% in the exposed population in this province (9–13). The majority of disease cases are under 10 years old children. *Leishmania infantum* infection rate (1–2%) among the vectors and its abundance (5–15%) in the three mentioned districts resulted in the annual transmission of 100 cases of disease to humans in recent years (9–13).

Accurate identification of phlebotomine sand flies as the disease's vectors is necessary to conduct epidemiological studies on all forms of leishmaniasis, study their infection status with parasites, and determine the pattern of transmission in the area (14).

Due to the similarity of their Morphological characters (isomorphic) in external and internal features, the females of *Ph. perfiliewi transcaucasicus*, *Ph. major/neglectus*, and *Ph. tobbi* (proven or probable vectors of visceral leishmaniasis) remain unidentified or difficult to identify and they are frequently categorized in identification keys based on morphology in a major complex/group without specific characteristics. Due to differences in vector capacity, resistance to insecticides, and current control strategies, this complex/group of sand flies requires precise identification to prevent and control leishmaniasis (15).

Landmark-based geometric morphometric is a tool that employs geometrical, biological, and statistical methods to study variations and fluctuations in the form (shape and size), body size, and complex structure of organisms and it is a strong, simple, and innovative instrument for analyzing various biological forms (16, 17). In this method, according to the defined significant variables, the shape is created and standard multivariate analyzes are used to describe patterns of change, similarity, and diversity within and between species while evaluating (16, 17). Many researchers selected and employed between 13 and 16 landmarks on the wings of arthropod specimens as part of the widely accepted Geometric morphometric protocol (16, 17).

DNA barcoding is a DNA-based taxonomic tool that is used to identify known and unknown species based on the sequence pattern of nucleotides in a segment of DNA of living organisms. It is an excellent complement to morphological methods and can be used to identify defective and incomplete morphological samples, immature forms of insects, and species with polymorphism in their life cycles and with isomorphism (18).

To identify and separate the females of three sand fly species, *Ph. perfiliewi transcaucasicus*, *Ph. major*, and *Ph. tobbi* in the northwest of Iran, we employed the geometric morphometric tool together with the DNA barcoding method to determine changes in wing shape, size, and wing indices as a morphological character in addition to other internal characters.

Materials and Methods

Study areas and sand fly collection

In this study, sand fly specimens collected with sticky paper and CDC light traps from three districts of Meshkin-Shahr (Our-Kandi, Nasir-Abad and Qort-Tapeh villages), Germi (MajidLoo, Qahramanloo, and Maqvan) and Bileh Savar (Masjedloo, TazehKand Chanagh-

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Bolag, and Engirloo) according to the presence of vectors and prevalence of visceral leishmaniasis diseases. The sand fly collection was carried out from collecting sites in the mentioned villages from April to September 2021 in collecting sites including rodent burrows, rock crevices, under bridges and bushes, caves, and places where people and animals rest with different biotopes (stables, barns, hen, nests, and yards) twice monthly (Fig. 1).

Preparation and preservation of sand fly specimens

The captured sand flies on sticky paper traps were gently separated by an entomological needle to prepare the samples for identification and carrying out morphological and morphometric studies. Surface contamination was removed by washing twice (once with 1% detergent for 2 minutes and again with distilled water twice), and the samples were conserved in 96% ethanol and kept at -20 °C.

Dissection, mounting, and identification of sand flies

For morphological identification, the head and the last two abdominal segments of each female specimen were dissected in a drop of sterile normal saline using sterile dissecting needles and were mounted on a slide in a drop of Puri's medium for geometric morphometric study, the right wing of each specimen were cut and transferred on another slide for subsequent processing. Finally, the rest of the body was transferred to 1.5CC microtubes for molecular studies.

Lewis (22), Seyedi-Rashti and Nadim (21), and the morphometric studies of Absavaran et al. (15) were utilized as valid identification keys for the morphological identification of sand flies at the species level.

Wing preparation, slide mounting, and imaging

The scales on the right wing of each female specimens of *Ph. perfiliewi transcaucasicus*, *Ph. major*, and *Ph. tobbi* were removed and prepared according to Belen et al. 2004 methods (19). The prepared wing was then placed on a slide and mounted with Puri's medium. All slides were photographed by using an Olympus SZX12 microscope with a DP12 camera, and all photographs with a magnification of 40× and visible vein were saved and uploaded to the computer. Images of wings with unclear characters or damage were removed from the study.

Wing Landmarks Selection, data acquisition, and statistical analysis

Photographs were first input to tpsUtil ver. 74 software to convert all documented photographs of the three species' wings to TPS format and two-dimensional Cartesian coordinates of 15 landmarks identified as the intersections of right-wing veins with the wing margin and then digitized by tpsDig2 software (Fig. 2 Part A). The coordinates were analyzed using tpsRelw software to calculate Eigen values for each principal warp. Landmark data after Generalized Procrustes Analysis (GPA), were analyzed using Relative warps and Principal Component Analysis (PCA) (tpsRelw ver. 1.65 software). Illustrating the difference among wing shapes was done using Morpho J as a deformation grid and Wireframe. Grouping of individuals along the coordinate axis of principal components (RW1 and RW2) was done using PAST ver. 2.17 software.

Analysis of the size and shape of the wings in three species was done in two sets (first set: with a large number of samples; second set: with a small number of samples that did not share any samples with another species in the first sets grouping).

DNA extraction and amplification of COI (Cytochrome C Oxidase subunit 1) gene:

DNA extraction of each specimen was carried out by using the Tissue Genomic DNA Extraction Mini Kit. Using universal primers including LCO1490 and HCO2198 created by

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Folmer et al. (20), polymerase chain reactions (PCR) were employed with the trademark Favorgen for the previously mentioned three species to amplify the 658 base pairs DNA barcode fragment of the COI gene.

Analysis of DNA barcode fragment sequences of COI gene

PCR products of each specimen were chosen for sequencing after being observed and examined by electrophoresis in 1% agarose gel to ensure their purity and the presence of the desired band. In Pishgam Biotechnology Company, sequence determination of the PCR product with forward primer (single-read sequencing) was carried out using the Sanger method. Then, using the ChromasPro v1.41 software, the results and nucleotide position in each species' sequence were checked and corrected. Next, a phylogenetic tree was constructed by using the Neighbor-Joining algorithm (NJ) based on the K2P distance model to demonstrate the pattern of clustering between species. Using the BLAST program (www.ncbi.nih.gov/BLAST/), the acquired sequences were compared to sequences of the same or closely similar species that are accessible in GenBank. The obtained sequences for each species in the subgenus *Larroussius* were compared to the barcode sequences listed in the database (BOLD: Barcode of Life Database) at www.boldsystems.org or www.barcodinglife.com to confirm the species' accurate identification. *Sergentomyia dentata* and some *Larroussius* species obtained from GenBank were employed as the study out-group and in-group identical species, respectively.

Results

Species composition

In this study, 96.73% (1923 of the total 1988) of sand flies belonged to the genus *Phlebotomus* from three districts in Ardabil Province: Meshkin-Shahr, Bileh Savar, and Germi Districts. The proportion of females in three particular species of *Ph. major* s.l., *Ph. tobbi*, and *Ph. perfiliewi transcaucasicus* were 22.5%, 6.1%, and 19%, respectively, among 1478 collected *Phlebotomus* specimens (Table 1).

To identify landmarks, a total of 286 specimens of *Ph. major* (n=100), *Ph. tobbi* (n=81), and *Ph. perfiliewi transcaucasicus* (n=105) were used (Table 1).

Consensus configuration and variation in wing

The average wing shape (Consensus configuration) of the right wing of the three species was estimated as Wireframe (Fig. 2, Part B).

Wing size and shape analysis

The first set

The three principal components (PC), which account for 53.30% of the average wing's eigenvalues and variance, are determined to have the most influence. The most effective landmarks in the two (PC) were 10, 9, 12, 1, and 11. In the third component, the most effective landmarks were 2, 3, 8, and 9, and Landmark 9 was a component in the three PC (Fig. 3).

Female species were grouped based on the software's examination of wing shape variations and principal component analysis (PCA) results. The samples in the two (PCA) overlap, as shown by the results in Fig. 4, part A.

The analysis of variance (ANOVA) revealed a significant difference in the centroid size of the wings between the three species ($P \geq 0.0012$ and $F= 5.46$), classifying all samples into three groups with no type. There has been no error found. Procrustean distances are provided as residuals in Table 2 of the first part since they also affect the population's wing shape in addition to classification.

The general comparison of the wing shapes of three species was done using the Morpho J software's (ANOVA) consideration of the outcome of the residual effects. F test (Goodall's F statistic) demonstrating the inequality in the variance of two communities with the values ($P< 0.0001$ and $F=1.73$) and ($P< 0.0001$) and Pillai's trace with the values ($P< 0.0001$) and

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Pillai's t= 0.46 demonstrates a statistical difference in the mean of the samples. These two tests reveal a highly significant difference (P< 0.0001) between the values of wing-shaped Procrustes in the females of the three species (Table 3).

