Geometric Morphometric Study of Honeybee (*Apis mellifera* L. 1758) Populations in Central Iran

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ARTICLE INFO	**ABSTRACT**
	The honeybee is one of the most important insect species for mankind due to its role in the pollination of crops and products that it makes. Western honeybee *Apis mellifera* is a cosmopolitan species due to human beekeeping practices. The geometric morphometric method was used to investigate the differences in wing size and shape of worker honeybee populations collected from 8 locations and apiaries from Isfahan and Chaharmahal & Bakhtiari provinces. Fore and hindwing shape and size variations were investigated based on 16 homologous landmark coordinates. In terms of wing size, data showed that Khansar's honeybees have the largest and Shahrekord honeybees have the smallest fore and hindwings. In terms of wing shape, more than half of the pairwise compared populations showed significant differences in both fore and hindwings. Also, allometry is not seen, meaning that variations in wing size and wing shape were independent from one another. This study provides information on the diversity of honeybee populations in the study area.

Introduction

Bees flew and pollinated flowering plants when dinosaurs walked on Earth more than 120 million years ago. Over the past 100 million years, bees and flowering plants have coevolved such that they cannot be found without one another. In addition to their critical role in pollination and food supply security, bees provide us with other valuable products such as honey, wax, propolis, and royal jelly that are used to make other valuable products such as medicines, syrups, candles, salves, ointments, make-up, hair products, etc. (Cardinal and Danforth, 2013; Kane and Faux, 2021).

Generally, what is called honeybee includes 10 species belonging to the Genus *Apis*, Family Apidae, and Order Hymenoptera. The Western honeybee, *Apis mellifera*, is naturally spanning western Asia, Europe, and Africa (Arias and Sheppard, 2005; Engel, 1999; Han *et al*., 2012)

but domestication and transport of the species by humans have made it a cosmopolitan species distributing around the world except for Antarctica and many oceanic islands (Hung *et al*., 2018; Ruttner, 1988). The other nine species of *Apis* exclusively appear in Asia (Han *et al*., 2012). Morphological and molecular investigations reveal five major evolutionary lineages of honeybees with more than 33 subspecies (also called geographic races). These lineages include A (Africa) and its sublineage Z, M (western and northern Europe), C (Eastern Europe), O (Near East and Central Asia), and Y (East Africa and Arabian Peninsula) (Ilyasov *et al*., 2020; Requier *et al*., 2019; Ruttner, 1988). Of these lineages and subspecies, *Apis mellifera meda* (the Persian honeybee) (Skorikov 1929b) and *Apis mellifera anatoliaca* (the Anatolian honeybee) (Maa 1953) belonging to lineage A (sublineage Z) as well as *Apis mellifera remipes* (The Armenian honeybee) (Gerstäcker 1862)

belonging to lineage O seems to occur in Iran (Ilyasov *et al*., 2020). Wild pollinators and honeybees are experiencing a global decline in varying levels due to human activities such as habitat destruction, pollution, pesticides, and diseases (Kükrer *et al*., 2021).

Additionally, native honeybees suffer local extinctions as a result of genetic mixing due to the honeybee trade, such as the replacement of local honeybees by non-native strains and beekeeping practices that move colonies between geographical regions (de La Rúa *et al*., 2009).

These challenges have negative impacts on honeybee populations and likely contribute to their extinction, but sometimes native populations better face these challenges due to their genetic make-up (Büchler *et al*., 2014).

Diversity is the key to adapting to these conditions. Therefore, studying honeybee genetic and morphological diversity at various levels, i.e., population and subspecies levels, is of great importance for the conservation of species, ecosystems, and the economic services that honeybees provide (Kükrer *et al*., 2021).

