

Geometric morphometric characteristics of *Apis mellifera* honeybee in Kazakhstan

ULZHAN NURALIEVA^{1,2,✉}, KADYRBAI TAJIYEV¹, ZHANAR SHERALIEVA¹,
MAXAT TOISHIMANOV^{2,✉✉}, GAUKHAR MOLDAKHMETOVA^{1,2}, KAMSHAT TEMIRBAYEVA^{1,3},
AIGUL TAJIEVA¹

¹Kazakh Research Institute of Livestock and Fodder Production. Zhandosov Str. 51, Almaty 050035, Kazakhstan. Tel.: +7778-9204862,

✉email: nuralieva0602@gmail.com

²Kazakh National Agrarian Research University. Abay Ave. 8, Almaty 050010, Kazakhstan. Tel.: +7707-9193922, ✉✉email: maxat.toishimanov@gmail.com

³Al-Farabi Kazakh National University. Al-Farabi Ave. 71, Almaty 050040, Kazakhstan

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Abstract. Nuralieva U, Tajiyev K, Sheralieva Z, Toishimanov M, Moldakhmetova G, Temirbayeva K, Tajieva A. 2023. Geometric morphometric characteristics of *Apis mellifera* honeybee in Kazakhstan. *Biodiversitas* 24: 4402-4409. This study was conducted in the whole beekeeping region of Kazakhstan. We researched morphological studies using (45 colony bees in 5 main regions in various geographical and climatic zone environmental conditions. The obtained data were compared with the *Apis mellifera carnica* reference samples, *Apis mellifera caucasica*, and *Apis mellifera mellifera*. Morphometric identification was carried out using 19 landmarks by IdentiFly software. Almost all colonies showed belonging to the lineage C (about 85%), by subspecies to *A. m. carnica*, *A. m. caucasica* showed 11%, *A. m. mellifera* belonged to only 4%. Furthermore, using multivariate principal component analysis compared honeybee lineages between reference samples. There were significant differences in wing landmarks between lineages and subspecies (Mahalanobis square distance). By Mahalanobis, distances between the evolutionary lineages M differ from line C by 15.93 and from line O by 19.03 units. In contrast, the distance between lines C and O is insignificant, with satisfactory reliability. The forewing wing size evaluated centroid size, which showed the highest degree of honeybees in the west and south Kazakhstan zones. The percentage of variation in wing centroids was relatively small between analyzed samples (less than 1%) but extremely changed for reference samples *A. m. carnica*, *A. m. caucasica*, *A. m. mellifera*, where log centroid sizes showed 1.33%, 4.18%, and 4.67%, respectively. The results showed significant differences between populations in different climatic zones.

Keywords: *Apis mellifera carnica*, *Apis mellifera caucasica*, *Apis mellifera mellifera*, geometric morphometric, honeybees, subspecies

INTRODUCTION

At different stages of beekeeping development in Kazakh land, bees were brought from the Carpathians, Ukrainian and Caucasian bees. Honey bees bred on the territory of the Republic of Kazakhstan are representatives of three evolutionary lineages, represented by the main three subspecies: *Apis mellifera carnica*, *Apis mellifera caucasica*, and *Apis mellifera mellifera* (Sheppard et al. 2003; Shimelkova et al. 2020).

Asia and the Middle East were considered possible centers of origin of *A. mellifera*, which was followed by the migration to Eurasia. According to Sheppard (2003), Kazakhstan's honey bees belong to the C, O lineage and require further clarification (Ruttner 1988; Sheppard et al. 2003; Kandemir et al. 2011). It has been established that currently, the *A. m. carnica*, *A. m. carpatica*, and *A. m. mellifera* subspecies are in all beekeeping farms and occupy the main honeybees in Kazakhstan, and they are acclimatized to the conditions of all climatic zones (Alpatov 1948; Sheppard et al. 2003; Shimelkova et al. 2020). There were about 200,000 bee colonies in the country in 2021, and beekeeping was developing dynamically; for 8 years, the population had increased 2 times (Information – Analytical System 2022).