In three species, the outcomes of the discriminating analysis were as follows: The covariance matrix uniformity is examined using the Box's M test. Indicating the non-uniformity of the covariance matrix, it was significant (P< 0.010242, Box's M= 16.926). The results of the canonical analysis demonstrate that many species overlap with one another. This was because the wing indices were so near to the average of the other two species and because the wing shapes were so similar (Fig. 5).

Mahalanobis distance, Procrustes distance, and T-square in three comparison groups had a significant P-value (parametric) (P< 0.05) in the two-by-two comparison of species. These indications showed a clear separation between *Ph. tobbi* and the other two species. In the comparison group, *Ph. tobbi* species had a greater Mahalanobis distance than the other two species (1.4947); nevertheless, this index was low when the profile species were compared to *Ph. major* s.l. (1.0784, 1.2310). These variations showed the variation in populations (Table 4).

The information obtained from the cross-validation table (Table 5), showed a positive predictive value for classifying two species apart was 119.04% for *Ph. major* vs. *Ph. tobbi* and 73.02% for *Ph. tobbi* vs. *Ph. major*. *Ph. tobbi* vs. *Ph. perfiliewi* had a positive predictive value of 78.81%, and vice versa, 89.86%; however, *Ph. major* s.l. vs. *Ph. perfiliewi* had a value of 83.75%, and vice versa, 76.25%.

The highest sensitivity rate and the lowest specificity rate in this method classifying and discriminating of species two by two, which has a high positive (true classification) predictive value, was found in the *Ph. tobbi* species from *Ph. major* s.l., which has higher validity than other comparisons.

The observed classification from analysis of species discrimination in cross table (Table 6) showed that 37.72% *Ph. major* s.l. from *Ph. perfiliewi* is separated and versa 36.27%. This index showed that *Ph. tobbi* and *Ph. major* s.l. were correlated with 47.82% and *Ph. perfiliewi* and *Ph. tobbi* were correlated with 40.76% and 35.87%, respectively. The residual of the individual is either diagnosed in another species' space or is placed outside of the separation space and further away from it.

The total discrimination percentage of the species is calculated by dividing the observed discrimination by the expected discrimination and it indicates the effectiveness of the discrimination method. The index *Ph. major* s.l. from *Ph. perfiliewi* is 74.47%, and versa 74.47% and *Ph. major* from *Ph. tobbi* is 86.55 % and versa 74.08 % and *Ph. tobbi* from *Ph. perfiliewi* is 82.37% and versa 72.12%.

The second set

The 53 female samples of right wings of three species including *Ph. major* s.l. (n= 19), *Ph. tobbi* (n=18) and *Ph. perfiliewi transcaucasicus* (n=16) were configured and analysed for two principal components (PC), the first PC of 24.96% of specimens and the second PC of 19.42% of specimens including 44.38% of the total changes, while in the first set of the analysis, the impact of the first two components were 40.6% of the total changes. The second set's component had a 3.78% greater effect than the first. In the second stage, changes can also be observed in landmarks 9 and 10, which are reaching towards the base of the wing. It appears that these deviations cause these species to isolate from the center of the accumulation of landmarks in the first stage. Additionally, it was discovered that the study's limited sample size contributed to an increase in the number of landmark deviations when comparing the variance of the landmarks in two stages. The landmarks 2, 3, 4, and 5 have had the greatest modifications in the second set, along with the alterations in

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landmarks 10, 12, 13, and 14 and 15, as in the previous set.

In the second set, the PCA-based grouping of the species into three species (green dots for *Ph. perfiliewi transcaucasicus*, blue dots for *Ph. tobbi*, and red spots for *Ph. major* s.l.) had some overlap in the species (Fig. 4, part B). It appears that these alterations were interspecies and were independent of the sample size. The ANOVA results showed a significant difference in the centroid size of the wings between the three species (P< 0.0005 and F= 5.99), classifying all samples into three groups with no type or errors. The increase in the F test by 0.53 and a decrease in p value from 0.0012 to P<0.0005 between the second and first analyses, respectively, demonstrated a significant change in the variance of wing size between the two sets (Table 2, part second set). The remaining effects of the comparison of wing form in three species were taken into account using MorphoJ software during (ANOVA) analyses (see second set in Table 3). In the second set, the F test (also known as Goodall's F statistic) revealed differences in variance among the three groups with the values (P< 0.0001 and F= 3.39), and Pillai's trace with the values (P< 0.0001 and Pillai's t= 1.53) revealed a statistical difference in the mean of the samples. These two tests showed a substantial difference in the values of the three species' wing-shaped Procrustes (P< 0.0001). Because there were fewer samples in the second set, the Pillai's trace effect increased despite the substantial difference, indicating a decline in the statistical difference between average wings among the species.

The second set of discriminating results were analyzed as follows: Box's M test, which was used to examine the covariance matrix uniformity, was significant (P< 0.010242, Box's M= 16.926), indicating that the covariance matrix was not uniform. Only two of the samples were common to the same grouping in Part B of Figure 5, where the canonical analysis of all three species showed that they could be distinguished from one another.

In the second part of Table 4, a two-by-two comparison, it is expected that the results of discriminating three species based on the Mean comparison with 1000 permutations have increased Procrustes distance, Mahalanobis distance, T-square, and P-value (parametric) in the comparison of species. Since the results of permutation tests with 1000 permutations have decreased when comparing *Ph. perfiliewi transcaucasicus* species with *Ph. major* s.l. and *Ph. major* s.l. with *Ph. tobbi*, and have increased when comparing *Ph. tobbi* with *Ph. perfiliewi transcaucasicus*, it can be inferred that the discriminating space decreases as the number of species increases. This index was less than 0.05 and significant when compared to two other species (*Ph. tobbi* and *Ph. perfiliewi transcaucasicus*). However, the difference between *Ph. tobbi* and *Ph. perfiliewi transcaucasicus* was 0.360, which was not statistically significant.

The information obtained from the cross-validation table (Table 7), regarding *Ph. tobbi* vs *Ph. major* and *Ph. major* versus *Ph. tobbi*, the positive predictive value for categorising two species apart was 60% and 80%, respectively. About *Ph. perfiliewi*, this value was determined to be 50%, and vice versa, for *Ph. tobbi*. The difference between *Ph. major* and *Ph. perfiliewi*'s positive predictive value was 68.42%, and the estimated amount of *Ph. perfiliewi* was 67.71%. The highest sensitivity rate and the lowest specificity rate in this method classifying and discriminating of species two by two, which has a high positive (true classification) predictive value, was found in *Ph. major* s.l. from *Ph. tobbi*, which has higher validity than other comparisons. The degree of validity has decreased compared to the first set.

The observed classification from analysis of species discrimination in the cross table (Table 8) showed that 54.28% of *Ph. major* s.l. from *Ph. perfiliewi* is separated and versa 45.72%. This index between *Ph. perfiliewi* and *Ph. tobbi* is 44.12% and versa 41.18% and be-

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tween *Ph. tobbi* and *Ph. major* s.l. is 48.65% and versa 37.84%. The residual individual is either diagnosed in another species' space or is placed outside of separation space and further away from it

The total discrimination percentage of the species is calculated by dividing the observed discrimination by the expected discrimination and it indicates the effectiveness of the discrimination method. The index *ph. major* from *Ph. perfiliewi* is 100%, and versa. and *Ph. major* from *Ph. tobbi* is 94.47% and versa 77.78 %. And *Ph. tobbi* from *Ph. perfiliewi* is 78.83 % and versa 93.69%. The degree of discrimination has increased compared to the first set.

Analyzing similarities and distinguishing in two sets

Through the use of MorphoJ software, the inter-species validation for this discrimination with the lowest required accurate recognition was computed, according to table 9, Average validation from another in each species decreased in the second set. This decrease was expected due to the decrease in sample size, and it is normal. However, the degree of discrimination has increased compared to the second set.

Based on the distance measurement (converted to rank), the similarity analysis test (One-way ANOSIM), a comparative non-parametric test, revealed a significant difference between the three species. The results of this categorization test are displayed in Figure 6. (R= -0.001471, P (same)= 0.4412), where negative R denotes group dissimilarity and pink was used to highlight the likelihood of separation and group dissimilarity.

The unweight pair group method with arithmetic mean (UPGMA) was used to determine the average of each landmark directly in clustering (all distances play a role in grouping), and it was derived from a combination of species and hierarchical clustering algorithms. Figure 7 displays the results of this method for two sets, showing that three species were classified individually by comparing the dendrogram distances between the two sets' results (first set=A, second set=B), which were not significantly different from one another.

Analysis of wing variation within species populations in different areas

Given that p (equal var-covar) was zero for all three species, it can be concluded that the covariance matrix was consistent among locations, the shape of the wings was not different, and integration was demonstrated by the Monte Carlo p test. The matrix of several sites within a species' population shows that the sample was random (P< 0.5).

