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Comparison of standard and geometric morphometric methods for discrimination of honey bees populations (*Apis mellifera* L.) in Iran

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Abstract
There are two methods of morphometric and geometric for discriminating of honeybee races and populations. Currently, geometric morphometric method uses as inexpensive, fast and precise method for identifying of honeybee races and populations. In standard morphometric, seven distances and three angles were measured from the forewings, proboscis, abdomen and hind legs of different honeybee populations. In geometric morphometric, 18 landmarks were located at wing vein junctions. Five areas were selected as representatives of main geographical points of the North, West, East, Center and South from Iran. Procrustes analysis, principal component analysis, and Canonical variate analysis (CVA) was conducted for discriminating of collected samples. Average length of forewing, proboscis and cubital index were 9.01 mm, 6.55 mm and 2.4 in Iran. Results showed that geometric and standard morphometrics discriminated area populations 77.5 and 70.7% respectively therefore the geometric morphometric method was more precise and easier than the standard morphometric because we used only forewing for discrimination of populations.

Keywords: standard morphometric, geometric morphometric, discrimination, *Apis mellifera*

1. Introduction

According to the one hypothesis, honeybees evolved in near Ethiopia in Africa and extended over Africa and then Middle East and Europe [19]. However the second hypothesis explains that bees belonging to the genus *Apis* were originally found only in the Old World, namely southern part of Caspian Sea then they were distributed to Africa and Europe [15]. According to Ruttner [15], there are at least 24 *Apis mellifera* subspecies grouped in three or four evolutionary branches, based on morphometric data. Ruttner [16] discriminated six local populations for *A. m. meda*, as follows: West and Central Iran (Azerbaijan-Iranian highlands), the subtropical coast of the Caspian Sea (Mazandaran), Northeast Iran (Mashad), Southeast Iran (Kerman), Iraq, and Southeast Anatolia, from Van Lake to Hatay.

The use of standard measurements in honeybee characterization and identification, also called traditional morphometrics, has been improved with multivariate analysis of morphometric characters. In standard morphometry on honeybee subspecies, wing shape has been studied by using angles and distances [15]. These angles and distances have generally been combined with other size characters (such forewing, proboscis, hind leg and abdomen) and analyzed through multivariate statistical analysis [4, 11].

In order to discriminate honey bee subspecies, geometric morphometrics (GM) was applied as a new method for the study of wing shape by using landmarks instead of standard morphometric in recent years. In honey bees, GM analyses of wing shape have provided many new insights, into either the characters or the identification of populations or lineages [7, 8, 10, 18]. Instead of distances and angles, it uses the coordinates of points called landmarks. The landmarks are superimposed by translation, scaling, and rotation. After superposition, the landmark configurations differ only in shape and can be analyzed by multivariate statistical methods [20].

The main aim of this study is to compare precise population discrimination by geometric morphometrics based on only forewing venation than standard morphometrics based on ten morphological characteristics. Furthermore, objective of this paper is to compare forewing, proboscis, abdomen and hind leg length of worker honeybees between collected samples from Iran.

2. Material and Methods

Five areas (three colonies in each area) were selected as representatives of main geographical points of the north, west, east, center and south of Iran (The north (Sari) 36° 33´ N; 53° 75´ E; The south (Shiraz) 29° 05´ N; 53° 30´ E; The west (Songhor) 34° 48´ N; 47° 17´ E; The east

(Birjand) 32° 52´ N; 53° 29´ E; The center (Esfahan) 32° 59´ N 51° 35´ E). Honey bees were preserved in preservative solution (30 parts distilled water, 15 parts 95% ethanol, 7 parts 38% to 40% formaldehyde and 2 parts acetic acid) to prevent sample deformation.

