Genetic structure of Caspian Sea southern area honeybee populations, based on microsatellite polymorphism

A. A. Gharahdaghi*, N. Asadi and M. H. Banabazi

Department of Biotechnology, Animal Science Research Institute of Iran, Karaj, Iran.

* Corresponding author, E-mail address: ghare.akbar4@gmail.com

Abstract Genetic diversity is a key component of ecosystems. The aim of the present study was to evaluate the genetic diversity of Iranian native honeybee colonies (*Apis mellifera meda* L.) located in the northern region. Colonies from 24 locations have been analyzed using microsatellite markers. Samples were collected from Caspian Sea southern area (north of Iran). Six microsatellite markers (A28, A29, A113, A24, A76, and A43) amplified them. Amplification was successful in four loci but not for A76 and A43. Polymorphism and diversity indices were high for all amplified loci and for total population. Average heterozygosity and average number of alleles were significantly high in these colonies. The phylogenies also revealed a genetic distance among colonies of this area. The results showed a reliable genetic diversity among *Apis mellifera meda* L. populations in the north of Iran (southern area of Caspian Sea). Based on the microsatellite genotypes, the honeybee population in the southern area of Caspian Sea can be clustered into two distinct subpopulations located in Gilan and Mazandaran area. These results support the previous morphological and biochemical evidences.

Keywords: genetic diversity, microsatellite, *Apis mellifera meda* L

Received: 5 Jun. 2014, *accepted:* 10 Sep. 2014, *published online:* 12 Nov. 2014

Introduction

The honeybee (*Apis mellifera* L.*)* is an agricultural and advantageous insect and also beekeeping is an industry that has been attracted many workers in worldwide. Genetic diversity serves as a way for populations to adapt to changing environments. Ruttner et al. (1985) morphologically classified 24 *Apis mellifera L.* subspecies into four major evolutionary biogeographic groups according to morphological characterizations. Iran's varied climate has provided a favorable condition for apiculture. Currently historical findings such as a Bronze dagger in the Lorestan province indicate the beekeeping there in 300 BC. The morphological and biochemical evidences have been verified Iranian native honey bees are relatives' of *Apis mellifera meda* L. (Ruttner et al., 1985 & Tahmasebi et al., 1998) as three distinctive ecotypes in north, west, and central region of Iran. They have also indicated that northern ecotype has been distinctively characterized more than two other populations. This is probably in accordance with environmental forces particularly climate. Therefore, it needs a specific attention and more investigation.

Microsatellites are the valuable genetic markers due to their dense distribution on the genome, great variation, co-dominant inheritance, and easy genotyping. Microsatellites, also known as simple sequence repeats (SSRs) or short tandem repeats (STRs), are repeating sequences of 2-5 base pairs of DNA. The multiple alleles are identified based on the number of tandem repeats and finally the length of a fragment amplified by the polymerase chain reaction (PCR). The primers are designed corresponding to the unique flanking regions surrounding of the tandem repeats. Due to a high mutation rate, microsatellites provide many alleles and great variation required for a range of genetics studies such as linkage analysis, parentage and assignment testing, domestication processes and so on (Barker, 1994). In the recent years, they have been extensively applied in population studies particularly phylogenetic inferences (Goldstein & Pollock, 1997; Goldstein & Schlotterer, 1999). They were greatly used as an auxiliary taxonomic tool in diverse populations (Takezaki et al., 1996; Sinacori et al., 1996; Hall et al., 1998; Bassam et al., 1993). They have also been used to studying of genetic structure and diversity among honeybee populations in Slovenia, Spain, Canary Islands, Balearic Islands, Italy, Sicily Island and Africa continent (Delarua et al., 2001; 2002 & 2003; Frank et al., 2001). Susnik et al. (2004*)* indicate that *Apis mellifera carnica* L. honeybees of Slovenia and Cr-

oatia are significantly the same and can be identified as one population. The aim of this study was to study the genetic structure of the *Apis mellifera meda* L. population located on southern area of Caspian Sea using microsatellite DNA markers.

Materials and methods

Sampling

Honey bee workers were collected from different colonies from southern Caspian Sea areas including Gilan and Mazandaran provinces. "A" indicates Gilan samples collected from Roudbar, Fouman, Talesh, Astara, Lahijan, Langroud and Roudsar cities. "B" includes Mazandaran samples collected from Noshahr, Tonekabon, Sari, Behshahr, and Ghaemshahr cities. Therefore, samples were initially assigned to twelve distinct geographical groups. The sampled honeybees were immediately killed by immersion in absolute ethanol and kept at –20ºC until DNA extraction.

