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Morphometric, Genetic and Competitive Study of the Grey Mountain Honeybee Populations (*Apis mellifera caucasia*) in Azerbaijan

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Abstract

We conducted a study on the mitochondrial DNA genetic diversity of honeybees from Caucasian Bee samples by morphometric analysis and molecular analyses of three regions of mitochondrial DNA (CytB, COI, and COI-COI intergenic regions). In morphometric study by principal component analysis 32 colonies were grouped in big cluster. ANOVA analyses showed significantly different among 4 groups of honeybees for most studied traits characters. Total of 488 bases in COI-COII region, 981 bases in COI region and 425 bases were obtained after trimming in partial CYT B region. This investigation showed that variation mtDNA regions can be used for the characterization of Azerbaijan honeybees.

Keywords: Azerbaijan honeybee, mtDNA, morphometric analysis, genetic variation

Introduction

The natural range of *Apis mellifera* includes Africa and Eurasia. In addition, this species has been introduced by humans to all other continents except Antarctica and is used intensively in pollination and honey production all over the world. Honeybees show considerable geographical variation, resulting in adaptation to regionally varying factors of climate and vegetation, but also to prevailing pests and pathogens. However, this natural heritage is increasingly subject to diffusion by human beekeeping efforts at a worrisome speed. The demand for high economic performance of bee colonies, combined with desirable behavioural characteristics, has led to considerable changes caused by systematic bee breeding. Thus, the original geographic distribution pattern is being dissolved EU-wide by mass importations and an increasing practice of queen trade and colony movements. These

activities endanger regional races and ecotypes by promoting hybridization (Marina *et al.,* 2015). Ruttner claimed that southwest Asia is a zone of high morphological diversification and evolution for honeybees. Many clearly distinct races have evolved within this region, which includes a diversity of habitats (Ruttner, 1988). Asia Minor, including Anatolia, appears to be the genetic center for these honeybee subspecies according to the mul tivariate statistical analysis of morphometric data. Honeybee races in this region include the subspecies *Apis mellifera anatoliaca*, *A. m. caucasica*, *A. m. meda*, and *A. m. syriaca*, which were considered by Ruttner (1988) to form a basal branch of the species. Honeybees show considerable geographical variation, resulting in adaptation to regionally varying factors of climate and vegetation, but also to prevailing pests and pathogens. However, this natural heritage is increasingly subject to diffusion by human beekeeping efforts at a worrisome speed (Marina *et al.,* 2015). The grey Caucasian mountain bee, *A. m. caucasica*, is a subspecies that has been used in beekeeping for more than 100 years in many places around the world. In 1916, the grey mountain honeybee was described by Gorbachev as *A. m. caucasica* and this name was adopted in apiculture (Farshineh *et al.,* 2015).

The objective of this research was to identify unique features of the grey mountain honeybee and compare morphometric characteristics of *anatoliaca* from Central Anatolia and *A. m. caucasica* from the Caucasus of Northeast Turkey.

Materials and methods

280 Caucasian Bee samples from 178 colonies were collected from apiaries of 28 villages of 14 regions of Azerbaijan Republic. All beehives were identified unique code and samples were collected 50ml plastic tubes which were labeled same as exceptional code of beehives. 30ml 70% ethanol was added into the tubes. The samples were sent to the Department of Biology, Middle East Technical University, Ankara, Turkey. A total of 32 colonies were mounted and from each hive 10 individual bees taken and their wings and legs were fixed between microscope slides. At the end 10 fore and hind wings from the same hive were fixed between two slides and sealed with plastic type around. Legs are mounted between slides using entellan. After finished they left out for a day to dry and later each individual wings and legs were photographed under a video camera attached to M16 LEICA microscope system. All the wings and legs were pictured with M16 LEICA microscope system. On the fore wings, wing length and wing width, cubital a and cubital b lengths, and 10 wing angles were going to measured. On the hind wing, a hamuli number is going

to be counted. On the hind leg, femur femur, tibia, metatarsus length, and metatarsus width are going to be measured.

Standard Morphometric Analysis

For standard morphometric study, a total of 320 forewing and hind leg images were measured with BEE2 software developed specifically for honeybees. At the same time from 320 hind wing images hamuli numbers were counted with Leica MZ16 stereomicroscope and also 2. 3. and 4. tergit colors were checked.

Morphometric measurements

Standard morphometric measurements were done with Bee2 (Meixner & Meixner, 2004) computer program. This program is specifically designed to make a morphometric study on honeybees and used in the Oberursel Beekeeping Institute, Germany where all the honeybee database was found. Scale were assigned by the computer and all the characters were measured with the aid of the Bee2 software. After scale is assigned than the necessary points were placed with the computer to do the measurements.