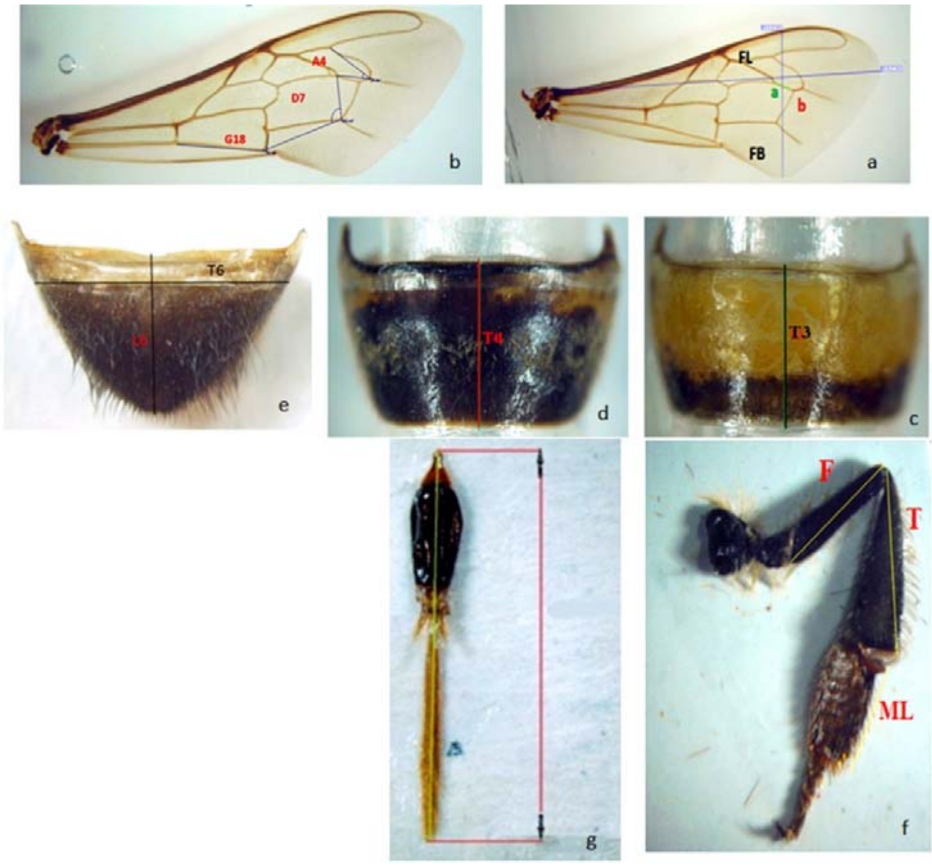

Fig 1: front wing of honeybee (*Apis mellifera meda*), FL= wing length, FB= wing width, $\frac{a}{B}$= cubital index; b: front wing angels, A4, D7, G18; **c**: third abdominal segment length dorsally; **d**: forth abdominal segment length dorsally; **e:** Index of sixth abdominal segment dorsally $\frac{T6}{L6}$; **f:** hind leg length (femur + tibia+ first tarsal segment); **g**: proboscis length

In standard morphometric, ten morphometric characters were biometrically measured with the tpsdig 2.16 [13]. Morphometric characters included forewing length (FL), forewing width (FB), cubical index ($\frac{a}{b}$), three angles of forewing (A4, D7 and G18,), proboscis length, hind leg length (femur + tibia+ first tarsal segment), Index of sixth abdominal segment dorsally $\frac{T6}{L6}$ and third + fourth abdominal segment length dorsally (Fig. 1) [15]. The value means morphometric characters of worker honeybees were calculated in each area. Honeybee populations were discriminated based on multivariate and canonical variate analyses (CVA). Statistical analyses were carried out with the softwares of SPSS 17.0 and SAS 12.0.