Kazakhstan has five geographical regions: north, west, east, central, and south. These regions' natural and climatic conditions differ significantly, characterized by a sharply continental climate where temperatures range from hot and arid to harsh winters with snowstorms (Frisch et al. 2009; Suleimenova et al. 2021; Salnikov et al. 2023).

Different morphometry and molecular genetics methods were used in beekeeping worldwide to identify subspecies. Based on Alpatov's classical morphometry technique (Alpatov 1948), which involves taking measurements of body structure and determining three indices of the right forewing. DuPraw method introduced the measurement of 17 angles between vein junctions in morphometry (DuPraw 1964, 1965), while Ruttner reduced this number to 11 (Ruttner et al. 1978) and is considered the founder and developer of morphometry standards (Ruttner 1988).

The prevalence of honey bees (*Apis mellifera*) across the globe has been described by leading scientists in beekeeping based on their research (Ruttner 1988; Sheppard et al. 1997; Kandemir et al. 2011; Meixner et al. 2013; Chen et al. 2016; Ilyasov et al. 2020; Dáttilo et al. 2022; Moldakhmetova et al. 2022). These described subspecies of honey bees have been divided and assigned to four evolutionary lineages based on their distribution areas through assessment using Ruttner's morphometric

method. Identification of subspecies of honey bees in the country with the determination of the level of breeding purity is important to preserve their diversity. Bouga et al. (2011) state that all identification methods for bee subspecies do not differ in the accuracy of research results. Several scientists (Nedić et al. 2014; Janczyk et al. 2021; Prabucki et al. 2022) noted that the Cubital index (Ci) and traditional methods were suitable for measuring and distinguishing subspecies in small quantities.

Therefore, Ruttner (1988) noted that classical morphometry is based on measuring 36 characteristics (wings, abdomen, legs), which is very laborious. For the first time, the concept of the shape of individual cells and the location of individual veins as a possible characteristic was introduced by Bykova et al. (2016). It was found that geometric morphometrics is based on the theory of form, using coordinates of markers located at the intersections of bee wing veins, which were later used for subspecies identification of bees (Tofilski 2004, 2008; Puškadija et al. 2020).

A computer program based on geometric morphometrics was developed to accelerate the process of identifying subspecies and evolutionary lineages of honeybees, which was based on the method of measuring the wings (Gerula et al. 2009; Kandemir et al. 2011; Oleksa et al. 2015; MacLeod 2007). Each researcher numbered the landmarks on the front wings of bees differently, and there was no regularity in their arrangement and order. Some used 18 points to identify subspecies of *Apis mellifera*, others used 19 (Tofilski 2017), and some increased their number to 23 (Berezin 2019).

In this study, the landmark-based geometric morphometric method was applied to study the biodiversity of Kazakhstani

honeybees. The aim of the research was to identify subspecies and linear affiliations of honeybees based on the more accurate use of the geometric morphometric method in different climatic zones of Kazakhstan.

MATERIALS AND METHODS

Data collection

According to the Bureau of National Statistics of the Republic of Kazakhstan (2022), the beekeeping industry in Kazakhstan was developing dynamically, as shown in Figure 1. In 2021, according to the Bureau, there were 198312 bee colonies in the country; for 8 years, the population had increased 2 times.

Sample collection

The object of the study was the honeybees *Apis mellifera* of the Kazakhstani population on the territory of Kazakhstan. A total of 675 bee samples were selected from 45 colonies, with 15 bee samples per colony. Samples from each apiary were selected randomly from 5 colonies of honeybees (Table 2). The samples were collected from eight farms located in eight geographical regions belonging to five regions of Kazakhstan. The choice of the five main geographical regions was not random, as such coverage, including the territories of eight regions of Kazakhstan, made it possible to obtain an overall picture of the level of spread of honeybee breeds and determine their breed. This was the main goal of our study. The research was conducted in eight apiaries located in five regions of Kazakhstan (Figure 1).