The number of studies focusing on the honeybee populations of Iran is increasing. These studies used morphological (traditional and geometric morphometric) (Boulhasani *et al*., 2018; Dadgostar, *et al*., 2020a and 2020b; Parichehreh Dizji *et al*., 2017; Tahmasebi *et al*., 1998) and molecular approaches (Rahimi *et al*., 2016, 2018, 2022; Salehi and Nazemi-Rafie, 2020) to investigate the structure of populations of *Apis mellifera meda,* the Persian honeybee. The geometric morphometrics method has been proven to be useful in detecting morphological differences and distinguishing different populations, subspecies, and species of honeybees (Abed *et al*., 2021; Boulhasani *et al*., 2018; Buala and Sopaladawan, 2022; Bustamante *et al*., 2020, 2021; Charistos *et al*., 2014; García *et al*., 2022; Masaquiza *et al*., 2023; Rodrigues *et al*., 2022; Salehi and Nazemi-Rafie, 2020; Santoso *et al*., 2018).

In the current study, the geometric morphometric method was used to investigate the differences between populations collected from different locations and apiaries from Isfahan and Chaharmahal and Bakhtiari provinces.

Materials and Methods

Sample preparation and imaging

With the cooperation of beekeepers during the spring and summer of 2022, a total of 278 worker bees were collected from eight research stations located in Isfahan and Chaharmahal and Bakhtiari provinces (Table 1), transferred to the laboratory, and placed in ethanol. The right forewing and hindwings of 196 bees were carefully removed and placed on the slide (the damaged or deformed wings were not used in making the slides). A coverslip was placed on the wings by adding a drop of ethanol to them and the wing slide was prepared. The prepared slides were immediately imaged by an image analysis system consisting of a Nikon SMZ-2B stereomicroscope (Nikon: 9-16, Ohi 3-Chome, Shinagawa-Ku, Tokyo 140, Japan) and digital microscope camera from Celestron (Celestron: 2835 Columbia St. Torrance, California 90503 U.S.A.) connected to a computer. The slides were placed in the same and similar direction under the stereomicroscope as much as possible. All the images were taken with a magnification of 0.6 and those related to the individuals of each population were saved in separate folders on the computer.

Digitizing images and statistical analysis

A total of 16 landmarks in the forewing and 5 landmarks in the hindwing were selected at the bifurcation of the wings. Landmarking was done on the images in the tpsdig2 64-bit version 2.32 software (Rohlf. F. James, 2015) environment and a tps landmark coordinate file was created for each population. Landmarking was done in the same order in all the images (Fig. 1) and those images that lacked the position of any of the landmarks for any reason were removed from the landmarking.
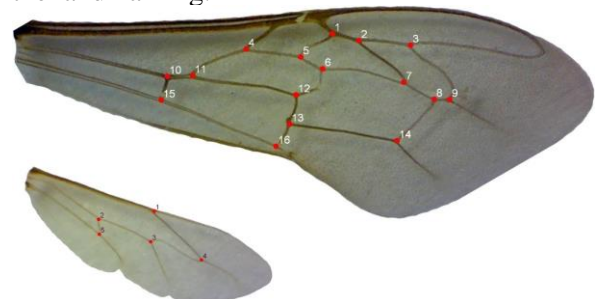

Fig. 1. Landmarking using tpsDig2 software: The Forewing has 16 landmarks and the hindwing has 5 landmarks.

Finally, the landmark data of all populations were saved in a single file in the order specified in Table 1 and prepared for analysis. The data matrix of all the landmark coordinates for the forewing and hindwing was entered separately into MorphoJ software package version 1.07a (Klingenberg, 2011) and evaluated for the possibility of outliers due to possible mistakes in

observing the order of landmarks in the landmarking process. Then, to remove non-shape changes and alignment and adaptation of landmarks, superimposition, Generalized Procrustes Analysis (GPA) was performed in MorphoJ and tpsRelw 64-bit version 1.75 software package (Rohlf. F. James, 2015).

Table 1. The sample information.