In geometric morphometric, the right forewings of 40 worker honeybees per colony were mounted by microscope slides. Digital photos were taken from mounted wings using a DP12 camera and SZX12 OLYMPUS stereomicroscope. Eighteen landmarks were digitized on forewings by tpsDig 2.16 software (Fig. 2). The vein junctions were used as homologous points for geometric morphometrics [13]. Later, landmarks were superimposed using a generalized procrustes analysis space [14]. All non-shape variations of these landmarks such as orientation (or rotation), scale, and size were removed. Next, digitized landmark outputs were analyzed by tpsRelw, tpsReg [13], and NtSys Pc. 2. Finally, samples of five areas were classified with Canonical variate analysis (CVA) by SPSS ver. 18. A multivariate analysis of variance (MANOVA) was carried out on the landmark data in order to compare honey bee populations.

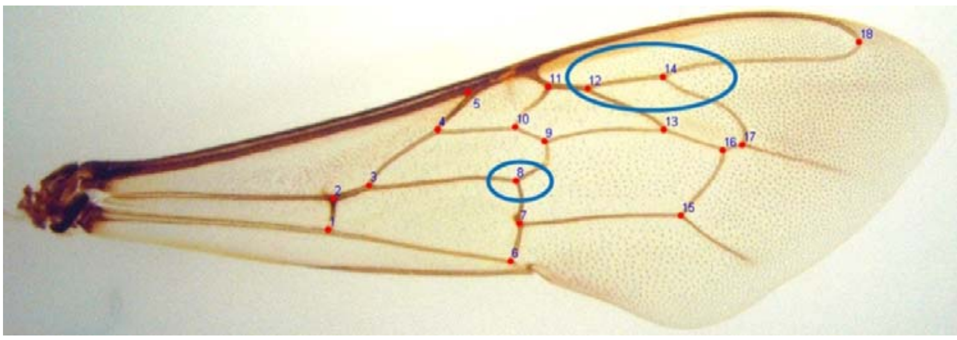

Fig 2: Eighteen landmarks on vein junctions of fore wing of *Apis mellifera*.

3. Results

Ten morphological characters were evaluated in Iran. Length and width means of forewings were 9.007±0.04 mm and 3.16±0.015 mm respectively in Iran. Also, cubital index and proboscis length means were 2.41±0.03 and 6.5±0.01 respectively (table 1). There was no significant difference between cubital indexes of five areas of Iran (F= 0.05, P=0.99). Honeybee samples of Songhor and Shiraz had the largest length of proboscis (6.8±0.009 mm) and forewing (9.21±0.21 mm) respectively (table 3). Correlations between measured characters were assessed. Results showed that there were positive correlations between length and width of forewing (r^2 =0.8, p=0.00), cubital index and forewing width (r^2 =0.56, p=0.014) and hind leg length and Index of sixth abdominal segment dorsally $\frac{T6}{L6}$ (r^2=0.6, p=0.006) (table 2).

Morphological characteristics of Honeybees were evaluated by principal component analysis (PCA). Three principal components (pc1, pc2 and pc3) were recognized and allocated 62.7% of sum of variations. Forewing length (FL), forewing width (FB), proboscis length, hind leg length (femur + tibia+ first tarsal segment) and third + fourth abdominal segment length dorsally related together in the first principle component. A4 and D7 Angels related together in the second principle component (table 4). Populations of five areas based on ten morphological characters were classified by canonical variate analysis (CVA). Cross-validation tests based on CVA correctly classified 70.7% of the colonies (Fig. 3). Statistical analysis results indicated that the honeybee populations of Songhor, Shiraz and Birjand were separated 100, 80 and 73.3 % from populations of other areas (table 5).

Relations of geographical populations based on ten morphological characters (standard morphometric) were evaluated using the UPGMA method. The cladogram resulting from the UPGMA cluster analysis showed that populations Birjand and Shiraz then Sari and Isfahan were classified together. Songhor population was separated completely. Cluster analyses divided populations into three main groups (Fig. 4).