Genotyping

Genomic DNA was extracted from whole bees using the method developed by Asadi et al. (2004). The quality and quantity of DNA samples were evaluated by spectrophotometer. They were amplified using four microsatellite markers (table 1) by Eppendorfe thermocycler (MJ research Inc, USA). We followed PCR conditions that have been already published for each loci (Estoup et al., 1995). The final reaction volume consisted 50 ng template DNA, 200 µM of each dNTPs, 10X PCR buffer (100 mM Tris-HCL, 50 mM KCL, 0.01% gelatin and 0.25% Tween 20), 1 µM of each primer pair, 5 units/µl Taq polymerase and $MgCI_2$ (0.7–1.5 mM). Amplification was done based on a thermal cycling program as one denaturation step at 94ºC for 10 min that then followed by 35 cycles of denaturation at 94°C for 30 s, annealing (according to table 1) for 30 s, extension at 72°C for 30 s and a final extension at 72°C for 10 min. The electrophoresis of PCR products were done on an 8% nondenaturing polyacrylamide gel for about 20 hours at 40 W. The individual alleles were visualized by silver staining (Bassam & Caetano-Anolles, 1993) and directly sized from two size markers (Roche, Germany) included on each gel using Gel-Pro Analyzer 3.1 software (BioRad, Italy).

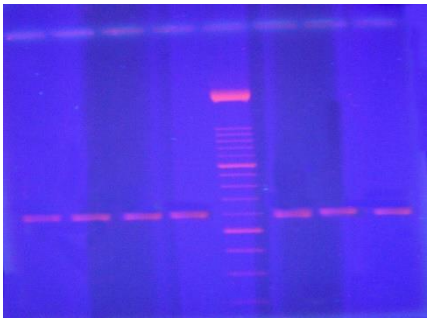

Fig 1. Quality of DNA extracted from samples

Data Analysis

The Hardy-Weinberg equilibrium was studied by Chi-square and Likelihood Ratio tests ($P < 0.005$). The number of alleles, the effective number of alleles, allele frequencies, observed heterozygosity (HO) and expected heterozygosity (HE) were estimated by POPGENE 1.31 software (Yeh et al., 1999). An Analysis of Molecular Variance (AMOVA) was done for determining of the contribution of each population into total diversity using GenAlex 6.0 software (Peakall et al., 2006). Genetic diversity and structure were measured as Cavalli-Sforza's genetic distances (Cavalli-Sforza & Edwards, 1967) and also phylogenies constructed from Neighbor-Joining (Saitou & Nei, 1987) and Unweighted Pair Group Method with Arithmetic Mean (UPGMA) algorithms. They were drawn from 1000 bootstrap iterations using MEGA 4 software (Tamura et al., 2006). In addition, polymorphic information content (PIC), that shows the variation per locus, was calculated using PIC 1.80 (Ott, 2001).

Results

DNA samples had an optical density ratio as A260/A280

Table 1. Microsatellite core sequence and corresponding primer sequences and PCR conditions

Loci	Core sequence	Primers	Tm (°C)
A113	$(TC)_2$ C $(TC)_2$ TT $(TC)_5$ TT $(TC)_8$ TT$(TC)_5$	5´CTCGAATCGTGGCGTCC3´ 5´CCTGTATTTTGCAACCTCGC3´	58
A24	$(CT)_{11}$	5´ CACAAGTTGCCAA CAATGC3´ 5´ CACATTGAGGATGAGCG3´	56
A29	(GT)24	5´AAACAGTACATTTGTGACCC3´ 5´CAACTTCAACTGAAATCCG3´	55
A28	$(CCT)_3$ GCT $(CCT)_6$ $(CT)_5$ TT $(CT)_4$	5´GAAGAGCGTTGGTTGCAGG3´ 5´GCCGTTCATGGTTACCACG3´	55

equal to 1.9-2.0 that indicates their high quality. In addition, they had enough concentration about 1700 ng/µl. Based on direct evaluations on 2% agarose gel, there are not any contamination included RNA and/or remained proteins on DNA samples (Fig. 1).

Microsatellite loci

All four microsatellites were successfully amplified by PCR. Total 31.5 alleles were found in these loci for *Apis mellifera meda* L. colonies in north of Iran. The average number of alleles per locus was 4.3±1.5 (Table 2), ranging from 5 for A24 to 9.5 for A113 and A28 loci. This was 7.3 and 8.2 for A and B populations, respectively.