Investigation results on wing indices

Table 11 displays the findings of the measurement of wing indices in pixels. Wing indices were measured, and it was discovered that *Ph. perfiliewi transcaucasicus* species had higher indices than *Ph. major* s.l. and *Ph. tobbi*. The wing indices in *Ph. major* s.l. was almost equal to the average, while those in *Ph. tobbi* species were higher than the average and lower than the other two species.

Analysis and sequencing of the DNA barcode fragment of the COI gene

The results of COI gene sequencing from 13 (5%) female specimens of the three mentioned species and three male samples from each species support the geometric morphometric analyses (Figs. 8, 9 and Table 12). The male populations of the species were used to verify the female genomic sequence. *Phlebotomus tobbi* and *Ph. perfiliewi transcaucasicus* male specimens were collected from sympatric regions, whereas *Ph. major* s.l. male specimens were collected from an allopatric region.

Figure 8 displays the results of the electrophoresis analysis of the three species of COI genes amplified using the PCR technique. For all three species, the estimated band was around 658 bp.

Through BLAST searches in the Genbank and BOLD databases, 13 sequences from the

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three mentioned species were compared to other mitochondrial sequences. All of the sequences from the current investigation were in agreement with the species that the PCR test had identified (similarity> 97% to reference sequences in the databases). Thirteen sequences from the three species had an average nucleotide composition percentage of 36.1% thymine, 30.5% adenine, 16.4% guanine, and 17% cytosine. A structure of trees was automatically generated to predict NJ values. For this calculation, the greatest Log likelihood was -1394.932. The transition/transversion ratios for purines and pyrimidines were K1= 0.174 and K2= 2.273, respectively. Figure 9 illustrates the phylogenetic tree based on the COI gene sequence and the 16 species of male and female sandflies from local populations connected to the main group by the NJ approach. *Phlebotomus tobbi* with an intra-species root from Turkey (GenBank accession number MN086652) was placed in one branch of the phylogenetic tree with 98% Bootstrapping value support. Additionally, populations from Italy with intra-species roots for the male and female *Ph. perfiliewi transcaucasicus* species (GenBank accession number MG948469) were grouped into one branch with 100% support in 500 times bootstrapping. The COI gene sequence of *Ph. major* species populations from sympatric and allopatric locations matched to *Ph. major* s.l. and *Ph. neglectus* with 97% and 96% coverage in the Blast method and also in Barcode of Life Data Systems (BOLD). As a result, the names of the sequences for phylogenetic tree design should be for both species. The COI gene sequence in this population was placed in a separate branch with 500 rounds of bootstrapping providing 100% support. *Phlebotomus major* s.l. from Turkey (GenBank accession number MN086544) was used in this branch as the root within the group. When comparing and constructing the phylogenic tree, *Sergentomyia dentata* was used as the root because sub-division may be identified in the species' branches.

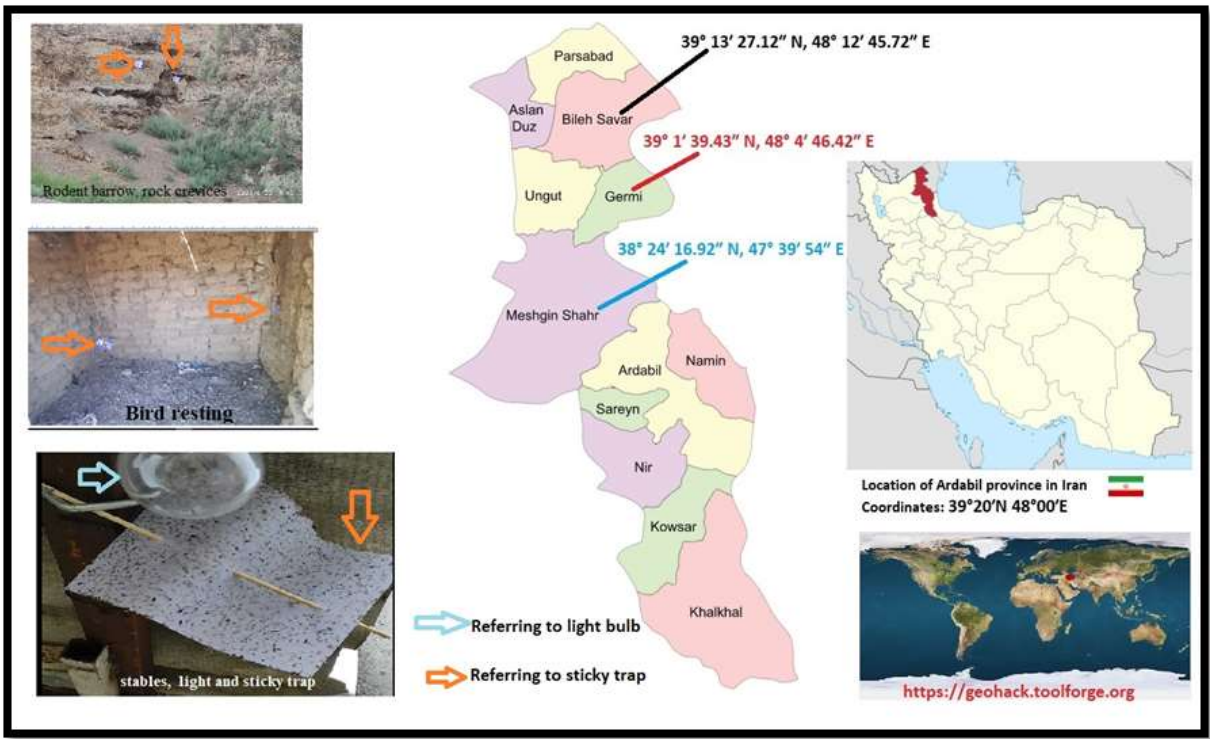

Fig. 1. Sand fly collection sites and map of the study areas with arrow indicators (personally designated)

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Table 1. Prepared slides as well as information on the population density of female sand flies of the subgenus *Larroussius* in the study area, Iran

County	No. (%)	Species	No.	Percentage	Slide mounted	Selected slide
Bileh Savar	93 (9.83)	*Ph. major* s.l.	28	2.96	24	6
		Ph. perfiliewi	49	5.18	3	3
		Ph. tobbi	16	1.69	9	9
Germi	840(88.8)	*Ph. major* s.l.	418	44.19	394	93
		Ph. perfiliewi	326	34.46	120	101
		Ph. tobbi	96	10.15	52	69
Meshkin-Shahr	13(1.34)	*Ph. major* s.l.	3	0.32	1	1
		Ph. perfiliewi	5	0.53	1	1
		Ph. tobbi	5	0.53	4	3
Total			946	100	608	286

Table 2. Results of ANOVA on comparison of wing size (Centroid size) in three species (first and second sets), Ardabil Province of Iran, 2022

Step Set	Effect	SS	MS	df	F	P (param.)
First	Individual	271860.2289	90620.0763	2	5.46	0.0012
	Residual	4729184.341	16593.62927	285		
Second	Individual	211479.5	105739.766	2	8.99	0.0005
	Residual	588049.2	11760.98488	50		

Table 3. The results of multivariate analysis of variance for the wing shape in three species on two sets, Ardabil Province of Iran, 2022

Step Set	Effect	SS	MS	df	F	P (param.)	Pillai tr.	P (param.)
First	Individual	0.00460469	5.90345E-05	78	1.73	<0.0001	0.46	<0.0001
	Residual	0.25352838	3.42144E-05	7410				
Second	Individual	0.006253	0.000120256	52	3.39	<0.0001	1.53	<0.0001
	Residual	0.046164	3.55107E-05	1300				

Table 4. Discriminant function analysis of three sand fly species based on two-by-two comparisons of right wing averages in Ardabil Province, Iran, 2022

Step Set	Comparison	Difference between means				P-values for permutation tests (1000 permutation runs)	
		Procrustes distance	Mahalanobis distance	T-square	P-value (parametric)	Procrustes distance	T-square
First	*Ph. major* s.l./*Ph. perfiliewi*	0.000568	1.0784	60.4745	0.0035	0.0360	0.00040
	Ph. major s.l. /*Ph. tobbi*	0.005405	1.4947	101.0201	0.0001	0.1510	<0.0001
	Ph. perfiliewi/*Ph. tobbi*	0.007058	1.2310	68.5215	0.0011	0.0090	<0.0001
Second	*Ph. major* s.l./*Ph. perfiliewi*	0.02316245	6.4267	370.5720	0.0213	0<0.0001	0.0160
	Ph. major s.l./*Ph. tobbi*	0.01571365	5.7516	296.8122	0.0435	0.0150	0.0480
	Ph. perfiliewi/*Ph. tobbi*	0.01646860	4.3421	160.2573	0.3607	0.0120	0.3750