Variance of coordinates of 18 landmarks (geometric morphometric method) on forewings as well as coordinates of landmarks of average shape were evaluated. Results indicated that the fourteenth landmark, the junction of the Rs and 2r-m veins, had the maximum variation (S^2=0.00006) in Iran; landmarks 18 and 12 had the next greatest variations (S^2= 0.00005 and 0.00004 respectively). Also, the eighth landmark, the junction of the 1m-cu and cu veins, had the minimum variation (S^2=0.00001) (Fig. 2). Furthermore, results of MANOVA of forewings (with 32 shape variables) in geometric morphometric method showed significant differences between populations of the five areas in Iran (F=4.16, p=0.000).

Allometry testing on forewings was performed. The results identified a significant difference between the size (centroid size) of the front wings and 32 shape variables (Wilks' lambda=0.54, p=0.00); therefore, the shape variations of the forewings were not uniform. Then, with the increasing of size of the front wings, wing shape changed. Area populations based on 32 shape variables of forewings were classified by canonical variate analysis (CVA). Cross-validation tests based on CVA of fore wings correctly classified 77.5% of the colonies (Fig. 5). Statistical analysis results indicated that the honeybee populations of Songhor, Shiraz and Birjand were separated 80, 82.5 and 85 % from populations of other areas (table 6). Relations of geographical populations were evaluated using the UPGMA method. The cladogram resulting from the UPGMA cluster analysis showed that populations of Sari and Isfahan were classified together. Birjand and Shiraz populations were separated completely. Cluster analyses divided populations into three main groups (Fig.6).