Table 1. Honeybee colonies by year in Kazakhstan's regions, according to the Bureau of National Statistics of the Republic of Kazakhstan

Region/year	2013	2014	2015	2016	2017	2018	2019	2020	2021
Abai region ¹	-	-	-	-	-	-	-	-	-
Akmola region	754	381	440	862	842	922	884	762	864
Aktobe region	-	-	-	299	464	457	457	476	2 093
Almaty region	14 984	15 833	16 056	16 209	17 781	19 746	20 686	18 399	22 973
West Kazakhstan region	108	175	162	324	325	320	370	320	1 439
Jambyl region	1 568	1 781	1 751	1 956	2 601	2 651	2 611	3 873	4 442
Zhetysu region ¹	-	-	-	-	-	-	-	-	-
Karaganda region	260	212	254	508	553	606	595	693	1 110
Kostanai region	576	412	438	407	477	512	906	903	929
Kyzylorda region	659	659	1 155	1 435	1 739	1 072	1 385	1 075	1 265
Pavlodar region	1 147	2 068	1 662	3 349	3 878	3 796	4 034	1 391	7 190
North Kazakhstan region	892	799	859	1 070	2 465	2 713	2 741	2 705	4 890
Ulytau region ¹	-	-	-	-	-	-	-	-	-
East Kazakhstan region	65 529	77 843	77 384	78 467	79 631	87 002	89 916	94 421	111 747
South-Kazakhstan region ²	6 085	13 419	13 412	14 473	15 953	-	-	-	-
Turkestan region ²	-	-	-	-	-	9 446	7 980	8 098	26 858
Shymkent city ²	-	-	-	-	-	500	750	500	12 456
Astana city	8	8	8	-	-	-	-	-	-
Almaty city	252	247	247	247	247	106	56	56	56
Kazakhstan	92 822	113 837	113 828	119 606	126 956	129 849	133 371	133 672	198 312

Note: ¹On 8 June 2022, the government announced that the Ulytau region split off from Karaganda region, Abai region from East Kazakhstan region, and t Zhetysu region from Almaty region. ²On 19 June 2018, Symkent City was taken out of South Kazakhstan Region and subordinated directly to the government of Kazakhstan. The region's administrative center was moved to Turkistan and renamed Turkistan Region (formerly South Kazakhstan Region)