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Table 5. Cross-validation analysis of species in two-by-two comparison of the right wing average in three sand fly specie from Ardabil Province, Iran, 2022

Cross-validation of *Phlebotomus major* s.l./*Ph. perfiliewi transcaucasicus*							
True species	**No. tested**	**Allocated to**		**Total**	**Sensitivity rate***	**Specificity rate****	**Positive predictive value *****
		***Ph. major* s.l.**	***Ph. perfiliewi***				
***Ph. major* s.l.**	100	67	37	104	64.42	35.58	83.75
Ph. perfiliewi	104	43	61	104	41.35	58.65	76.25
Cross-validation of *Phlebotomus major* s.l./*Ph. tobbi*							
True species	**No of tested**	**Allocated to**		**Total**	**sensitivity rate***	**specificity rate****	**Positive predictive value *****
		Ph. major	***Ph. tobbi***				
Ph. major	100	75	29	104	72.16	27.84	119.04
Ph. tobbi	80	34	46	80	42.5	57.5	73.02
Cross-validation of *Phlebotomus tobbi*/*Ph. perfiliewi transcaucasicus*							
True species	**No. tested**	**Allocated to**		**Total**	**Sensitivity rate***	**Specificity rate****	**Positive predictive value *****
		Ph. perfiliewi	***Ph. tobbi***				
Ph. perfiliewi	104	62	42	104	59.62	40.38	89.86
Ph. tobbi	80	27	53	80	33.75	66.25	76.81

*= The true diagnostic (positive) rate (TPR, also called sensitivity) is calculated as TP/TP+FN
**The true negative rate (also called specificity)= TN/TN+FP.
*** (true classification) It's calculated as TP/TP+FP

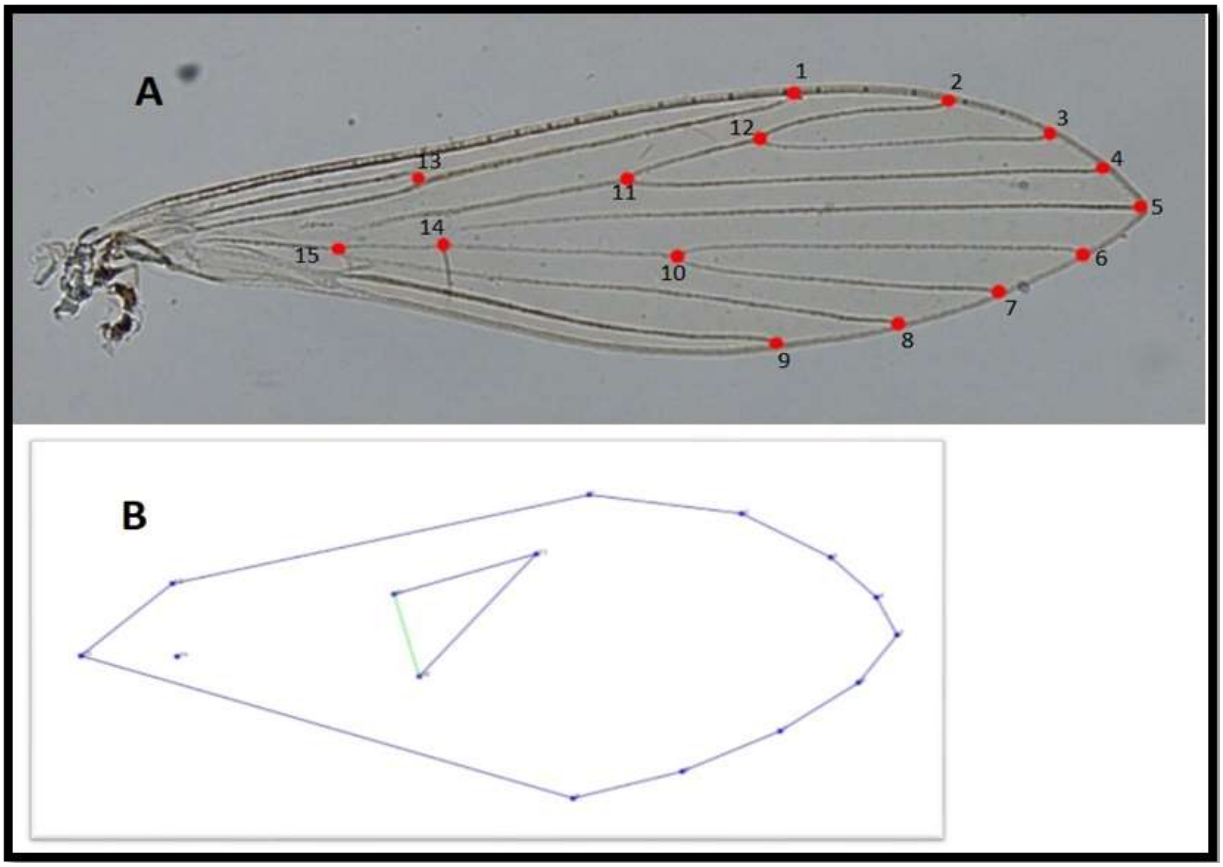

Fig. 2. A: Digitization of 15 landmarks of the right wing and B: Consensus configuration of the right wing of the female sand flies as wireframe using software tpsDig, Ardabil Province of Iran, 2022

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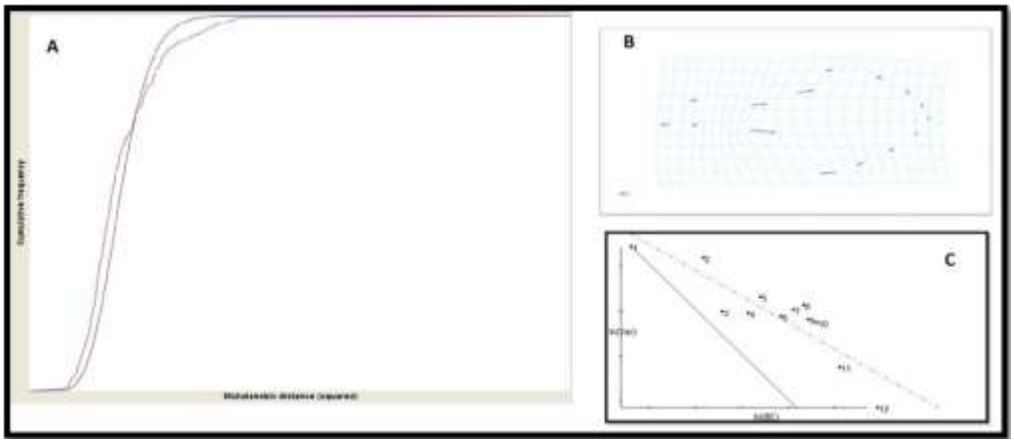

Fig. 3. A) Mahalanobis distance diagram in the size of the right wing, B) pattern changes in the shape of the right wing due to the displacement of landmarks in PC1, C) the report of the variance of each partial deflection against the report of the bending energy of each principle by the Bookstein method, Ardabil Province of Iran, 2022

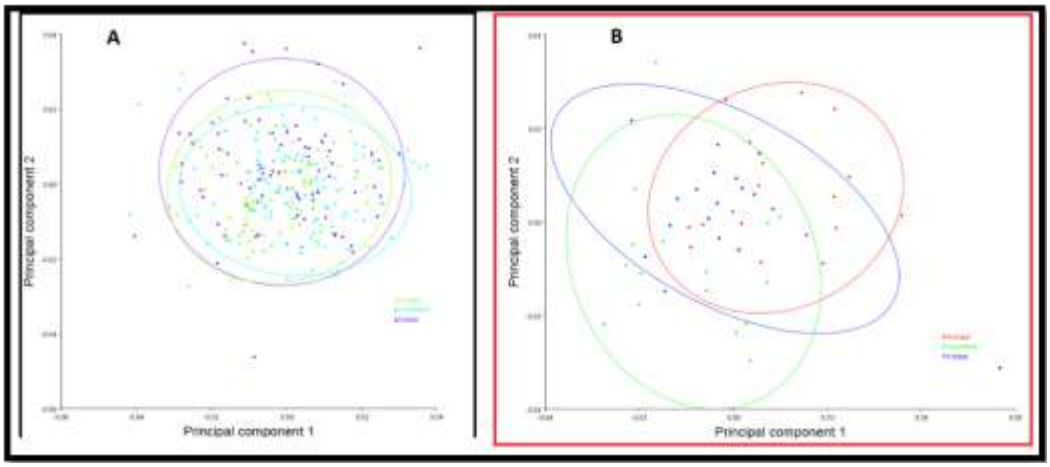

Fig. 4. Principal component analysis (PCA) diagram of the shape of the right wing and its grouping in three species: Part A is related to the first set; Part B is related to the second set, Ardabil Province of Iran, 2022