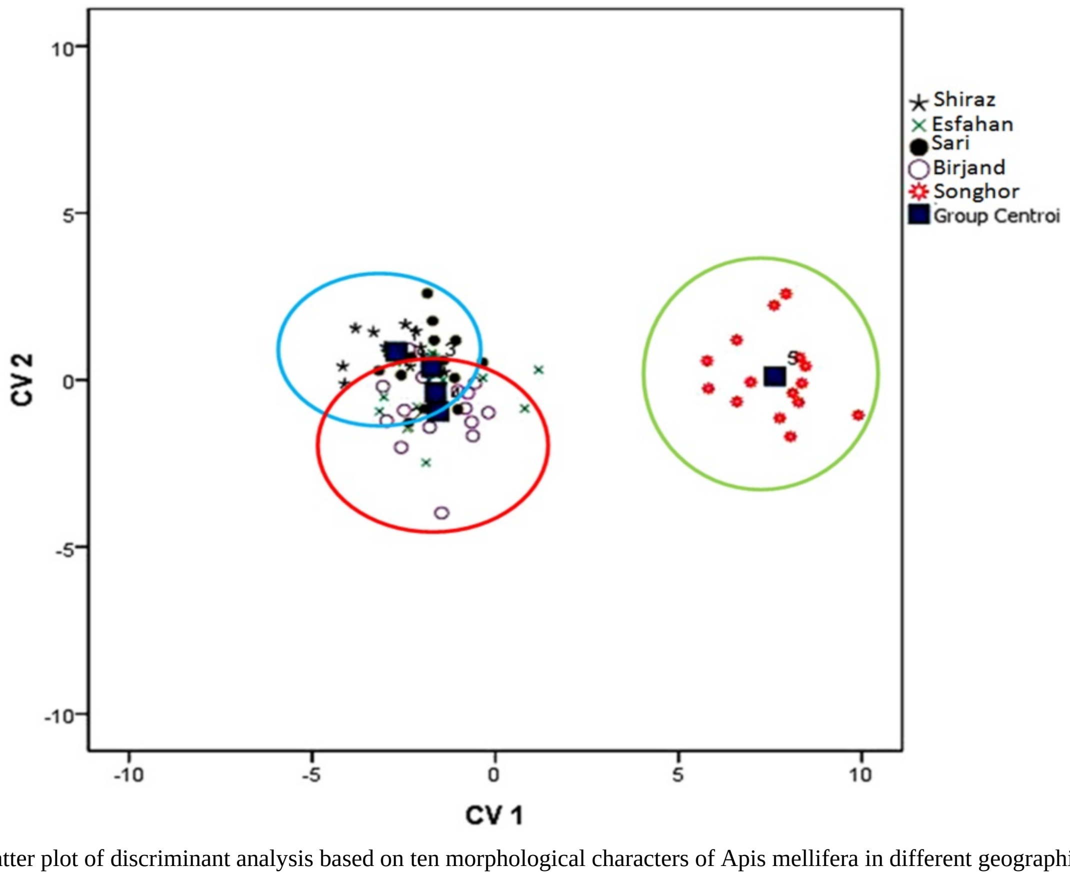

Fig 3: Scatter plot of discriminant analysis based on ten morphological characters of Apis mellifera in different geographical areas.

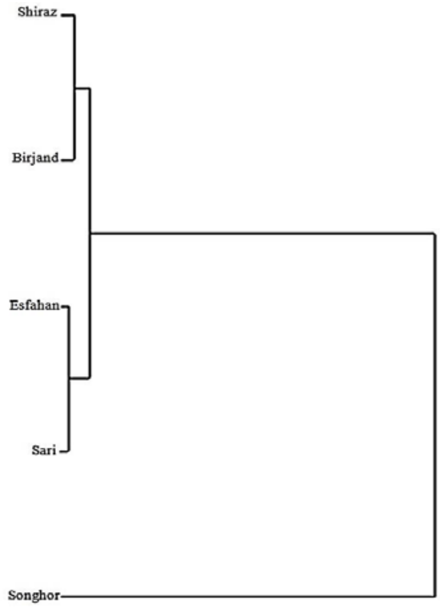

Fig 4: Dendrogram resulting from a UPGMA cluster analysis of samples from geographical populations using ten morphological characters of *Apis mellifera*.

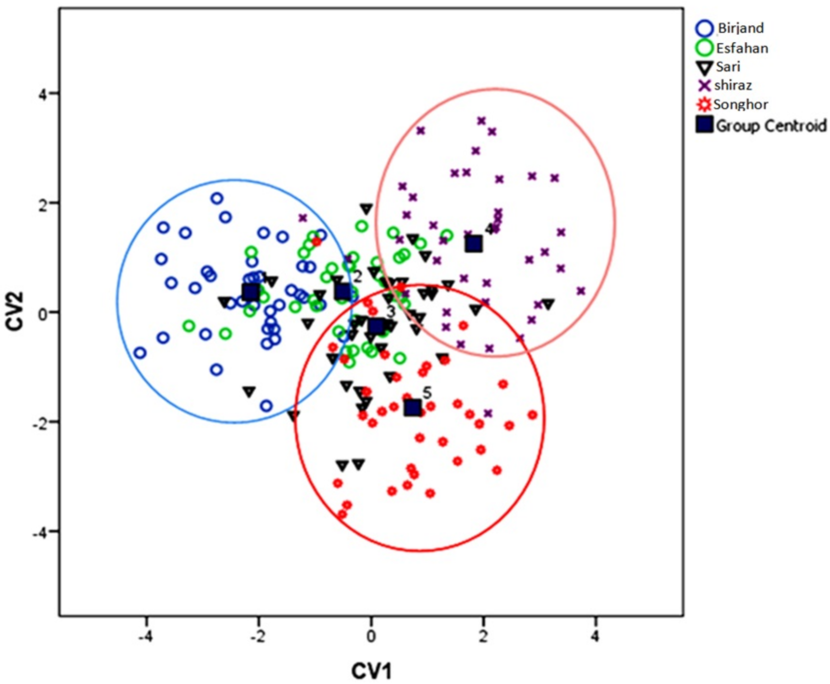

Fig 5: Scatter plot of discriminant analysis based on 18 landmarks on fore wings of *Apis mellifera* in different geographical areas.