No.	Township	Sampling location	Geographical coordinates	Elevation	Total number of samples	Number of prepared slides	Forewing landmarking	Population position in the data matrix	Hindwing landmarking	Population position in the data matrix
1	Semirom	Semirom	31°24'51" N 51°34'10" E	2400 m	17	13	13	1-13	11	1-11
2	Dehaghan	Golshan	31°55'50.5" N 51°45'04.3" E	1950 m	40	30	30	14-43	30	12-41
3	Khansar	Khansar	33°13'30.1" N 50°18'22.4" E	2215 m	70	30	24	44-67	30	42-71
4	Kashan	Kashan	33°59'N 51°26'E	970 m	35	30	29	68-96	30	72-101
5	Ardestan	Goonian	33°15'43.8" N 52°14'06.6" E	2000 m	40	30	30	97-126	29	102-130
6	Shahreza	Shahreza road	31°57'09.7" N 51°46'33.6" E	1925 m	14	13	13	127-139	12	131-142
7	Ardal	Do polan road	31°55'03.4" N 50°36'19.8" E	1480 m	22	20	20	140-159	20	143-162
8	Shahrekord	Hashtgerd	32°19'32" N 50°51'51" E	2060 m	40	30	29	160-188	30	163-192

The centroid size and weight matrix calculated by the tpsRelw software package for forewing and hindwing were saved separately for further analysis. The allometric effect was investigated by Regression Analysis using weight matrix and centroid size to see if there is a relationship between changes in shape and size using the tpsRegr 64-bit version 1.50 software package (Rohlf. F. James, 2015). Principle Component Analysis as a tool to investigate patterns of diversity in the population using a variance-covariance matrix was carried out using MorphoJ. Canonical Variate Analysis as a tool to analyze and test the difference between populations was carried out using NTSYSpc version 2.02 (Rohlf. F. James, 2000). Kruskal-Wallis non-parametric univariate analysis of variance was performed on centroid size data using PAST v4.12 software package (Hammer and Harper, 2001) to detect differences in wing size in populations along with the post hoc test (Dunn's test) to investigate possible differences between each pair of compared populations. One-way multivariate analysis of variance, PERMANOVA, on superimposed landmark coordinate data by Procrustes analysis was performed to detect differences in wing shape in

populations using PAST v4.12 software package with 9999 permutations along with the Bonferroni-corrected post hoc analysis. Permutation tests do not require assumptions such as data normality that parametric versions of MANOVA require. Clustering or cluster analysis was carried out on the weight matrix data to detect the phenetic relationships between the studied populations using the NTSYSpc software package.

Results

Wings size variation

The centroid size, which is calculated with the second root of the square of each Landmark to the Landmark Space Arrangement Center, is introduced as the geometrical size of each sample. The average centroid size in the forewing showed that the population of Khansar's honeybees has the largest and the population of Shahrekord honeybees has the smallest forewings (Fig. 2A). In addition, the average centroid size in the hindwing showed that the population of Khansar's honeybees has the largest and the population of Shahrekord honeybees has the smallest hindwings (Fig. 2B).
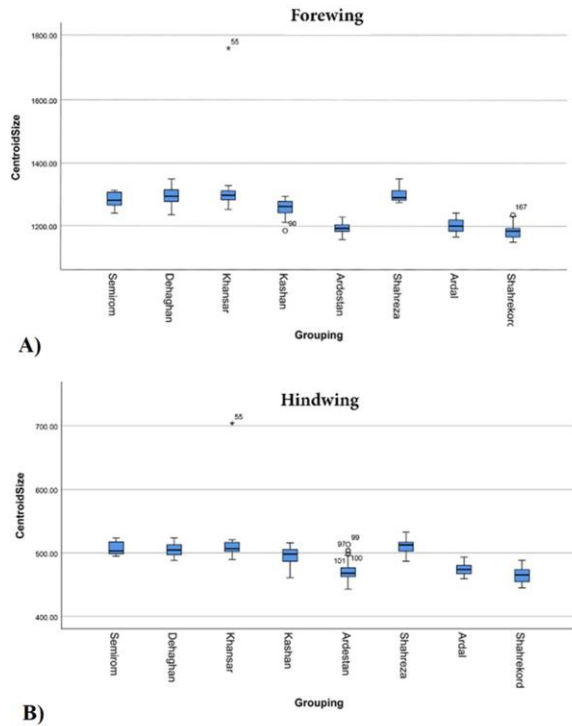
Fig. 2. The mean centroid size of wings: A) The mean centroid size in the forewings; B) The mean centroid size in the hindwings.