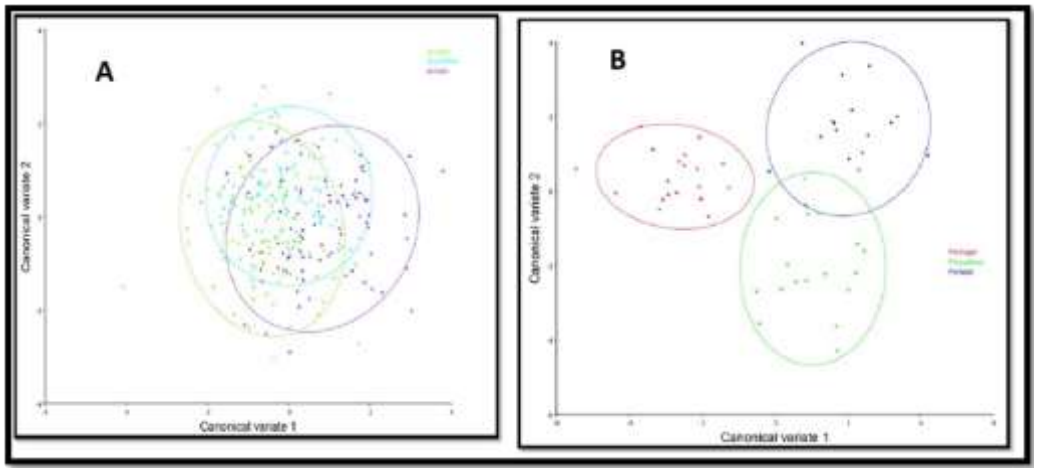

Fig. 5. Canonical analysis (CA) diagram of the shape of right wing and the species grouping in individual: Part A is related to the first set; Part B is related to the second set, Ardabil Province of Iran, 2022

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Table 6. Analysis of species through cross discrimination of right wing averages in three sand fly species using past software in Ardabil Province, Iran, 2022

The discriminant function of *Phlebotomus major* s.l./*Ph. perfiliewi transcaucasicus*								
Species	**No. tested**	**Expected discrimination ***	**Allocated to**		**Total**	**Detection percentage ****	**Percent Classification of observed *****	**Total discriminating percentage ********
			***Ph. major* s.l.**	***Ph. perfiliewi***				
***Ph. major* s.l.**	100	49.02	79	25	104	79	38.72	**74.15**
Ph. perfiliewi	104	50.98	30	74	104	71.15	36.27	**74.47**
Discriminant function of *Phlebotomus major* s.l./*Ph. tobbi*								
Species	**No. tested**	**Expected discrimination ***	**Allocated to**		**Total**	**Detection percentage ****	**Percent Classification of observed *****	**Total discriminating percentage ********
			***Ph. major* s.l.**	***Ph. tobbi***				
***Ph. major* s.l.**	100	55.55	88	16	104	88	47.82	**86.55**
Ph. tobbi	80	44.45	19	61	80	76.25	33.15	**74.08**
Discriminant function of *Phlebotomus tobbi*/*Ph. perfiliewi transcaucasicus*								
Species	**No. tested**	**Expected discrimination ***	**Allocated to**		**Total**	**Detection percentage ****	**Percent Classification of observed *****	**Total discriminating percentage ********
			Ph. perfiliewi	***Ph. tobbi***				
Ph. perfiliewi	104	56.52	75	29	104	72.12	40.76	**72.12**
Ph. tobbi	80	43.48	14	66	80	82.5	35.87	**82.37**

*Expected discrimination= Number of species tested/total species *100
**True Allocated/individuals tested of the same species *100
***percent Classification of observed= true Allocated/the total number of individuals tested from different species *100
****= true Classification of observed/percent of expected Classification *100

Table 7. Cross-validation analysis of species in two-by-two comparison of right wing average in three sand fly specie by past software in the second set, Ardabil Province of Iran, 2022

Cross-validation of *Phlebotomus major* s.l./*Ph. perfiliewi transcaucasicus*							
True species	**No. tested**	**Allocated to**		**Total**	**Sensitivity rate***	**Specificity rate****	**Positive predictive value *****
		***Ph. major* s.l.**	***Ph. perfiliewi***				
Ph. major	19	13	6	19	68.42	50	68.42
Ph. perfiliewi	16	6	11	17	35.29	50	64.71
Cross-validation of *Phlebotomus major* s.l./*Ph. tobbi*							
True species	**No. of tested**	**Allocated to**		**Total**	**Sensitivity rate***	**Specificity rate****	**Positive predictive value *****
		***Ph. major* s.l.**	***Ph. tobbi***				
Ph. ma jor	19	12	7	19	63.16	46.67	80
Ph. tobbi	18	8	9	17	52.94	53.33	60
Cross-validation of *Phlebotomus tobbi*/*Ph. perfiliewi transcaucasicus*							
true species	**No. tested**	**Allocated to**		**Total**	**Sensitivity rate***	**Specificity rate****	**Positive predictive value *****
		Ph. perfiliewi	***Ph. tobbi***				
Ph. perfiliewi	16	8	9	17	47.06	52.94	50
Ph. tobbi	18	8	9	17	52.94	47.06	50

*=The true diagnostic (positive) rate (TPR, also called sensitivity) is calculated as TP/TP+FN
**The true negative rate (also called specificity)= TN/TN+FP.
*** (true classification) It's calculated as TP/TP+FP

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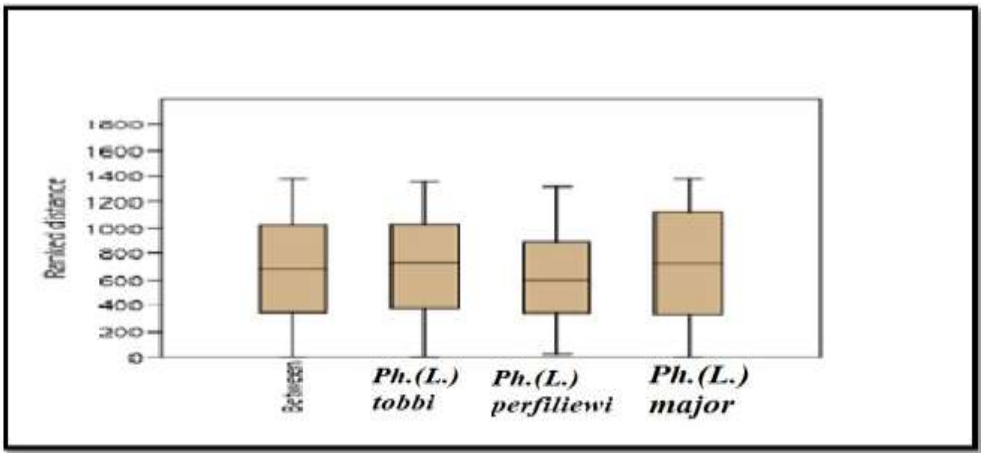

Fig. 6. Similarity and differentiation between species based on the distance from the reference landmark using the One-way ANOSIM test, Ardabil Province of Iran, 2022

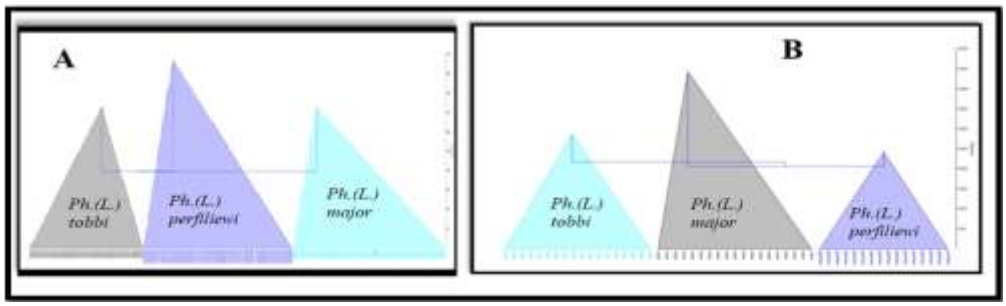

Fig. 7. Hierarchical clustering in mix of three species based on the coordinates of all 15 landmarks without pairing their weights (UPGMA), Ardabil Province of Iran, 2022