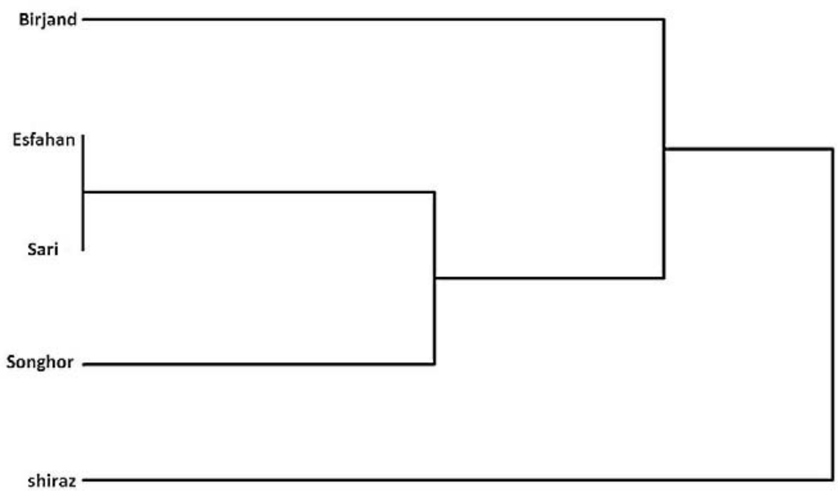

Fig 6: Dendrogram resulting from a UPGMA cluster analysis of samples from geographical populations using 18 landmarks on fore wings of *Apis mellifera*.

Table 1: Mean of measured characters of *Apis mellifera meda* in areas of Iran

area	Index of sixth abdominal segment dorsally	third and fourth abdominal segment length dorsally (mm)	proboscis length (mm)	hind leg length (mm)	cubital index	G18 (Degree)	D7 (Degree)	A4 (Degree)	forewing width (mm)	forewing length (mm)
Shiraz	0.76±0.016	4.47±0.088	6.49±0.01	7.87±0.054	2.41±0.02	93.47±1.99	98.58±0.99	28.88±4.76	3.23±0.18	9.21±0.22
Esfahan	0.76±0.022	4.49±0.087	6.48±0.01	7.77±0.05	2.38±0.027	96.23±0.82	97.36±0.77	31.02±0.45	3.21±0.02	9.15±0.04
Sari	0.76±0.019	4.54±0.09	6.49±0.01	7.91±0.065	2.44±0.028	93.79±1.06	97.70±0.72	30.21±0.41	3.25±0.012	9.2±0.026
Birjand	0.76±0.036	4.40±0.079	6.48±0.01	7.730.063	2.41±0.033	92.32±0.76	98.47±1.12	31.74±0.51	3.22±0.019	9.11±0.032
Songhor	0.78±0.028	4.67±0.097	6.81±0.01	8.21±0.039	2.41±0.33	92.73±0.73	97.75±0.92	32.66±0.59	2.93±0.019	8.34±0.036
Mean	0.766±0.015	4.51±0.039	6.55±0.003	7.90±0.031	2.41±0.02	93.71±0.43	97.97±0.4	30.90±0.26	3.16±0.016	9.007±0.04

Table 2: correlations between ten morphological characters of *Apis mellifera meda* in Iran (p values are in parentheses)

	forewing length	forewing width	A4	D7	G18	proboscis length	hind leg length	Index of sixth abdominal segment dorsally	cubital index	third and fourth abdominal segment length dorsally
forewing length										
forewing width	0.83 (0.00)									
A4	-0.16 (0.28)	-0.07 (0.39)								
D7	-0.38 (0.08)	-0.30 (0.13)	0.25 (0.18)							
G18	-0.027 (0.46)	-0.045 (0.4)	0.329 (0.1)	0.263 (0.1)						
proboscis length	-0.1 (0.3)	-0.019 (0.4)	0.031 (0.4)	0.23 (0.1)	-0.15 (0.2)					
hind leg length	0.052 (0.4)	-0.14 (0.3)	-0.04 (0.4)	-.0.04 (0.4)	-0.02 (0.4)	0.25 (0.1)				
Index of sixth abdominal segment dorsally	-0.19 (0.2)	-0.18 (0.2)	-0.25 (0.17)	-0.07 (0.4)	0.089 (0.3)	0.37 (0.08)	0.6 (0.008)			
cubital index	0.4 (0.05)	0.5 (0.014)	0.16 (0.2)	-0.11 (0.3)	0.35 (0.09)	-0.29 (0.14)	-0.22 (0.2)	-0.32 (0.11)		
third and fourth abdominal segment length dorsally	0.42 (0.05)	0.27 (0.1)	-0.31 (0.21)	0.30 (0.13)	-0.17 (0.26)	0.35 (0.09)	0.37 (0.08)	0.17 (0.2)	0.13 (0.3)	