Besides these characters, hamuli number and the color of 2, 3 and 4th tergites were checked with MZ16 stereo microscope.

All measured morphological characters were analyzed by SPSS statistical package. Then discriminant function analysis is carried out to see the groupings of the honeybee samples. We compared the samples of the Azerbaijanian samples by itself and then compared them with Anatolian and Caucasian samples.

Molecular analysis

Out of 40 colonies 32 of them used in morphometric analysis but for molecular analysis all of them utilized. 3 mtDNA genes were amplified and sequences were done. Until now only COI-COII results arrived. Later 6 msat locus will also be analyzed. DNA was extracted using the CTAB protocol. The standard PCR is utilized and three regions (CytB, COI, and COI-COI intergenic regions) were amplified. For three mtDNA region used primer pairs were showed in table 1.

After PCR, all amplified samples were run at 1% agarose gel for the visualization of the amplification products. Three regions were amplified and send to sequencing of the regions.

Table 1. Primers used for three regions of mtDNA

Region	Primer	Sequence
CO-I	CO-I F	5'-TTA AGA TCC CCA GGA TCA TG-3'
	CO-I R	5'-TGC AAA TAC TGC ACC TAT TG-3'
COI-COII	E2	5'-GGC AGA ATA AGT GCA TTG-3'
	H2	5'-CAA TAT CAT TGA TGA CC-3'
Cyt B	BglII-F	5'-TAT GTA CTA CCA TGA GGA CAA ATA TC-3'
	BglII-R	5'-ATT ACA CCT CCT AAT TTA TTA GGA AT-3'

Results

Azerbaijanian Honeybee Standard Morphometric analysis

32 colonies were subjected to principal component analysis for their homogeneity and it is seen all colonies in one big cluster (Figure 1-2). In principle component analysis the colony scatter is seen later they are compared with other populations of Anatoliaca and Caucasica.