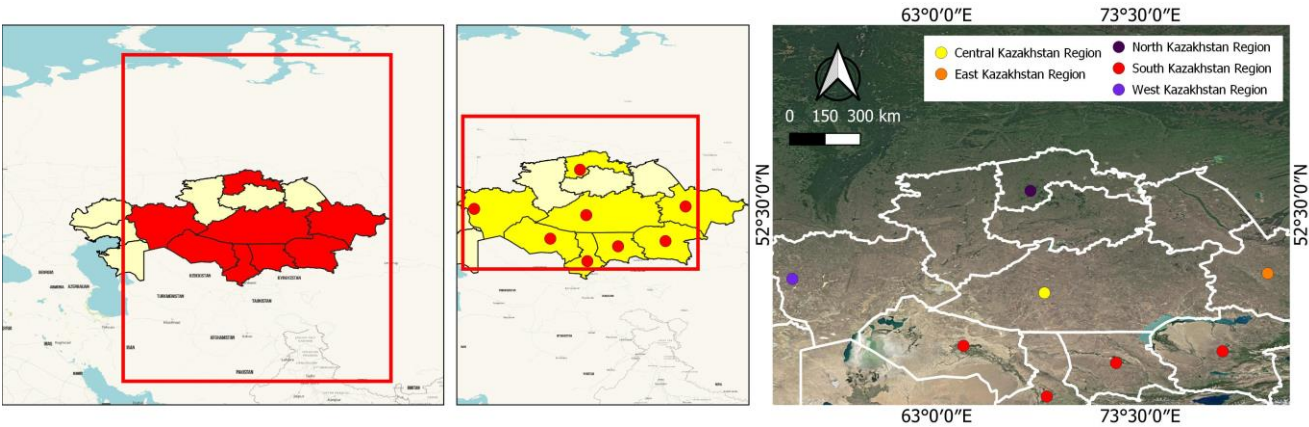


Figure 1. Geographical localization of the studied honey bees in Kazakhstan

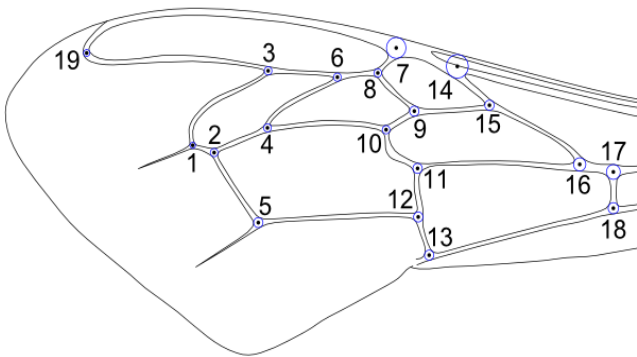


Figure 2. 19 landmarks on the right forewing of working honey bees

Table 2. The number of studied bee colonies in Kazakhstan

Zone	Region	Coordinates	Number of bee colonies
North	North Kazakhstan	53°41'24.6"N 61°55'58.6"E	5
West	West Kazakhstan	49°09'25.4"N 47°53'21.7"E	5
East	East Kazakhstan	50°06'23.7"N 81°32'55.9"E	5
Center	Karagandy	49°35'01.0"N 72°34'23.5"E	5
South	Kyzylorda	44°42'54.8"N 62°31'34.6"E	5
	Turkestan	41°51'45.7"N 69°34'45.5"E	5
	Zhambyl	42°58'10.0"N 71°28'24.2"E	5
	Zhambyl	42°55'00.8"N 75°22'37.0"E	5
	Almaty	43°22'07.0"N 79°36'33.6"E	5
Total			45

Samples of bees for the study were selected during the full activity period of bees in the autumn before the wintering of 2022 (south, north, east, west, center). The samples were collected using the commonly accepted

Alpatov method (1948). They were fixed with 70% ethanol, and each batch was marked with the number of the bee family. Preparation was made from the right front wing by fixing it on a transparent adhesive tape, scanning it, and obtaining an electronic version. They were scanned using an Epson V600 Photo scanner. The resolution of the images was 3200 dots per inch (image size: 5782x3946 pixels). The research methodology was based on the results of scientists from different countries' studies on determining linear affiliation by geometric morphometric method (Tofilski 2004, 2008).

In their research, Nawrocka et al. (2018) used the computer software Identify to identify honeybee subspecies and evolutionary lines based on geometric morphometry, using 19 coordinates of the right forewing of honeybees. They established a specific positioning of evolutionary lines in the discrimination of honeybee forms and subspecies based on canonical variational analysis of the wing shape. They achieved results in the development of a fast and easy identification method based on the analysis of the wing vein structure of honeybees using geometric morphometric methods.

The measurement of the right forewings was marked with 19 characteristic landmarks by IdentiFly software. Figure 2 shows 19 characteristic landmarks marked by the IdentiFly software (Miguel et al. 2011; Oleksa et al. 2015).

The wing data were compared with *A. m. carnica*, *A. m. caucasica*, and *A. m. mellifera* reference samples from the Morphometric Bee Data Bank in Oberursel, Germany. The resolution of the reference images was 1096 dpi.

Statistical analysis

The average wing's centroid sizes were calculated for each colony. The calculation of the Mahalanobis distance was performed using the R program, where the coordinates of the means in each group and the overall covariance matrix were calculated. Then, the distances between the means of all groups were calculated. Differences in wing size were based on calculating centroid size and analyzed by Analysis of Variance (ANOVA). Discriminant analysis was used to visualize the differences between bee colonies. Statistical data analysis was performed using R studio, JMP, and Statistica 7 (StatSoft).

RESULTS AND DISCUSSION

Centroid size was calculated from the square root of the sum of the squared distances between the center of the polygon and each landmark. Wing size, shown by centroid size (Figure 4), differed significantly between districts and reference sample (ANOVA: F ratio=25.56, $P<0.001$). The reference *A. m. carnica* (6.69) wing showed the smallest size, and *A. m. caucasica* (7.06) and *A. m. mellifera* (7.07) showed the highest size. According to Figure 3, East Kazakhstan (6.79) samples showed the largest among local honey bee samples, then south Kazakhstan zones-Kyzylorda (6.77), Zhambyl (6.77), Almaty (6.76), Turkestan (6.76) regions showed approximately same results. The smallest centroid sizes are shown in the west and central zone samples. The means, standard deviations, and standard errors for each bee colony wing are given in Table 3. In Figure 4, canonical variances explained 42.68% of the total variation, with CV1 30.08% and CV2 12.60%.