Results of MANOVA of ten morphological characters indicated significant differences between populations of the five areas (F=9.04, p=0.000).

Table 3: measurement means of ten morphological characters of *Apis mellifera meda* in Iran

Characteristics morphometrics	Mean	SD	SE	Max	Min
forewing length (mm)	9.007	0.375	0.041	9.38	8.16
forewing width (mm)	3.168	0.138	0.015	3.38	2.80
A4 (degree)	30.906	2.266	0.261	37.97	25.13
D7 (degree)	97.979	3.487	0.402	108.13	90.07
G18 (degree)	93.711	3.779	0.436	101.86	84.52
cubital index	2.41	0.33	0.038	3.41	1.93
hind leg length (mm)	7.904	0.270	0.031	8.64	7.32
proboscis length	6.55	0.171	0.019	7.03	6.21
third and fourth abdominal segment length dorsally (mm)	4.51	0.130	0.015	4.82	4.22
Index of sixth abdominal segment dorsally	0.766	0.029	0.003	0.83	0.70

Table 4: coefficients of three principle components of ten morphological characters of *Apis mellifera meda* in Iran

characteristic	first principle component	second principle component	third principle component
forewing length	-0.81	-0.26	0.25
forewing width	-0.8	-025	0.28
A4	0.29	0.7	0.019
D7	-0.043	0.76	0.1
G18	-0.06	0.12	0.54
proboscis length	0.85	0.13	-0.09
hind leg length	0.78	-0.10	0.03
Index of sixth abdominal segment dorsally	0.18	-0.02	-0.75
Cubital index	0.16	-0.47	0.56
third and fourth abdominal segment length dorsally	0.77	-0.10	0.06

Table 5: summary of discriminant analysis of ten morphological characters of *Apis mellifera meda* in Iran (Percent classifications are in parentheses)

area	Songhor	Birjand	Sari	Esfahan	Shiraz
Songhor	15 (100%)	-	-	-	-
Birjand	-	11 (73.3%)	-	-	-
Sari	-	-	6 (40%)	-	-
Esfahan	-	-	-	9 (60%)	-
Shiraz	-	-	-	-	12 (80%)

Table 6: summary of discriminant analysis of *Apis mellifera meda* with geometric morphometric method in Iran (Percent classifications are in parentheses)

area	Songhor	Birjand	Sari	Esfahan	Shiraz
Songhor	32 (80%)	-	-	-	-
Birjand	-	34 (85%)	-	-	-
Sari	-	-	26 (65%)	-	-
Esfahan	-	-	-	30 (75%)	-
Shiraz	-	-	-	-	33 (82.5%)