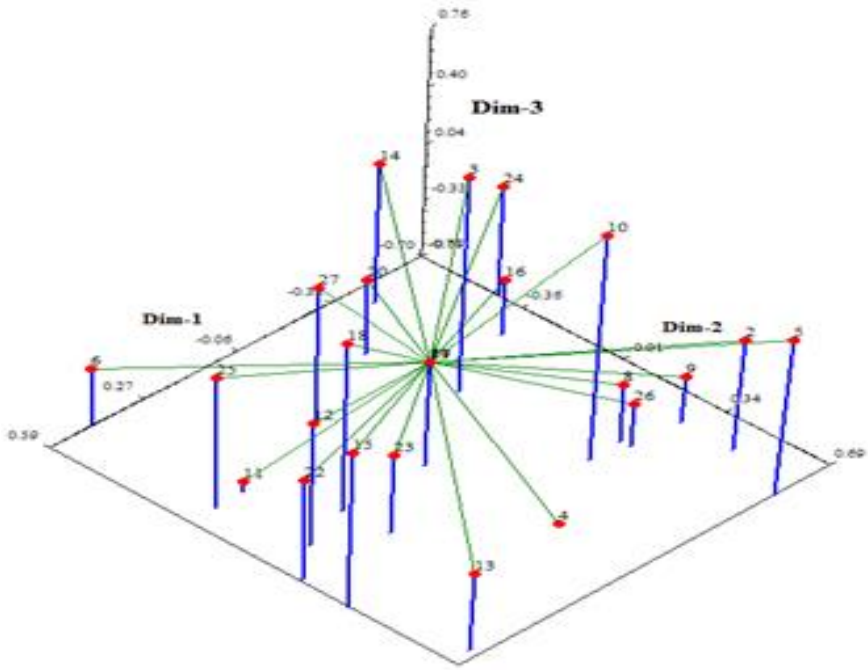

Figure 1. Principle component scatter plot of Azerbaijan honeybee colonies

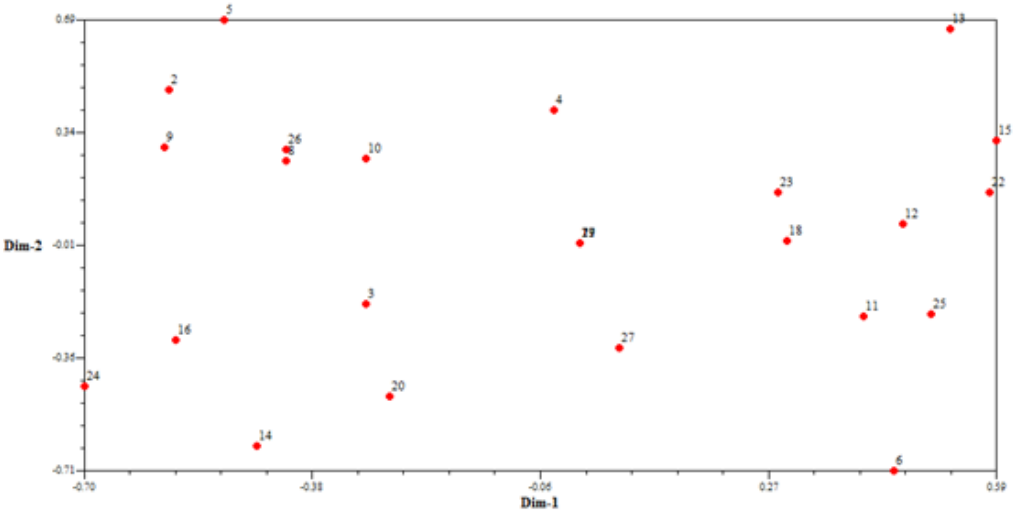

Figure 2. All colonies formed one big cluster with no separation.

Comparison of the different honeybee populations-Turkey and Azerbaijan

Azerbaijanian samples (averages of villages) were compared with two honeybee subspecies from Turkey (Anatolian honeybees-Apis *mellifera anatoliaca*, and two Caucasus honeybees from Camili and Posof-Apis *mellifera* caucasica). In this analysis averages of tergite color also utilized. For checking the 27 morphological characters with ANOVA only two showed non-significant results which were CUB1 and LTAR. All other characters were significantly different among 4 groups of honeybees.

Summary of discrimination (Eigenvalues, % variance and cumulative variance) were given in Table 3. All of the morphological variation is explained in 3 axes. First axis explained all of the variation (100%).

Table 3. Eigenvalues, % variance and cumulative variance of the discrimination in analyses

Function	Eigenvalue	% of Variance	Cumulative %	Canonical Correlation
1	28.046(a)	88.5	88.5	0.983
2	2.521(a)	8.0	96.5	0.846
3	1.120(a)	3.5	100.0	0.727

Success of the classification is 98% which shows how the groups are different from each other. *A. m. anatoliaca*, and *A. m. caucasica Artvin* were 100% separated from each other. However, *A. m. caucasica Posof* is 90% placed in its original group but 5% percent shared with Artvin and 5% shared with Azerbaijan group. Azerbaijan samples on the other hand clustered 96.9% with its own group only 3.1% shared with Posof samples.

The results were also displayed as scatter plot in Discriminant Function Analysis (Figure 3). As it is observed there are two big groups one is *A. m. anatoliaca* and the other group is *A. m. caucasica*. However, *A. m. caucasica* is further divided according to the geography and it is well seen that the Azerbaijan groups were formed a cluster with *A. m. caucasica* from Posof.

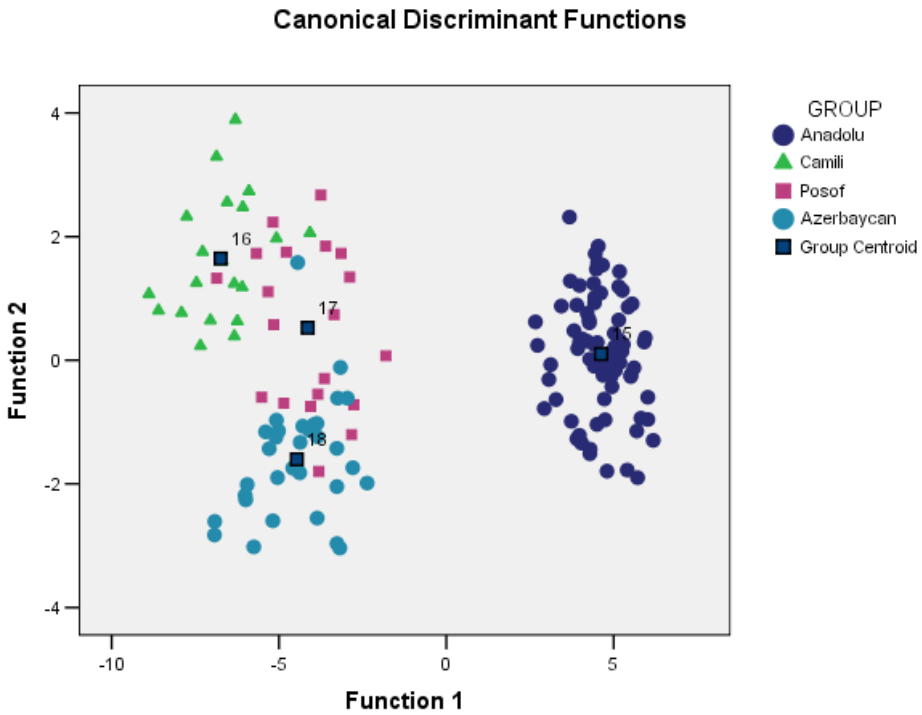

Figure 3. Discriminant Function Analysis of four honeybee groups.