Table 2. Number of detected alleles, observed and expected heterozygosities (H_O and H_E)

Locus	Sample size	Total no. of alleles	Ho	H_E
A24	80	5	0.203	0.686
A29	90	7	0.468	0.809
A28	90	9	0.090	0.826
A113	80	10	0.052	0.850
Mean	85	7.75±1.5	0.203±0.362	0.638±0.121

The observed allele sizes were in accordance with Estoup et al (1995) and Delarua et al (2002). Although Estoup et al (1995) could successfully amplified A43 and A76 loci; they weren't replicated in the present study even by several optimizations. This may be resulted from mutations in sequence primer binding sites in the studied population. All individuals were distinctively genotyped in four loci. Table 3 shows our allele sizes compared with two previous reports (Estoup et al., 1995; Delarua et al., 2002).

Table 3. The allelic range of studied loci

Loci	A24	A113	A28	A29
allelic range (bp)				
Observed	96-115	220-246	122-148	132-166
Reported	96-118	200-238	122-145	134-163

The highest average number of alleles was for A28 and A113 loci and for A24 locus was the lowest (Table 4). Delarua et al (2002) and Estoup et al (1995) have reported the lowest average number of allele's for A24 locus.

Table 4. Average number of microsatellite alleles for studied loci

Loci	A29	A28	A113	A24
Average				
Observed alleles	7.5	9.5	9.5	5.0
Effective alleles	5.2	5.4	6.5	3.1

In addition, the average effective number of alleles obtained in Mazandaran population was more than Gilan (Table 5). This difference mainly originates from more values for A28 and A113 loci. These average observed and effective alleles may reflect genetic diversity between two subpopulations into studied population as more diversity for Mazandaran than Gilan.

Table 5. The average number of alleles of Mazandaran and Gilan honeybee subpopulations

population	Mazandaran	Gilan
Average		
Observed allel	7.5	8.2
Effective allel	5.0	5.1

Genetic diversity

Both populations showed a departure from Hardy-Weinberg equilibrium both by Chi-square test and Likelihood Ratio test ($P<0.01$). This can mainly be due to sampling that disturbs equilibrium laws. The highest and lowest average of observed and unbiased expected heterozygosity were in A (0.243, 0.786) and B (0.164, 0.799) populations (Table 2). The Shannon's index also showed the same trend for A and B populations. The average observed and expected heterozygosities in total population were ranged from 0.243 to 0.164 and from 0.786 to 0.799, respectively. A24 locus showed the lowest Shannon index in total population. However, A113 and A24 had the highest in Mazandaran and Gilan, respectively (table.6).

Table 6. Average Shannon information index

loci	A24	A113	A28	A29
Shannon index	1.313	2.008	1.864	1.744

Analysis of molecular variance (AMOVA) showed that 92% of total genetic diversity is significantly due to diversity within populations and only 8% is resulted from diversity among populations. This may be resulted from strain sharing among populations. In view of loci, the lowest and highest observed heterozygosity was estimated for A29 and A113 loci in Gilan population, and for A24 and A113 loci in Mazandaran population, respectively (table 7). The same trend was observed for the unbiased expected heterozygosity (table 7).

Table 8 shows that the lowest and highest average of observed heterozygosity (H_O) and unbiased expected heterozygosity (H_E) were for A29, A113, A24, and A113 loci, respectively.

In conclusion, the above diversity indices indicate more genetic diversity in Mazandaran honeybee population than Gilan. (0.243 and 0.799 compared with 0.164 and

Table 7. Observed heterozygosity (H_O) and unbiased expected heterozygosity (H_E)

Loci	A24		A113		A28		A29	
population	H_O	H_E	H_O	H_E	H_O	H_E	H_O	H_E
Gilan	0.240	0.714	0.020	0.817	0.061	0.818	0.652	0.848
Mazandaran	0.166	0.166	0.085	0.884	0.120	0.834	0.285	0.770

0.786) (Table 9).

Table 8. The observed heterozygosity average and unbiased expected heterozygosity for studied loci

Loci	A24	A113	A28	A29
Average				
H_O	0.203	0.052	0.090	0.468
H_E	0.686	0.850	0.826	0.809

Table 9. The average observed heterozygosity and average unbiased expected heterozygosity of studied populations

Population	Mazandaran	Gilan
Average		
H_O	0.243	0.164
H_E	0799	0.786

The Nei's Standard genetic distances (DS) and assignment test based on Likelihood Ratio Distance (DLR) showed a correlation of 95 %. This high correlation was drawn on a graph that shows parallel changes in logarithms of both statistics. The genetic distance between A and B populations was low. Table 10 shows Nei's standard and modified Cavalli-Sforza's genetic distance matrices.