Beekeeping apiaries from eight regions of five regions of Kazakhstan were covered to determine the presence of subspecies of honey bees and their breed affiliation. Identification of breed affiliation and evolutionary lineages of honey bees in Kazakhstan were studied using the method of geometric morphometry based on 19 coordinates of the right forewing, and the results were identified using the IdentiFly program.

According to IdentiFly software (Tofilski 2017), it has been established that representatives of three evolutionary lineages, C, O, and M, are present in the territory of Kazakhstan. The results of geometric morphometric analysis, discriminant analysis of the coordinates, and the percentage ratio of existing representatives of evolutionary lineages and subspecies of honey bees have been determined. It was found that about 84.5% of honey bees in Kazakhstan belong to the C lineage, 11.1% to the O lineage, and 4.4% to the M lineage (Table 4).

The study's results on determining the subspecies bred in the territory of all natural-climatic zones show the presence of representatives of three evolutionary lines, as seen in Table 4. Of the 45 bee colonies surveyed, 38 colonies belonged to lineage C, 5 colonies to lineage O, and 2 colonies to lineage M. All Lineage O bee colonies were found in Zhambyl province (South Kazakhstan). 2 colonies of lineage M belong to East Kazakhstan province.

According to the geometric morphometric analysis results, three main subspecies of honeybees have been identified in different regions of Kazakhstan. The distribution of samples along three main evolutionary lines (C, O, M) and subspecies of honey bees in the space of the first and second discriminant functions are shown in Figure 5.

It can be seen that three comparative subspecies of honey bees are located separately from each other. Subspecies *A. m. carnica*, belonging to the evolutionary line C, are located in one strip of two discriminant functions. In contrast, subspecies *A. m. caucasica* are located below and above in the left corner of the plane of representatives of the evolutionary line C. Subspecies *A. m. mellifera* is located at a considerable distance from the two compared subspecies of honey bees.

Reference honey bee populations were compared with analyzed samples by discriminant analysis to verify the accuracy of the separation of representatives of the C, O, and M lineages (Figure 6).

When determining the discrimination of evolutionary lines, reference populations of subspecies belonging to lines C, O, and M were used together. All representatives of lines C, O, M, and reference populations were located in the correct plane, confirming the correctness of the discriminant analyses conducted.

Table 3. Mean, standard deviation, and standard error values for wing centroid size

Zones	Mean	Std Dev	Std Err Mean
<i>A. m. carnica</i> ref	6.69	0.04	0.012
<i>A. m. caucasica</i> ref	7.06	0.02	0.004
<i>A. m. mellifera</i> ref	7.07	0.03	0.007
Almaty	6.76	0.02	0.002
East Kazakhstan	6.79	0.02	0.002
Karagandy	6.74	0.02	0.002
Kyzylorda	6.77	0.02	0.002
North Kazakhstan	6.76	0.02	0.002
Turkestan	6.76	0.02	0.002
West Kazakhstan	6.75	0.02	0.002
Zhambyl	6.78	0.02	0.002
Zhambyl 2	6.76	0.02	0.002

Table 4. The percentage ratio of three evolutionary lines determined from the studied samples of the right wing of bees

Evolutionary line	Number of bee colonies	Percent %
C	38	84.5
O	5	11.1
M	2	4.4

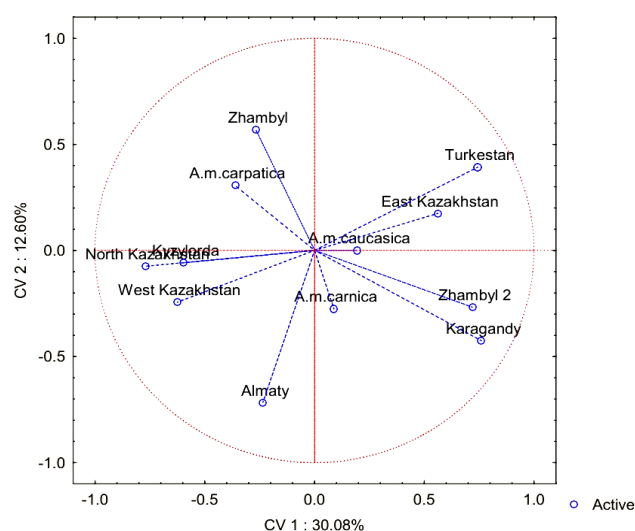


Figure 3. Analysis of canonical variation of centroid size of the honeybee wings from each region, including the reference samples

The Mahalanobis squared distance between the evolutionary lines of honeybees and the statistical significance of pairwise comparisons between the lines verified the results' reliability.