Table 4. Discriminant function classification result of 4 honeybee groups.

Classification Results[a]

			Predicted Group Membership				
		GROUP	15	16	17	18	Total
Original	Count	15	78	0	0	0	78
		16	0	20	0	0	20
		17	0	1	18	1	20
		18	0	0	1	31	32
	%	15	100,0	,0	,0	,0	100,0
		16	,0	100,0	,0	,0	100,0
		17	,0	5,0	90,0	5,0	100,0
		18	,0	,0	3,1	96,9	100,0

a. 98,0% of original grouped cases correctly classified.

The results of the analysis of Azerbaijan colonies showed close affinity to Caucasian honeybees and they are 96.9% original and only show some similarity to Posof honeybees.

The morphometric values of these bees are tabulated and shown in Table 5.

Table 5. Morphometric comparison of the 4 honeybees. Measurements are in mm

	Central Anatolia *A. m. anatoliaca*		Camili-Artvin *A. m. caucasica*		Posof-Ardahan *A. m. caucasica*		Azerbaijan *A. m. caucasica*	
Characters	Mean	Std. Deviation	Mean	Std. Deviation	Mean	Std. Deviation	Mean	Std. Deviation
EM	2,572	0,071	2,643	0,048	2,596	0,049	2,583	0,068
TIB	3,136	0,076	3,236	0,066	3,164	0,076	3,164	0,090
LTAR	1,967	0,128	1,973	0,055	1,983	0,065	1,969	0,064
WTAR	1,128	0,025	1,203	0,024	1,178	0,036	1,150	0,029
LEG	7,654	0,140	7,855	0,137	7,742	0,161	7,716	0,206
LWMTAR	58,137	1,585	60,668	2,228	59,602	2,962	58,599	1,974
LFW	8,778	0,096	9,089	0,224	8,974	0,169	8,897	0,107
WFW	2,963	0,045	3,073	0,109	3,077	0,041	3,024	0,047
LWFW	0,338	0,003	0,337	0,009	0,346	0,019	0,340	0,003
CUB1	0,492	0,026	0,481	0,024	0,498	0,017	0,490	0,028
CUB2	0,312	0,021	0,310	0,018	0,302	0,020	0,285	0,016
CIND	1,624	0,154	1,593	0,138	1,690	0,149	1,768	0,173
TER2	7,295	0,367	3,230	0,516	3,950	0,435	3,638	0,618

TER3	6,183	0,339	2,320	0,533	2,910	0,402	2,925	0,945
TER4	4,140	0,263	1,610	0,442	1,970	0,478	1,900	0,296
A4	32,428	1,394	36,251	1,282	34,341	1,127	34,534	1,264
B4	99,411	3,044	93,694	1,607	96,089	2,614	97,509	3,316
D7	99,459	2,581	100,913	1,573	100,724	1,362	101,640	1,830
E9	19,437	0,817	19,667	0,830	19,981	0,758	19,975	0,763
G18	89,061	2,274	92,596	2,542	91,122	2,011	90,892	2,368
J10	52,054	2,889	50,694	1,808	53,357	1,604	52,719	2,749
J16	88,861	2,225	86,499	1,784	87,116	2,453	89,097	2,084
K19	78,402	2,341	76,003	1,762	77,800	1,677	75,009	1,902
L13	14,273	1,082	14,001	0,884	14,308	0,776	14,821	1,025
N23	86,995	2,553	85,130	2,267	85,268	2,294	87,849	1,907
O26	42,625	2,205	41,127	1,274	40,872	3,155	40,049	2,500
HOOKS	20,736	0,703	21,189	0,845	21,360	0,776	21,303	0,719

After PCR check all the samples prepared to be sent to the sequencing company with proper primer pairs. All sequences were done with both primer pairs (figure 4). Until now only COI-COII intergenic sequences were received from the company. When the sequences were received they are opened with Chromas computer software (Figure 5) to see the chromatogram files and the sequences were checked with eye.