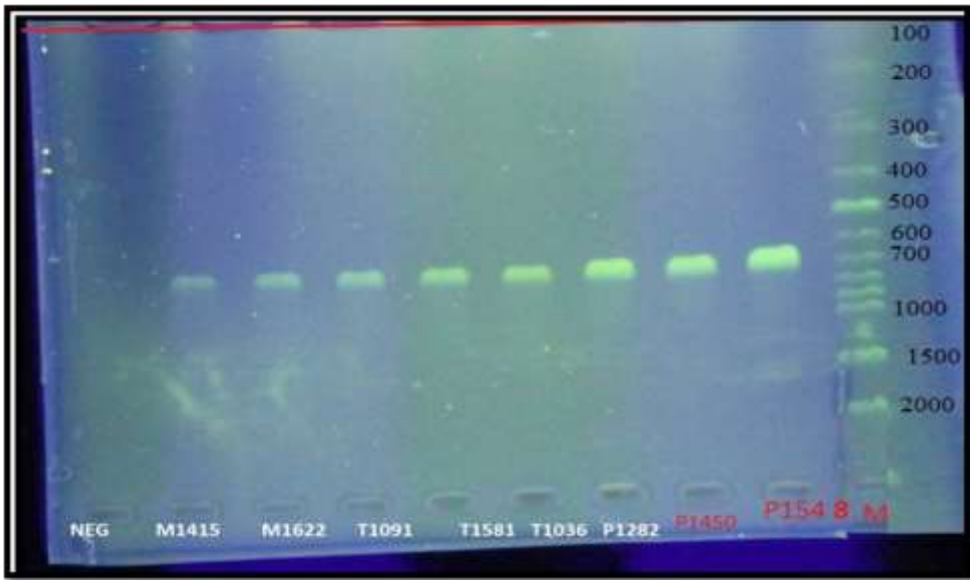

Fig. 8. PCR product from COI gene assay on a 1.5% agarose gel for 3 species. Lane 1: 100bp molecular ladder, lanes 2–9 (p1548, p1450, p1282= *Phlebotomus perfiliewi transcaucasicus*), (T1036, T1581, T1091 = *Ph. tobbi*), (M1622, M1415= *Ph. major* s.l.) and lane 10: negative control

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Table 8. Cross discrimination analysis of species in two-by-two comparison of right wing average in three sand fly species by past software in second set

Discriminant function of *Phlebotomus major* s.l./*Phlebotomus perfiliewi transcaucasicus*								
Species	**No. tested**	**Expected discrimination ***	**Allocated to**		**Total**	**Detection percentage ****	**Percent Classification of observed *****	**Total discriminating percentage ********
			***Ph. major* s.l.**	***Ph. perfiliewi***				
Ph. major	19	54.28	19	0	19	100	54.28	**100**
Ph. perfilliewi	16	45.72	0	16	16	100	45.72	**100**
Discriminant function of *Phlebotomus major* s.l./*Phlebotomus tobbi*								
Species	**No. tested**	**Expected discrimination ***	**Allocated to**		**Total**	**Detection percentage ****	**Percent Classification of observed *****	**Total discriminating percentage ********
			***Ph. major* s.l.**	***Ph. tobbi***				
Ph. major	19	51.5	18	0	18	94.74	48.65	**94.47**
Ph. tobbi	18	48.65	0	14	14	77.78	37.84	**77.78**
Discriminant function of *Phlebotomus tobbi*/*Phlebotomus perfiliewi transcaucasicus*								
Species	**No. tested**	**Expected discrimination ***	**Allocated to**		**Total**	**Detection percentage ****	**Percent Classification of observed *****	**Total discriminating percentage ********
			Ph. perfilliewi	***Ph. tobbi***				
Ph. perfilliewi	16	47.09	15	1	16	93.75	44.12	**93.69**
Ph. tobbi	18	52.91	1	14	15	77.78	41.18	**78.83**

*Expected discrimination= Number of species tested/total species *100
**True Allocated/individuals tested of the same species *100
***percent Classification of observed= true Allocated/the total number of individuals tested from different species *100
****= true Classification of observ /percent of expected Classification *100

Table 9. Comparison of validation and discrimination in two set in the population of three sand fly species, Ardabil Province of Iran, 2022

Species	**Average validation from other species**		**The difference between two stages (stage 2 - stage 1)**	**Result**
	First set	**Second set**		
***Ph. major* s.l.**	101.4	74.21	-27.19	Decrease
Ph. tobbi	74.92	55	-19.92	Decrease
Ph. perfiliewi trancaucasicus	83.06	57.36	-25.7	Decrease
	Average discrimination from other species		**The difference between two stages (stage 2 - stage 1)**	**Result**
	First set	Second set		
***Ph. major* s.l.**	80.35	97.24	16.89	increase
Ph. tobbi	78.23	78.31	0.08	increase
Ph. perfiliewi trancaucasicus	73.3	96.85	23.55	increase

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Table 10. One-way analysis of variance for centroid size observed between the study area and species, Ardabil Province of Iran, 2022

	Effect	SS	MS	df	F	P (param.)
***Ph. major* s.l.**	**Individual**	114148.6	38049.52782	3	1.89	0.1367
	Residual	2016601	20166.01482	100		
Ph. perfiliewi transcaucasicus	**Individual**	49074.27	9814.854483	5	0.64	0.6685
	Residual	1499220	15298.16767	98		
Ph. tobbi	**Individual**	87741.71	21935.42827	4	1.69	0.1609
	Residual	972636.9	12968.49178	75		

Table 11. Mean and standard deviation of wing indices in females of three species in pixels' size, Ardabil Province of Iran, 2022

Species	Branching of R1+2+3 (LM11) to branching of M1+2 (LM10)	End of R1 (LM1) to end of CuA1 (LM9)= width	Branching M1+2 to M2	Delta index	Gamma index	Alpha to beta ratio	Beta index	Alpha index
Ph. tobbi	166.88	561.73	648.73	124.04	889.1	1.586877	246.9	391.8
Ph. perfiliewi	164.28	643.3	790.73	189.86	1012.77	1.500096	313.16	469.77
***Ph. major* s.l.**	187.13	664.55	713.26	145.6	962.66	1.356492	303.12	411.18
Mean	172.76	623.19	717.57	153.17	954.84	1.48	287.73	424.25
Standard deviation	12.51	54.28	71.10	33.56	62.20	0.12	35.71	40.59

Table 12. Genbank accession code of three species samples and their location and source in this study used for phylogenetic tree, F= female, M= male

Species	Location	Sample code	Genbank accession number
***Ph.* (*Lar.*) *major* s.l.**	TazehKand	153-F	Under study
	TazehKand	950-F	Under study
	TazehKand	978-F	Under study
	Qahramanloo	1106-F	Under study
	Qahramanloo	1408-F	Under study
	Karaj	Kord2-M	Under study
	Turkey	MN086544	MN086544
Ph.* (*Lar.*) *perfiliewi	Qahramanloo	1450-F	OR391745
	Qahramanloo	1548-F	Under study
	Qahramanloo	1700-M	Under study
	Italy	MG948469	MG948469
Ph.* (*Lar.*) *tobbi	Qahramanloo	1581-F	Under study
	TazehKand	9328-F	Under study
	Qahramanloo	1036-F	OR391747
	TazehKand	969-F	OR391746
	TazehKand	1639-M	OR391748
	TazehKand	936-F	Under study
	TazehKand	943-F	Under study
	Turkey	MN086652	MN086652

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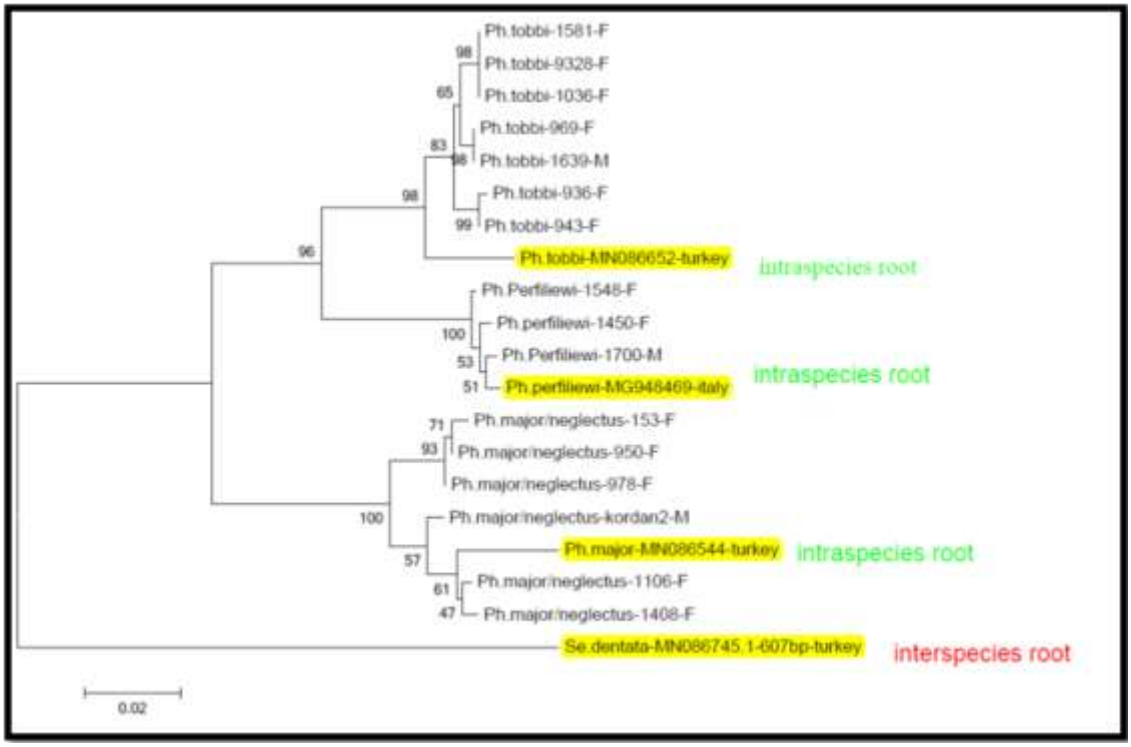