Table 5 shows significant differences in the Mahalanobis distances between the evolutionary lines C, M, O. Line M differs from line C by 15.93 and from line O by 19.03 units, while the distance between lines C and O is

insignificant with satisfactory reliability. These figures once again confirm the overheating area between lines C and O. This is confirmed by the data presented in Table 6, where the largest Mahalanobis squared distances between the subspecies *A. m. mellifera* and the other two subspecies range from 17.98 to 22.66 conditional units, depending on the ratio of the compared honey bee subspecies with high significance.

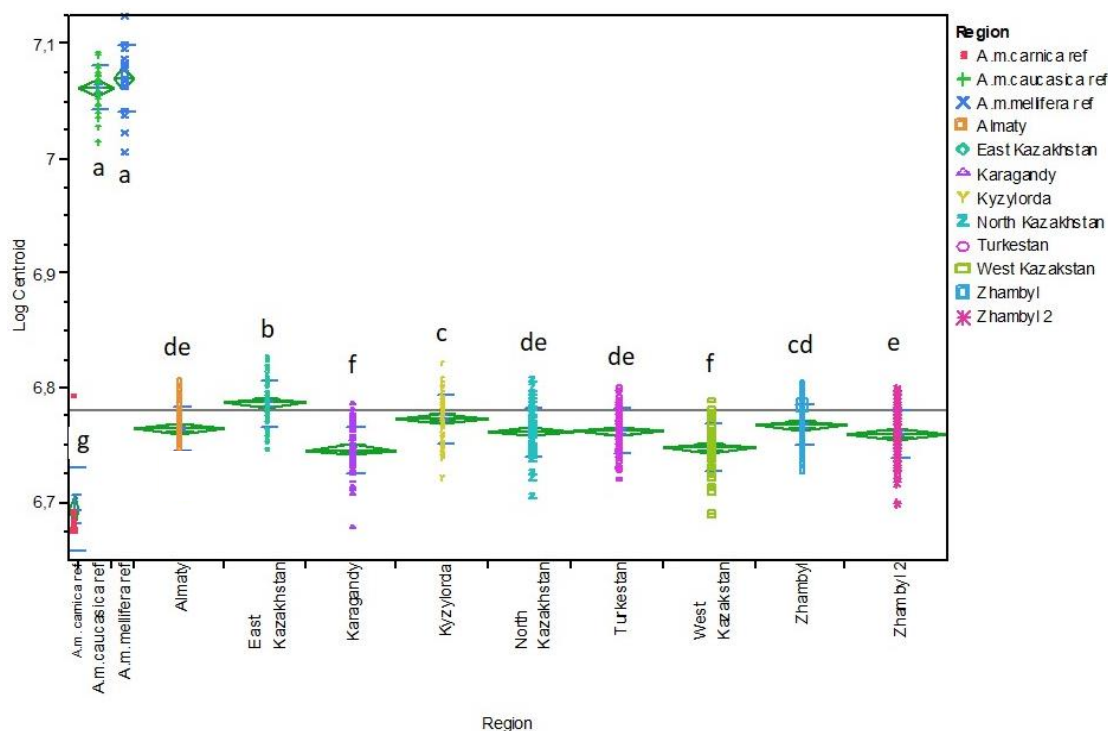


Figure 4. Analysis of variation of centroid size of the honeybee wings from each region, including the reference samples. Each green rhombus showed centroid size means, blue lines-standard deviations. Comparison circles visualize each district wings mean by All pairs, Tukey HSD test ($p<0.05$). Values within each column, followed by different letters, are significantly different ($p<0.05$)