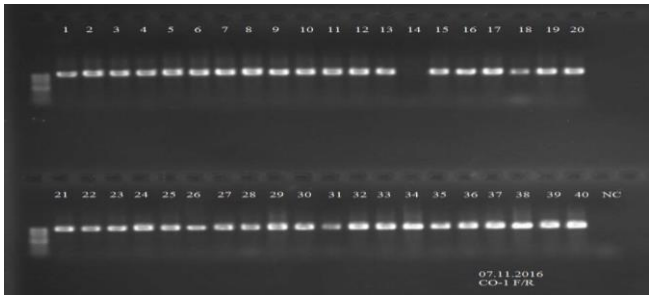

Figure 4. Agarose gel of PCR check: mtDNA COI region.

All sequences were checked and the forward sequences were exported as Fasta file. Reverse sequences were first transformed by Reverse-Complement and then exported to Fasta file. Later both forward and the reverse sequence were combined in txt file to align in Clustal X software.

For all sequences the same procedure repeated, and all 38 sequences cleaned, trimmed and ready for analysis with MEGA software.

MEGA (Molecular Evolutionary Genomic Analysis) software used for further analysis to find out the variation and the sites show differences in different honeybee

colonies. This is done only for COI-COII sequences and there are three sites that showed variation. All sequences were aligned with MEGA.

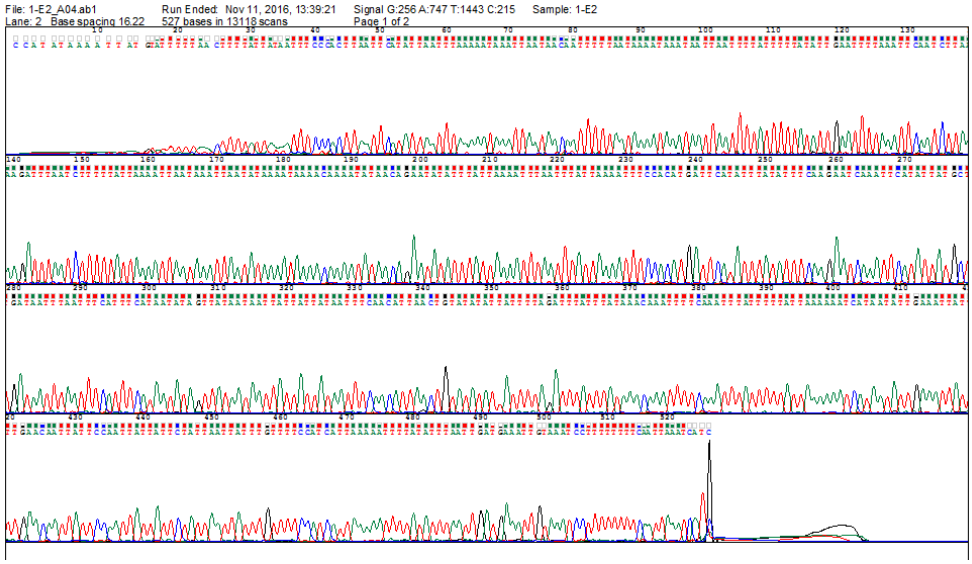

Figure 5. Sequence chromatogram as it is viewed by Chromas programme.

Total of 488 base were obtained after trimming in COI-COII region and a total of 3 different haplotype were obtained. These haplotypes were different in 3 different sites. According to the sequence we obtained these three sites were in 141, 206 and 341 sites.

Total of 981 base were obtained after trimming in COI region from 29 Azerbaijan honeybee samples and a total of 4 different haplotypes were obtained. These haplotypes were different in 3 different sites. According to the sequence we obtained these three sites were in 94, 388 and 517 sites.

Among 29 sequences done, 20 of them belong to Haplotype 1, 4 belong to Haplotype 2, 2 of them belong to Haplotype 3 and 3 of them belong to Haplotype 4.

Total of 425 base were obtained after trimming in partial CYT B region from 40 Azerbaijan honeybee samples (Figure 16) and a total of 4 different haplotypes were obtained. These haplotypes were different in 4 sites. According to the sequence we obtained these four sites were in 130., 209., 322. and 394. sites.

Among 40 sequences done, 20 of them belong to Haplotype 1, 16 belong to Haplotype 2, 3 of them belong to Haplotype 3 and 1 of them belong to Haplotype 4. Those four haplotype gene sequences given below.

Four different haplotype CYTB sequences were given below Different bases shown in red:

This small mtDNA study including three partial gene sequences showed a remarkable genetic variation that can be used for the characterization of Azerbaijan honeybees. Several haplotypes were found for COI-COII intergenic region, COI and CytB partial gene sequences. This shows the amount of genetic variation found in Azerbaijan honeybee population with respect to the studied mtDNA gene sequences.

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