Fig. 9. Neighbor-Joining phylogenetic analysis based on COI gene sequences of 3 species in the present study and reference species (yellow highlight) from GenBank as an outgroup and ingroup to root in the tree, Bootstrap values (500 replicates) neighbor-joining appeared near the branches, the optimal tree with the sum of branch length= 0.38306711 is shown Evolutionary analyses were conducted in MEGA7

Discussion

Accurate identification of sand flies specimens is required for a correct determination of the relevant bionomics, and a better understanding of the dynamics, pathogens, and risk of acquiring diseases transmitted by them. In the present study, 949 specimens of the subgenus *Larroussius* were collected, with a combination of *Ph. major* s.l. (47.52%), *Ph. tobbi* (12.75%), and *Ph. perfiliewi transcaucasicus* (39.73%), and their species composition were matched with previous studies (3, 4, 8, 11, 21). The female specimens of the subgenus *Larroussius* have morphologically close-related species of sandflies that are difficult to identify and many attempts have been made to distinguish these complex specimens using cytochrome b (mtDNA) and elongation factor- 1 (nDNA) nuclear genes, morphological characteristics including the length and number of spermatic bands, the structure of the pharyngeal armature, but the majority of them have did not succeed (15). In the present study area, these complex specimens represent the confirmed or suspected vectors of visceral leishmaniasis (1–2). Most studies have reported on the prevalence of *Leishmania* parasite infection in recent years, particularly *Leishmania infantum*, which can infect humans and cause disease in up to 100 cases per year, especially in children. (5, 6, 7, 12, 13). Amplification of the COI gene by the PCR method using universal primers and its sequencing has recently been used to identify complex species, and it was utilized for the first time for phlebotomine sand flies in Iran (16, 17, 18, 25). The geometric morphometric method was used to compare the size and shape of the right wing in two sets of 286 (31.17%) of the subgenus

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specimens of three female sand fly species that are members of the *Ph. major* complex. The two sets were created according to the descriptions in the materials and methods (25, 27), which were separated into two stages. The right wings of three species of *Ph. major* s.l., *Ph. tobbi*, and *Ph. perfiliewi transcaucasicus* sandflies with 15 landmarks have been successfully employed for analysis. Most researchers have found that they come within the same range. The decrease in sample size and the removal of overlapping samples from the first set were both attributed to an estimated increase in the impact of this two-principal component, which was 3.78%. The researchers' suggestion for sample size for each species is around the number of landmarks. Landmarks 10, 12, 13, 14, and 15 had the most variations in both sets, but in the first set, 1, 9, and 11 in the second set, 2, 3, 4, and 5 had the most changes. The landmarks in the middle and close to the base of the wing appear to be the main areas where the species differ from one another. Alpha, beta, gamma, and delta sizes were more effective for Landmarks 10 and 12. These effects are confirmed in Table 10 of this investigation and are also compatible with findings from investigations on sibling species of *Lutzomyia cruciata* performed in Mexico (31). Two analytical sets were used for the categorization, each with a 95% confidence interval, and the first set included 286 specimens from three different species that initially overlapped. The second biggest classification concerned *Ph. tobbi*, however, in the second group with 53 specimens of three different species, the classification based on PC1 and PC2 differentiated the species and reduced their overlap. The results of the CS analysis using the ANOVA approach in two adjusted sets with a test (Fset 1=5.46, Fset 2=8.99) and the significant parameter was P > 0.0012 for the first set, P> 0.0005 for the second set, demonstrating that different species have different wing sizes. The findings of this study were in agreement with research on female sand fly specimens of the *Ph. caucasicus* group in Iran's cutaneous leishmaniosis endemic areas (29), as well as with geometric morphometric findings on *Lutzomyia* specimens in Brazil (28) and *Phlebotomus sergenti* in Morocco (30). There was significant variation in wing shape across the three species, according to the F test and Pillai's trace done in an ANOVA on the wing shape for the two sets. The significance level of the F test and Pillai's trace test was comparable in Mexico to research done on *Lutzomyia crusiata* sibling species (set 1: F= 1.73, P>0 .0001, Pillai tr=1 .53, P> 0.0001, and set 2: F= 3.39, P> 0.0001) (31). By examining the uniformity of the wing covariance matrix with a test, it was determined that the wing canonical discriminating in 3 species was significant: BOXM= 16.926, P> 0.010242 in two sets. Three samples were placed in overlap during discriminating analyses, and further samples were positioned at a specific distance from one another. The Mahalonobis distance and the Procrustes distance in the two-by-two comparison differed between the two sets, and the T-Square test was significant in all comparisons except for one with a P> 0.05. A 1000-permutation permutation test showed significant results. Due to the overlap of 3 samples in the second set of the *Ph. tobbi-Ph. Perfiliewi transcaucasicus* comparison, the difference in averages in the T-Square test was not significant. The distance between Procrustes and Malanobis in the second set was increasing in comparison to the previous set, and we were expecting that by removing the samples from one another, this gap would also grow. In the first set, the range of total species discrimination was calculated to be 77.12–86.55, and in the second set, 77.80–100. It may be stated that the geometric morphological method allows for more accurate classification and identification of the species with a smaller sample set. When the validity of the discriminability between the two sets is compared, *Ph. major* s.l. and *Ph. perfiliewi transcaucasicus* exhibit increased

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validity whereas *Ph. tobbi* shows decreased validity. Based on matrix ranking, the one-way (ANOSIM) approach revealed differences between the three species. The difference between species is revealed in two sets by hierarchical clustering of mixed species based on the coordinates of landmarks that all landmarks serve as crucial for categorization. The ANOVA approach was used to analyze the shape and size of the wing in each population of each species, separately from the places where data were collected. The results had a low F test and a P value of 0.05, which was not significant. It is determined that there are no significant variations in the size and shape of the wings among the sites investigated.

The estimated size for each species was different and varied significantly from one another. The total effects of changes in landmarks on the distance of alpha, beta, ratio of alpha to beta, gamma and delta, width and length of wings were tested independently for all three species. *Phlebotomus tobbi* indicated sizes are less than those of the other two species, meaning its wing was smaller *Phlebotomus major* s.l. was almost in the middle of the other two species while *Ph. perfiliewi transcaucasicus* exhibits larger sizes.

This result was supported by the clustering of the phylogenetic tree based on the COI gene in this study, which was also in agreement with findings from related investigations on Mexican *Lut. crusiata* siblings (31). The COI gene barcode sequence has been effectively tested in multiple processes within the *Ph. major* complex in the study area's districts. The major complex group of sand flies was collected (males and females) in three districts as well as from allopatric locations, without considering the species delimitation algorithm into account. The COI gene tree phylogeny based on neighbor-joining indicated that the COI barcode was a useful tool to identify between members of the *Ph. major* complex since all species were placed in one cluster and each species in one branch. Nuclear gene Elongation Factor 1-alpha (EF-1), which demonstrated the existence of different genes between sympatric geographic groups (15), and mitochondrial gene Cytochrome b (Cyt b), which was identified in the same species, confirm earlier research. In this work, three species from the study area were used to distinguish among species using the COI gene with high similarity and more than 98% support, which is consistent with studies done using the same method with comparable samples in the region near Iran in Turkey (26). Obtained sequences from GenBank were placed in the same branch as the in-group and out-group (26). The geometric morphometric results of this study were in agreement with genetic analysis, which confirmed species classification within species level. A similarity report of more than 97% based on the COI gene in the BLAST databases in the Genbank and BOLD Biolife system indicates that *Ph. neglectus* is present in the study area as a member of sibling and sympatric species. *Phlebotomus neglectus* was also widely observed in Bileh Savar and Germi counties via the COI gene. The existence of this species had previously been reported from different sites with question and possibility (23, 24). With the conclusion of this investigation, we are closer to reporting the definitive existence of *Ph. neglectus*, and numerous morphometric studies for female specimens in the study area were necessary.