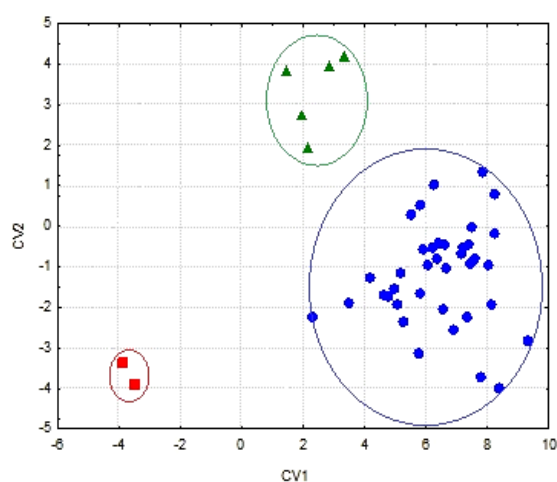


Figure 5. The location of honey bees depends on the evolutionary line C, M, and O

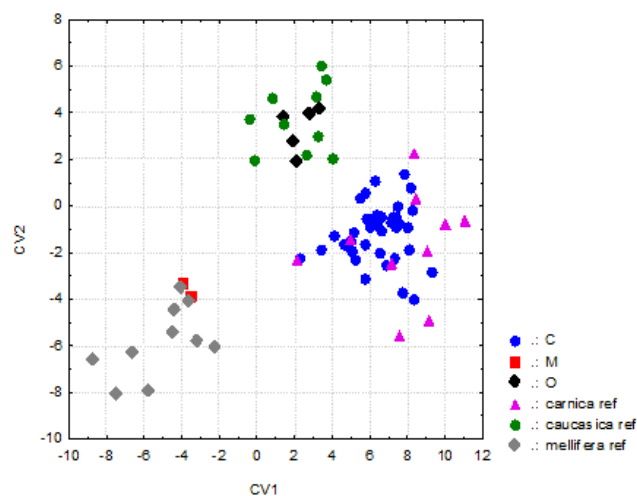


Figure 6. Discrimination of honey bees of three evolutionary lines compared with reference populations of the *Apis mellifera carnica*, *Apis mellifera caucasica*, and *Apis mellifera mellifera*

Table 5. Mahalanobis square distances between evolutionary lines of honey bees

Lineage	C	O	M
C	-	*	**
O	7.04	-	**
M	15.93	19.03	-

Note: * $p < 0.05$, ** $p < 0.01$ **Table 6.** Mahalanobis squared distances between honey bee subspecies and statistical significance of pairwise comparisons between subspecies

	<i>A. m. carnica</i>	<i>A. m. caucasica</i>	<i>A. m. mellifera</i>
<i>A. m. carnica</i>	-	*	**
<i>A. m. caucasica</i>	6.97	-	**
<i>A. m. mellifera</i>	17.98	22.66	-

Note: * $p < 0.5$, ** $p < 0.01$

Based on the conducted analyses, we believe that the presence of the percentage of *A. m. caucasica* lineage is a result of unplanned, random importation of bee packages from different countries both near and far abroad during different periods of the development of beekeeping in Kazakhstan. Different subspecies of honey bees were imported to the southern regions of Kazakhstan, including the grey Caucasian bee from the Caucasus, the Ukrainian steppe bee from Ukraine, and the European dark bee from Russia. Later, Italian and Carniolan breeds were imported from the North Caucasus (Bilash 1991). In addition, during the adaptation testing, hybrids of the European dark bee, Caucasian, Italian, and Carniolan breeds were used, which were widespread in other geographical areas of Kazakhstan. Even now, bee packages continue to be imported from neighboring countries. However, as a result of the research, it has been established that representatives of three evolutionary lines are currently present and bred within the population of honey bees in Kazakhstan.