One-way ANOVA analysis of centroid size

ANOVA is a statistical method for testing the null hypothesis in which several single-variable specimens have been taken from populations with the same average. It is assumed that the samples are close to the normal distribution and have similar variances. If the number of individuals in the population is equal, these two assumptions are not vital. However, if the assumptions are severely violated, the Kruskal-Wallis one-way ANOVA should be used instead. The results of the Shapiro-Wilk normality test and Levene's homogeneity test on centroid size data of the forewing and hindwing showed that some populations do not follow the normal data distribution and homogeneity of the averages; so due to the unequal sample size of the populations, the common one-way ANOVA are not usable. Kruskal-Wallis one-way ANOVA on the total centroid size of the forewings and hindwings was performed separately to investigate the possible differences between populations and within the population. Additionally, Dunn's post hoc test was performed to examine the possible differences between each pair of comparable populations. The results of Kruskal-Walli's analysis on centroid size showed that there is a significant difference between populations in forewings (p = 3.016E-28) and hindwings (p = 1.498E-24) and the results of the post hoc test demonstrated that most of the compared populations have significant differences (Table 2). Among the populations studied about the centroid size of the forewings, except for the populations of Semirom-Dehaghan, Semirom-Khansar, Semirom-Kashan, Semirom-Shahreza, Dehaghan-Khansar, Dehaghan-Kashan, Dehaghan-Shahreza, Khansar-Shahreza, Kashan-Shahreza, Ardestan-Ardal, Ardestan-Shahrekord, and Ardal-Shahrekord, other populations had a significant difference. Moreover, among the populations studied concerning the centroid size of the hindwing, except for the aforementioned populations and Khansar-Kashan populations, other comparisons of populations were significant with an almost similar pattern (Table 2).

Wings shape variation

Principal component analysis

By principal component analysis (PCA) of all forewing samples using the MorphoJ software package, about 32% of the variation in shape between samples was explained by the first two components extracted from the variance-covariance matrix (the first component 17.83% and the second component 14.17%). Also, 84.52% of the variation is explained by the first 10 components out of 28 components. In the hindwing, the first two components explain about 69.357% of the shape variation between samples (the first component 40.830% and the second component 28.527%) and 6 components explain 100% of the variation. Scatter plots of PCA of populations are shown in Fig. 3.

Canonical variate analysis

The results of canonical variate analysis on the forewing and hindwing are shown in Fig. 4. The plates in this diagram show the mean shape (consensus) configuration and the height of the bars shows the distance of each population with the consensus configuration and the distance of

143

the bars from each other shows the distance of the populations from each other.
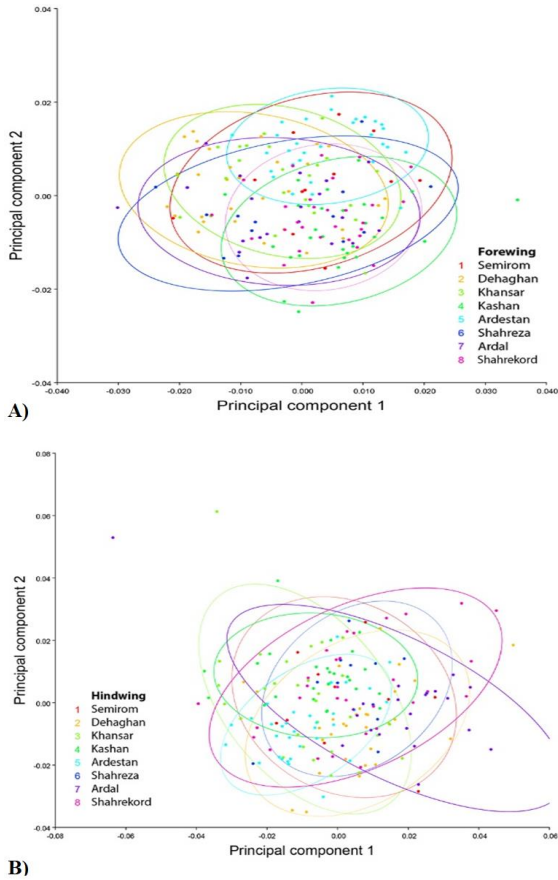

Fig. 3. Wing PCA: A) Forewing; B) Hindwing.