Table 10. Genetic distance DS (numbers below the diagonal matrix)

Matrixes of genetic distances (DS)	Mazandaran,	Gilan
Mazandaran,	****	0.776
Gilan	0.253	****

Discussion

Based on the previous studies using morphological characteristics (Ruttner et al., 1978; Tahmasebi et al., 1998), Iranian honeybee population in northern Iran is unique with distinct features such as a larger body size and length of the trunk. The morphological studies have also indicated that this population is close to Caucasians honeybee. In the present study, the nuclear DNA data (microsatellite markers) supported morphological evidences. In addition, Iranian honeybee population in northern area can be divided into two diverse subpopulations in Mazandaran and Gilan regions based on our present results.

The genetic diversity indices within and between these two subpopulations also showed a significant difference. The mentioned previous studies that were based on morphological and productive characterizations may be affected by environmental conditions. However, the molecular markers data can be more accurate and stable. Suazo and Mctiernan (1998) in their study on European and African honeybees using RAPD markers could find a specific band (539) for East Europe honeybees group and a specific one derived from primer (652) for African honeybee. Genetic diversity in the studied population can be vital in reduction of sex allele's homozygosity and survival of honeybee colonies. This diversity among colonies can cause by random mating of a queen with males of the neighboring apiaries. Another factor affecting the genetic diversity within subpopulations can be due to the continued migration of colonies within one area with the same climate. According to our results, honeybee colonies in Mazandaran were more diverse than Gilan colonies. It can be reflected some different climates in these regions. Mazandaran has extensive citrus gardens and migration of honeybees throughout province can be effective in this diversity. Despite of high genetic diversity observed within each subpopulation, the genetic variation between two populations was relatively low. This sets these populations in one phylogeny. Some populations studies have been conducted among other honeybee populations also show a lack of diversity within populations and low genetic distance among populations. Delarua et al. (2003) have reported a similar heterozygosity and allelic diversity observed in the Balearic honeybee populations, Iberian honeybees, and Canary Islands (Canarian) populations. A climate somewhat different in the north of Iran may be caused this distinct branch including Mazandaran and Gilan compared with the other regions. Alborz Mountains has made over the years these two subpopulations bee, genetically very similar together and distinct from others.

Conclusions

Mazandaran honeybee population is more diverse than Gilan. This may be due to multiple annual migrations, climate variability, and larger honeybee population size in Mazandaran. In sum, Iranian honeybee population in the northern area can be considered as a unique and more distinct population than others. Based on this finding, it is recommended a special queen-breeding program

for the northern area of Iran. The intra and inter population diversity can play an important role to avoid punitive genetic disorders and loss of colonies production. In conclusion, molecular markers data are a more reliable source to studying of evolutionary and population studies on honeybee compared with morphological and productive characterizations, because they do not affect by environmental effects.

References

Asadi, N., Esmaeilkhanian, S., Nejati javaremi, A., Gharahdaghi, A.A., Mirhadi A., 2004. Genomic DNA extraction from honeybee using a modified procedure in Iran, Proceedings of Fifth Iranian Honeybee Seminar Karaj, Iran.

Barker, J. S. F., 1994. A global protocol for determining genetic distances among domestic livestock breeds. Proceedings of the fifth World Congress on Genetics Applied to Livestock Production. University of Guelph, Canada. 21, 501-508.

Bassam, B. J., Caetano-Anolles, G., 1993. Silver staining of DNA in polyacrylamide gels. *Applied Biochemistry and Biotechnology* 42, 181-188.

Delarua, P., Galian, J., Serrano, J., Moritz, R. F. A., 2001. Genetic structure and distinctness of *Apis mellifera* L. populations from the Canary Islands. *Molecular Ecology* 10, 1733-1742.

Delarua, P., Galian, J., Serrano, J., Moritz, R. F. A., 2002. Microsatellite analysis of non-migratory colonies of *Apis mellifera iberica* from South-Eastern Spain. *Journal of Zoological Systematics and Evolutionary Research* 40, 164-168.

Delarua, P., Galian, J., Serrano, J., Moritz, R. F. A., 2003. Genetic structure of Balearic honeybee populations based on microsatellite polymorphism. *Genetics Selection Evolution* 35, 339-350.

Estoup, A., Garnery, L., Solignac, M., Cornuet, J. M., 1995. Microsatellite variation in honeybee (*Apis mellifera* L.) population: Hierarchical genetic structure and test of the infinite allele and stepwise mutation models. *Genetics* 140, 679-695.

Frank, P., Garnery, L., Loiseau, A., Oldroyd, B. P., Hepburn, H. R., Solignac, M., Cornuet, J.-M., 2001. Genetic diversity of the honeybee in Africa: Microsatellite and mitochondrial data. *Heredity* 86, 420-430.