Discussion

The morphological characteristics of honeybees are important in the identification and discrimination of honeybee subspecies (Tofilski 2008; Abou-Shaara 2013; Henriques et al. 2020; Abed et al. 2021; Masaquiza et al. 2023). Wing size depends not only on genetics but also on environmental conditions (Janczyk et al. 2021; Aglagane et al. 2022). The centroid size of the investigated samples was between 6.75 and 6.79, whereas the identified *A. m. carnica* centroid size was between 6.74–6.76. At the same time, the analyzed *A. m. carnica* reference sample from the Oberursel database showed 6.69, which results were the same as the results by Puškadija et al. (2020). Identified lineage O colonies from East Kazakhstan region centroid size showed 6.78. Our analyzed *A. m. caucasica* reference samples showed more than 7.06. Beekeepers in this area have similar habits of rotation and replacement due to

various environmental conditions (Čápek et al. 2016; Kambur et al. 2018; Kekeçoğlu et al. 2020).

Geometric morphometric methods have been used to identify subspecies of honeybees by scientists from different countries, such as Tofilski (2008), who is the developer of the IdentiFly program, as well as Gerula et al. (2009), Kandemir et al. (2011), Berezin (2019), Bustamante (2021) and García (2022). Polish scientists studied the diversity of honeybees in Poland, Turkish scientists studied the distribution of honeybees in the Mediterranean region, and Berezin studied the territory of the Russian Federation. The method of geometric morphometric used by them yielded positive results in identifying bees using 18–23 coordinates of the right forewing of different subspecies of honeybees (Tofilski 2004, 2008; Bustamante et al. 2020; Dukku et al. 2020; Zahara et al. 2022).

Landmark identification analysis of wing shape might be reliable for discriminating between honeybee subspecies (Barour et al. 2016). Using geometric morphometric methods based on 19 landmarks by IdentiFly on the right forewing of honeybees allowed determine their breed and the presence of other breeds. Discriminant analysis of the orientation coordinates on the forewing showed that the most common line in Kazakhstan is line C, which mostly includes the subspecies *A. m. carnica*.

During the research to determine the subspecies affiliation of honey bees to the O-lineage, it was found that a higher percentage of the subspecies of Caucasian origin and a newly presumed subspecies of the Kazakhstani honey bee population, whose presence was established by Sheppard et al. (2003) based on their research in the Tien Shan Mountains and natural-climatic zones of Kazakhstan.

The appearance of *A. m. caucasica* is explained by the early importation of Caucasian subspecies and their spread throughout the territory of Kazakhstan. This is confirmed by the presence of Caucasian subspecies in the apiaries of Kazakh bee populations. Additionally, in their research, Sheppard et al. (2003) exclude the presence of representatives of the Caucasian subspecies belonging to the C-lineage.

In Kazakhstan, for the first time, the identification of honey bees with the determination of their genetic admixture is being carried out, including the proportion of admixture of subspecies of honey bees of Caucasian and Carpathian origin. In the future, using this obtained data, we plan to conduct more thorough studies to determine the genetic admixture of subspecies that are not typical for Kazakhstan and to determine the direction of beekeeping, conduct breeding work, and preserve the gene pool of valuable and effective bee subspecies for Kazakhstan's populations of honey bees.

IdentiFly software is a timely developed tool for determining the honey bee subspecies using geometric morphometric methods based on 19 coordinates on the right forewing. This is supported by Tofilski (2004, 2008), Gerula (2009), Kandemir et al. (2011), and Gomeh et al. (2016) that geometric morphometric methods have advantages over traditional methods.

Oleksa et al. (2015) argued that similar results could be obtained by morphometric and molecular genetic methods in determining the breed of honey bees. Further studies will be conducted to determine the purity of honey bee breeds in Kazakhstan based on geometric morphometric methods, with parallel use of molecular genetic methods. A promising plan for the development of beekeeping in Kazakhstan has been identified, which involves using honey bee breeds with high acclimatization, productivity, and resistance to invasive diseases.

It has been established that around 85% of honey bees in Kazakhstan belong to the C-lineage. The presence of small proportions of other breeds on beekeeping farms in Kazakhstan has been identified, which can be explained by the early hybridization of local bees imported from the Caucasus, Ukraine, Russia, and Uzbekistan. Three subspecies have been identified for zoning honey bees, taking into account the natural climatic zones of Kazakhstan: *A. m. carnica*, *A. m. mellifera*, and *A. m. caucasica*. The obtained results will allow the development of an effective breeding zoning program and a national program for the development of beekeeping in Kazakhstan.

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