The results in the forewing reveal that the Shahrekord and Ardel populations show the highest and lowest differences with the consensus configuration, respectively. The results in the hindwing also explain that Ardel and Ardestan populations show the highest and lowest differences with the consensus configuration, respectively.
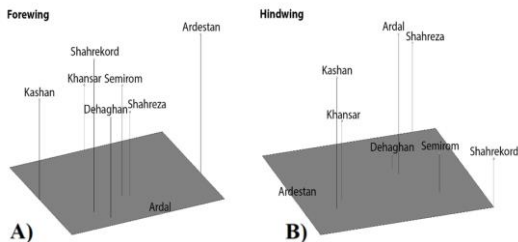

Fig. 4. Wings CVA: A) Forewing; B) Hindwing. The plates in the diagram show the consensus configuration and the bars show the distance of each population with the consensus configuration. The distance of the bars from each other also shows the distance of the populations from each other.

Multivariate analysis of variance

A version of Multivariate analysis of variance (MANOVA) which uses the Permutation Test to determine the significance of differences (PERMANOVA) was performed on the data of landmark coordinates superimposed by Procrustes analysis using PAST v4.12 software package with 9999 permutations. The results of this test showed that there are significant differences between the population of honeybees in terms of the shape of the forewing (F= 5.441, p (same): 0.0001) and the hindwing (F= 5.514, p (same): 0.0001). The results of the post hoc test showed that in the forewing, except for the population pairs of Semirom-Dehaghan, Semirom-Khansar, Semirom-Ardestan, Semirom-Shaherza, Semirom-Ardel, Dehaghan-Shahreza, Khansar-Shahreza, Kashan-Shahreza, Kashan-Shahrkord, Shahreza-Ardel, and Shahreza-Shahrkord, other pairwise comparisons of populations have significant differences. In the hindwing, except for the comparisons of Dehaghan-Khansar, Dehaghan-Kashan, Dehaghan-Ardestan, Khansar-Ardal, Khansar-Shahrkord, Kashan-Ardestan, Kashan-Ardal, Kashan-Shahrkord, Ardestan-Shahreza, Ardestan-Ardal, and Ardestan-Shahrekord, other pairwise population comparisons do not show significant differences (Table 3).

Clustering analysis

Clustering or cluster analysis (CA) is the process of grouping people with similar characteristics or similar variable sizes. As seen in Fig. 5, in the clustering of populations based on forewing the populations of Semiram, Dehaghan, Shahreza, Kashan, and Shahrekord form a cluster and are separate from other populations. In addition, the clustering of populations according to similarity in the hindwing shows three separate clusters. The populations of Semiram and Shahrekord form a cluster and the populations of Dehaghan, Ardel, and Shahreza form another cluster. The populations of Khansar, Kashan, and Ardestan also form the third cluster.

Allometry vs Isometry

The relationship between changes in the size and shape of the wings was investigated by regression analysis. The results of the statistical analysis suggest no significant allometric

changes in both forewing (Wilk's Lambda= 0.82843001, P= 0.1107) and hindwing (Wilk's Lambda= 0.99543797, P= 0.6491), meaning that size and shape changes do not depend on each other in both forewing and hindwing.

Table 2. Dunn's post hoc test results on centroid size of the forewing and hindwing. The Bonferroni Corrected P-Values are on the left and the Z Statistic values are on the right side of the table. Significant values have been highlighted.