4. Discussion

Morphological characteristics of honeybees effect on foraging efficiency; For example, proboscis and forewing lengths play important roles on nectar collection and flight distance respectively. Increase of hind leg length causes enlargement of pollen basket therefore honeybee can collect more pollen [5]. According to the obtained results and statistical analysis, cubital index mean was 2.41±0.03 in Iran therefore race of honeybees was recognized *Apis mellifera meda*. Thahmasebi [17] collected 1320 honeybee workers from 25 provinces throughout Iran. At least ten morphological characters (forewing length (FL), forewing width (FB), cubical index ($\frac{a}{b}$), three angles of forewing (A4, D7 and G18,), proboscis length, hind leg length (femur + tibia+ first tarsal segment), Index of sixth abdominal segment dorsally $\frac{T6}{L6}$ and third + fourth abdominal segment length dorsally) were measured. He obtained cubital index mean of 2.4 from Iran and conclude that there was Iranian race (*Apis mellifera meda*) in sampled areas. Furthermore, sampled areas separated completely using morphological characters. Rahimi and Asadi [12] studied honeybees and obtained cubital index mean 2.4 from northern areas of Iran. Thamasebi [17] obtained significant correlations between forewing length and width, forewing and proboscis length, hind leg and proboscis length. Attal [1] evaluated Morphometric and genetic markers for discriminating of honey bee subspecies of Saudi Arabia, morphometric analysis using 24 characteristics separated two subspecies of the Saudi honeybees, *Apis mellifera jemenitica* and *Apis mellifera litorea*. Also, he suggested that the mtDNA COI–COII region and the morphometric variations can discriminate the honey bee of Saudi Arabia and subspecies. Farshineh [6] compared *A. mellifera meda* populations of Iran (Orumieh, Tbriz, and Tehran) with populations in different zones of Turkey (Kiseher and Beypazari) and *A. mellifera carnica* of northern Turkey. They found that honeybee populations in Iran were smaller than honeybee populations in Turkey. Amssalu [2] evaluated Ethiopian honeybees at five locations (northeast, west, east, southeast, and central Ethiopia). Results showed that *A. mellifera woyi-gambell* and *A. m. monticola* were cited in the southeast and the North Mountains in dry and semi-humid climates, respectively.

Our research indicated that geometric morphometric was more precise than standard morphometric; because Cross-validation tests based on CVA of shape variables fore wings (geometric morphometric method) correctly classified 77.5% of the colonies whereas Cross-validation tests based on CVA of ten morphological characteristics (standard morphometric) classified 70.7% of the colonies. Also, cluster analysis separated almost two populations Birjand and Shiraz in geometric morphometric method whereas cluster analysis of standard morphometric separated only Songhor population completely. Charistos [3] confirmed that the use of geometric morphometrics was already more known than mtDNA studies; He mentioned that Greek honey bee populations are not classified clearly using only morphometrics analysis referred to in Ruttner [15]. Great hybridization exists due to beekeeping practices (commercial breeding and migratory beekeeping). Furthermore, Francoy *et al*. (2008) discriminated two races of European and Africanized honeybees well; they confirmed that geometric morphometric method was faster and easier than standard morphometric.

Tofilski [18] demonstrated that geometric morphometrics (84.9%) yielded better discrimination of three honeybee subspecies (*A. m. mellifera, A. m. carnica and A. m.*

caucasica) than standard morphometrics (83.8%). Kandemir and Ozkan [9] compared standard morphometrics and geometric morphometrics ability to discriminate the honey bee populations distributed throughout Turkey. The results showed that geometric morphometrics was more successful (81.5%) than standard morphometrics (70.4%). Miguel [10] used three methods of geometric and standard morphometrics and mitochondrial data for discriminating of M branch subspecies *A. m. iberiensis* and the North African subspecies (A branch); they concluded that wing geometric morphometrics appears more appropriate than mitochondrial DNA analysis or traditional morphometrics in the screening and identification of the Africanization process in honeybees. Zelditch [20] confirmed that geometric morphometric method was more practical and easier and accomplished in a much shorter time because all procedures are based on computer-assisted technology.

5. Conclusions

Geometric morphometrics method is more precise and easier compared to the standard morphometrics Therefore, the discrimination ability of geometric morphometrics on the honeybee populations is better than standard morphometrics. This application is in line with the reports of successful use of geometric morphometric in discriminating of *Apis mellifera* populations by Zelditch [20] and Charistos [13].

6. Acknowledgment

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