Conclusion

In this study, the efficacy of wing geometric morphometry in conjunction with molecular techniques was tested for the first time to distinguish the three major group species in the area. In addition to the identification keys, the current study showed that the DNA barcode and geometric morphometric studies can classify three species in the endemic areas of visceral leishmaniosis in the districts of Germi, Bileh Savar, and Meshkin-Shahr. These taxonomic tools can also be used to identify

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vectors, evaluate their risk, and help in their control. The COI and DNA barcode of *Ph. perfiliewi transcaucasicus* and *Ph. tobbi* in the study areas were extracted, sequenced, and registered in GenBank on the NCBI database. These species were also added to the extensive DNA barcode library of sandflies in Iran, and the presence of *Ph. neglectus* species in this area was confirmed. It is advised that the best way to differentiate among the region's female specimens of major group sand flies was to identify each species using a DNA barcode with universal COI primers. In remote Laboratory centers without access to molecular biology tools or with limited resources, the GM method is a viable alternative. It can be utilized as a supplement to earlier morphological efforts in various target locations and circumstances to prevent mistakes brought on by incorrect specimen diagnosis.

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Ethical considerations

This experiment was carried out under the guidance of the Ethics Committee of the Tehran University of Medical Sciences (IR.TUMS. REC.1400.044).

Conflict of interest statement

The authors declare there is no conflict of interest.

References

1. Alvar J, Velez ID, Bern C, Herrero M, Desjeux P, Cano J, Jannin J, den Boer M (2012) Leishmaniasis worldwide and global estimates of its incidence. PLOS One. 7 (5): e35671.
2. Mohebali M (2013) Visceral leishmaniasis in Iran: Review of the Epidemiological and Clinical Features. Iran J Parasitol. 8(3):348–358.
3. Rassi Y, Javadian E, Nadim A (2005) *Phlebotomus* (*Larroussius*) *kandelakii* the principal and proven vector of visceral leishmaniasis in Northwest of Iran. Pak J Biol Sci. 8(12): 1802–1806.
4. Rassi Y, Javadian E, Nadim A (2009) *Phlebotomus perfiliewi transcaucasicus*, a vector of *Leishmania infantum* in northwestern Iran. J Med Entomol. 46(5): 1094–1098.
5. Rassi Y, Abai MR, Oshaghi MA, Javadian E, Sanei A, Rafidzadeh S, Arzamani K (2012) First detection of *Leishmania infantum* in *Phlebotomus kandelakii* using molecular methods in north-eastern Islamic Republic of Iran. EMHJ. 18(4): 387–392.
6. Rassi Y, Sanei Dehkordi A, Oshaghi MA, Abai MR, Mohtarami F, Enayati A, Javadian, E (2012) First report on natural infection of the *Phlebotomus tobbi* by *Leishmania infantum* in northwestern Iran. Exp Parasitol. 131(3): 344–349.
7. Azizi K, Rassi Y, Javadian E, Motazedian MH, Asgari Q, Yaghoobi-Ershadi MR (2008) First detection of *Leishmania infantum* in *Phlebotomus* (*Larroussius*) *major* (Diptera: Psychodidae) from Iran. J Med Entomol. 45(4): 726–731.
8 .Yaghoobi-Ershadi M (2012) Phlebotomine sand flies (Diptera: Psychodidae) in Iran and their role on Leishmania transmission. J Arthropod-Borne Dis. 6(1): 1–17.
9. Rostamian M, Bashiri H, Yousefinejad V, Bozorgomid A, Sohrabi N, Raeghi S, Rezaeian S (2021) Prevalence of human visceral leishmaniasis in Iran: A systematic review and meta-analysis. Comp Immunol Microb. 75: 1–10.

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https://doi.org/10.18502/jad.v18i3.18573

10. Adham D, Moradi-Asl E, Dorosti A, Khaiatzadeh S (2020) Spatial autocorrelation and epidemiological survey of visceral leishmaniasis in an endemic area of Azerbaijan region, the northwest of Iran. PLoS One 15(8): e0236414.
11. Moradi-Asl E, Rassi Y, Adham D, Hanafi-Bojd AA, Saghafipour A, Rafizadeh S (2018) Spatial distribution of sand flies (Diptera: Psychodidae; *Larroussius* group), the vectors of visceral leishmaniasis in Northwest of Iran. Asian Pac J Trop Biomed. 8(9): 425–430.
12. Oshaghi MA, Rassi Y, Hazratian T, Fallah E, Rafizadeh S (2013) Natural infection of wild caught *Phlebotomus tobbi* to *Leishmania infantum* in East Azerbaijan Province, northwestern Iran. J Arthropod-Borne Dis. 50(1): 9–24.
13. Oshaghi MA, Ravasan NM, Hide M, Javadian EA, Rassi Y, Sadraei J, Mohebali M, Sedaghat MM, Hajjaran H, Zarei Z, Mohtarami F (2009) *Phlebotomus perfiliewi transcaucasicus* is circulating both *Leishmania donovani* and *L. infantum* in northwest Iran. Exp Parasitol. 123(3): 218–225.
14. Killick-kendrick R (1990) Phlebotomine vectors of leishmaniasis: a review. Med Vet Entomol. 4(1): 1–24.
15. Absavaran A, Rassi Y, Parvizi P, Oshaghi M, Abaie M, Rafizadeh S, Mohebali M, Zarea Z, Javadian E (2009) Identification of Sand flies of the Subgenus *Larroussius* based on Molecular and Morphological Characters in North Western Iran. J Arthropod-Borne Dis. 3(2): 22–35.
16. Daly HV (1985) Insect Morphometrics. Annual Review of Entomology. 30(1): 415–438.
17. Bookstein FL (1982) Foundations of morphometrics. Annu Rev Entomol. 13(1): 451–470.
18. Pentinsaari M, Salmela H, Mutanen M, Roslin T (2016) Molecular evolution of a widely adopted taxonomic marker (COI) across the animal tree of life. Sci Rep. 6(1): 352–375.
19. Belen A, Alten B, Aytekin AM (2004) Altitudinal variation in morphometric and molecular characteristics of *Phlebotomus papatasi* populations. Med Vet Entomol. 18(4): 343–350.
20. Folmer O, Black M, Hoeh W, Lutz R, Vrijemnhoek R (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol Mar Biol Biotechnol. 3(5): 294–299.
21. Seyedi-Rashti MA, Nadim A (1992) The genus *Phlebotomus* (Diptera: Psychodidae: Phlebotominae) of the countries of the Eastern Mediterranean Region. Iran J Public Health. 21(1–4): 11–50.
22. Lewis DJ (1982) A taxonomic review of the genus *Phlebotomus* (Diptera: Psychodidae). Bulletin of the British Museum (Natural History) Zoology. (Ent). 45(2): 121–209.
23. Badakhshan M, Sadraei J, Moin-Vaziri V (2011) Morphometric and morphological variation between two different populations of *Phlebotomus major sl* from endemic and non-endemic foci of visceral leishmaniasis in Iran. J Vector Ecol. 36 (1): 153–158.
24. Badakhshan M, Moin-Vaziri V, Sadraei J (2022) *Phlebotomus neglectus* (Diptera: Psychodidae): new insights on its presence in Iran based on three independent genetic loci. J Med Entomol. 59(1): 233–239.
25. Giordani BF, Andrade AJ, Galati EAB, Gurgel-Goncalves R (2017) The role of wing geometric morphometrics in the identification of sandflies within the subgenus *Lutzomyia*. Med Vet Entomol. 31(4): 373–380.
26. Kasap OE, Linton YM, Karakus M, Ozbel Y, Alten B (2019) Revision of the species composition and distribution of

https://doi.org/10.18502/jad.v18i3.18573

Turkish sand flies using DNA barcodes. Parasit Vectors. 12(1): 1–20.
27. Zelditch ML, Swiderski DL, Sheets HD (2012) Geometric morphometrics for biologists: a primer. Second edition. Academic Press. USA.
28. Giordani BF, Andrade A.J D, Galati EAB, Gurgel-GonÇalves R (2017) The role of wing geometric morphometrics in the identification of sandflies within the subgenus *Lutzomyia*. Med Vet Entomol. 31 (4): 373–380.
29. Absavaran A, Mohebali M, Moin-Vaziri V, Zahraei-Ramazani A, Akhavan AA, Mozaffarian F, Rafizadeh S, Rassi Y (2019) Morphometric discrimination between females of two isomorphic sand fly species, *Phlebotomus caucasicus* and *Phlebotomus mongolensis* (Diptera: Phlebotominae) in endemic and non-endemic foci of zoonotic cutaneous leishmaniasis in Iran. Asian Pac J Trop Biomed. 12(4): 153–162.
30. Abou-Elaaz FZ, Sereno D, Himmi O, Ghamizi M, Guernaoui S (2023) Descriptive and Geometric Morphometry of the Wings of *Phlebotomus sergenti* Populations in Central Morocco. J Arthropod-Borne Dis. 17(1): 36–50.
31. Mikery OF, Rebollar-Téllez EA, Cruz-López LC, Marina CF, Castillo A (2019) Traditional and geometric morphometry analyses of *Lutzomyia cruciata* (Diptera: Psychodidae: Phlebotominae) populations of Chiapas, Mexico. J Med Entomol. 56 (3): 697–707.