	Study locations	Semirom	Dehaghan	Khansar	Kashan	Ardestan	Shahreza	Ardal	Shahrekord
Forewing	**Semirom**	-	0.7169	1.077	1.503	4.746	0.8794	4.142	5.376
	Dehaghan	1	-	0.4805	2.826	7.025	0.3219	5.936	7.804
	Khansar	1	1	-	3.172	7.178	0.06797	6.146	7.924
	Kashan	1	0.132	0.04246	-	4.077	2.531	3.317	4.868
	Ardestan	5.82E-05	6.00E-11	1.97E-11	0.001278	-	5.784	0.347	0.8393
	Shahreza	1	1	1	0.3185	2.04E-07	-	5.11	6.409
	Ardal	0.0009642	8.16E-08	2.23E-08	0.02548	1	9.01E-06	-	1.097
	Shahrekord	2.13E-06	1.67E-13	6.45E-14	3.15E-05	1	4.09E-09	1	-
Hindwing	**Semirom**	-	0.1744	0.377	1.372	4.581	0.4915	3.976	5.276
	Dehaghan	1	-	0.7527	1.606	6.098	0.7806	4.957	6.965
	Khansar	1	1	-	2.324	6.874	0.2116	5.63	7.718
	Kashan	1	1	0.5641	-	4.147	1.998	3.32	5.035
	Ardestan	0.0001296	3.00E-08	1.75E-10	0.0009422	-	5.343	0.3428	1.081
	Shahreza	1	1	1	1	2.56E-06	-	4.649	6.046
	Ardal	0.001965	2.01E-05	5.04E-07	0.02519	1	9.34E-05	-	1.273
	Shahrekord	3.69E-06	9.20E-11	3.32E-13	1.34E-05	1	4.17E-08	1	-

Table 3. The results of the Bonferroni-Corrected post hoc test on superimposed landmark coordinate data with Procrustes analysis on forewing and hindwing. The P-values are on the left and the F-value is on the right side of the table. Significant values have been highlighted.

	Study locations	Semirom	Dehaghan	Khansar	Kashan	Ardestan	Shahreza	Ardal	Shahrekord
Forewing	**Semirom**	-	1.962	2.635	3.779	2.633	1.21	2.936	2.852
	Dehaghan	0.9604	-	3.759	9.428	9.479	1.778	5.001	7.198
	Khansar	0.1512	0.0056	-	5.81	6.473	2.07	7.482	5.145
	Kashan	0.0084	0.0028	0.0028	-	12.76	2.355	8.287	2.427
	Ardestan	0.056	0.0028	0.0028	0.0028	-	5.114	10.27	10.73
	Shahreza	1	1	0.728	0.3416	0.0028	-	2.253	1.766
	Ardal	0.0532	0.0028	0.0028	0.0028	0.0028	0.6048	-	8.078
	Shahrekord	0.0308	0.0028	0.0028	0.21	0.0028	1	0.0028	-
Hindwing	**Semirom**	-	1.37	1.774	2.644	4.201	0.8568	2.6	0.81
	Dehaghan	1	-	5.855	10.16	7.715	2.182	2.806	4.145
	Khansar	1	0.0056	-	2.279	3.509	2.796	9.492	5.645
	Kashan	1	0.0028	1	-	8.427	3.287	11.35	6.397
	Ardestan	0.1344	0.0056	0.1232	0.0028	-	7.802	14.95	13.37
	Shahreza	1	1	0.8736	0.5012	0.0112	-	1.837	1.464
	Ardal	1	0.8092	0.0028	0.0028	0.0028	1	-	4.918
	Shahrekord	1	0.2492	0.0112	0.0224	0.0028	1	0.0952	-

Discussion

The honeybee is one of the most important insects due to its role in the pollination of flowering plants and crops and in producing honey. The global market size of honey was USD 8.17 billion in 2021 and is expected to grow to USD 12.69 billion by 2029 (Fortune Business Insights, 2022). It is estimated that the honeybee is responsible for 12 out of 16 billion USD annual pollination value in the United States (Calderone, 2012; Khalifa *et al.*, 2021; Rader *et al.*, 2016). Experts are reporting a significant annual decline in honeybee colonies since 2006. This decline has been attributed to many reasons such as pests, pesticides, disease, habitat loss, and reduced species or genetic