Goldstein, D.B. & Pollock D.D. (1997) Launching microsatellttes: a review of mutation processes and methods of phylogenetic inference. *Journal of Heredity* 88, 335-342.

Goldstein, D.B. & Schlotterer C. (1999) Microsatellites, evolution and applications, First edition. Oxford University Press, New York, NY, USA.

Hall, H. G. 1998. PCR amplification of a locus with RFLP allele specific to African honeybees. *Biochemical Genetics*, 36, 351–361.

Nei, M. 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics*. 89, 583-590.

Nei, M., Tajima, F. Tateno, Y., 1983. Accuracy of estimated phylogenetic trees from molecular data. *Journal of Molecular Evolution*. 19,153-170.

Ott, J. 2001. Program HET version 1.80, Utility programs for analysis of genetic linkage. Rockefeller University. New York, USA.

Peakall, R., Smouse, P. E., 2006. GENALEX 6: Genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Note*. 6, 288-295.

Ruttner, F., Pourasghar, D., Kauhausen, D., 1985. Honeybees of Iran: *Apis mellifera meda skorikow*, The Persian bee. *Apidologie*. 16, 241-264.

Saitou, N., Nei, M. 1987., The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*. 4, 406-425.

Sinacori, A., Rinderer, T. E., Lancaster, V., Sheppard, W. S. (1998). A morphological and mitochondrial assessment of *Apis mellifera* from Palermo, Italy. *Apidologie*, 29, 481–490.

Suazo, A., Mctiernan, R. 1998. Differences between African and European Honeybee in Random Amplified DNA. *Heredity*. 89, 32-36.

Susnik, S., Kozmus, P. Janez P., Vladimir M. 2004. Molecular characterisation of indigenous *Apis mellifera carnica* in Slovenia. *Apidologie*. 35, 623-636

Tahmasebi, GH. Ebadi, R., Esmaili, M., Kambousia, J., .1998. Morphological Study of honeybee (*Apis mellifera* L.) in Iran. *Journal of Science and Technology of Agriculture and Natural Resources Water and Soil Science* 2, 89-101.

Takezaki, N. and Nei, M. 1996. Genetic distances and reconstruction of phylogenetic trees from microsatellite DNA. *Genetics*. 144, 389-399.

Yeh, F. C., Yang, R., Boyle, T., 1999. POPGENE version 1.31. Microsoft windows-based free ware for population genetic analysis. University of Alberta, Edmonton, Canada.

Communicating editor: *Ali K. Esmailizadeh*

ساختار ژنتیکی جمعیت زنبور عسل مناطق جنوبی دریای خزر بر اساس چند شکلی نشانگرهای ریزماهواره

ع.ا. قره داغی*، ن. اسدی و م. ح. بنابازی

نویسنده مسئول، پست الکترونیک: ghare.akbar4@gmail.com

چکیده تنوع ژنتیکی از اجزای کلیدی اکوسیستم ها است. این تحقیق به منظور ارزیابی تنوع ژنتیکی کلنی های زنبور عسل بومی ایران (*Apis mellifera meda* L.) در مناطق شمالی کشور انجام شده است. کلنی های جمع آوری شده از ۲۴ محل، توسط نشانگرهای ریزماهواره مورد تجزیه و تحلیل قرار گرفته است. نمونه ها از مناطق جنوبی دریای خزر (شمال ایران) جمع آوری شده بود. به منظور بررسی ساختار ژنتیکی این نمونه ها از ۶ جایگاه ریزماهواره ای (A28، A29، A113، A24، A76 و A43) استفاده شد. تکثیر در دو جایگاه A76 وA43 موفقیت آمیز نبود. ۴ جایگاه باقیمانده چندشکلی بالایی از خود نشان دادند به طوری که بین ۳ تا ۷ آلل بازاء هر جایگاه بدست آمد. میانگین هتروزایگوسیتی و میانگین تعداد آلل ها به طور معنی داری در کلنی ها بالا بود. همچنین درخت فیلوژنیک به دو روش UPGMA و NJ فواصل ژنتیکی بین کلنی های این منطقه را نشان داد. هر ۴ جایگاه ریزماهواره ای تکثیر شده در جمعیت زنبوران عسل بومی این منطقه به میزان زیادی چندشکل بودند. نتایج مطالعه حاضر حاکی از تنوع ژنتیکی در بین کلنی های جنوب دریای خزر است. بر اساس آزمون تمایز ژنتیکی، جمعیت های زنبور عسل جنوب دریای خزر در دو خوشه (گیلان و مازندران) دسته بندی می شوندکه با نتایج مطالعات ریخت شناختی و بیوشیمیایی قبلی مطابقت دارد.