diversity (Nowierski, 2021). The genetic diversity of a species helps it better cope with variable environmental conditions and disease (Dillon & Lozier, 2019; Forsman, 2014; Somero, 2012); therefore, it is important to monitor the diversity and conserve it. Phenotypic diversity is sometimes projecting genetic diversity. Therefore, the current research used the diversity of wing structure to evaluate the diversity of honeybee populations in the studied areas. It is reasonable to assume that beekeeping practices such as moving bee colonies around and bee trade may gradually reduce bee diversity. Therefore, this study hypothesizes that there is not much diversity in the bee populations in the study locations. The present research used the centroid size of the wings as a criterion to check the size variation among the bee populations. The average centroid size of the forewing and hindwing showed that Khansar's honeybee population has the biggest and the Shahrekord honeybee population has the smallest forewing and hindwing.
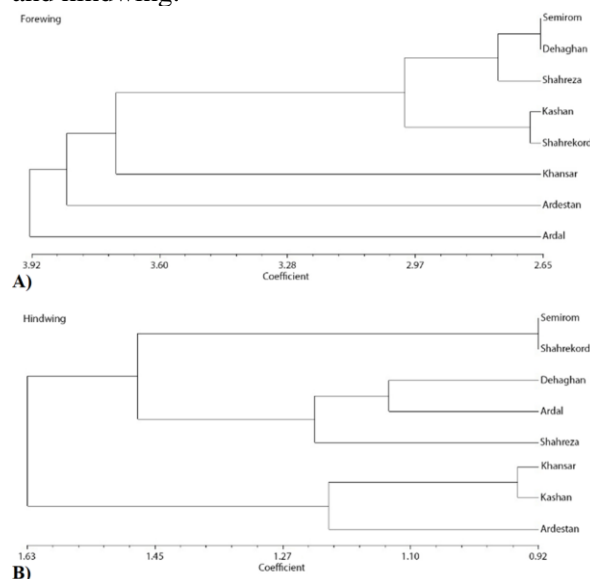

Fig. 5. Results of cluster analysis of the populations: A) In the forewing; B) In the hindwing.

The ANOVA and post hoc analyses showed that 16 out of 28 pairwise comparisons and 15 out of 28 pairwise comparisons are significant in the forewing and hindwing, respectively. This study also used superimposed landmark coordinates to evaluate significant differences in wing shape using MANOVA and post hoc analyses. The

analyses of data showed that in the forewing, 17 out of 28 pairwise comparisons and in the hindwing, 11 out of 28 pairwise comparisons are significant. By closer look at the data, no strict relationship was found between geographical distance/elevation and the significance of the differences between populations. However, often the populations that were geographically distant from one another tend to differ significantly in wing size and shape. Isolation by distance (IBD) theory states that genetic differences can accumulate locally when dispersal (i.e., gene flow) between regions or populations is restricted geographically (Slatkin, 1993; Wright, 1943) and isolation by environment (IBE) theory states that genetic differences increase with differences in environment (Sexton *et al.*, 2014; Wang & Bradburd, 2014; Wang & Summers, 2010). Therefore, it is likely that those distant populations that do not differ significantly might have the same origin due to beekeeping practices or similar environmental conditions. The analyses of data also suggest that changes in wing size and wing shape among the populations were independent of one another and an allometric effect is not seen. Morphological and molecular characteristics can be studied to evaluate differentiation among populations (Dutech *et al.*, 2005). Wing morphology evaluated by geometric morphometric method have been used to identify and discriminate different species (Ndungu *et al.*, 2023; Santoso *et al.*, 2018), subspecies (Abed *et al.*, 2021; Henriques *et al.*, 2020), and populations (Aglagane *et al.*, 2022; Boulhasani *et al.*, 2018; Buala & Sopaladawan, 2022) of honeybee. Machine learning and web-based applications have also been developed to facilitate capturing wing morphology to facilitate further classification and analysis (Bustamante *et al.*, 2021; Rebelo *et al.*, 2021; Rodrigues *et al.*, 2022). The present study used the geometric morphometric method to evaluate the diversity of honeybee populations in a few locations in Isfahan and Chaharmahal and Bakhtiari provinces in Iran. Overall, the findings show that even though all the study stations are located in less than 54000 square

kilometers, there is a considerable amount of diversity in wing size and wing shape in both forewing and hindwing projecting the diversity of honeybee populations in the area.

Authors' contributions

RA conceived the study, carried out the data analysis, and wrote the manuscript. ZZ collected the samples, did the experimental work, and collected the landmark coordinate data. The authors read and approved the final manuscript.

Acknowledgments

Conflict of Interest

The authors declare no conflict of interest.

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