GENETIC DIVERSITY OF HONEY BEE POPULATIONS IN TURKEY BASED ON MICROSATELLITE MARKERS: A COMPARISON BETWEEN MIGRATORY VERSUS STATIONARY APIARIES AND ISOLATED REGIONS VERSUS REGIONS OPEN TO MIGRATORY BEEKEEPING

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ABSTRACT

GENETIC DIVERSITY OF HONEY BEE POPULATIONS IN TURKEY BASED ON MICROSATELLITE MARKERS: A COMPARISON BETWEEN MIGRATORY VERSUS STATIONARY APIARIES AND ISOLATED REGIONS VERSUS REGIONS OPEN TO MIGRATORY BEEKEEPING

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The honey bee (*Apis mellifera* L.) is a globally significant species of apparent economic and ecological importance. Recent reports from Spain, Italy and Greece point to an intense admixture of honey bee populations signified by a loss of population structure. This is mostly attributed to migratory beekeeping practices and replacement of queens or colonies with commercial ones that are usually from non-native races or hybrids of different subspecies. These two practices are also heavily carried out in parts of Turkey where almost three-quarters of the 6 million colonies are transferred seasonally from one region to other.

Past research using microsatellite and RAPD markers, mtDNA, allozymes and geometric morphometry revealed the presence of five different subspecies of honey bees (*meda*, *syriaca*, *caucasica*, *anatoliaca* and an ecotype from Carniolan subspecies group) in Turkey. Here, we carried out an analysis of population structure of Turkish honeybees sampled from six different regions (n = 250) during the period 2010-2012. A total of 29 microsatellite markers were used in four multiplex reactions. The results show that population structure is preserved in general although there are signs of gene flow between the clusters.

Overall F_{ST} between stationary colonies was calculated as 0,067. For migratory colonies the value was 0,015 and for all the 250 samples the value was 0,047. Four different clusters corresponding to geographical distributions of four subspecies were revealed in structure analysis. The differentiation between the clusters was also apparent in PCA and FCA as well as phylogenetic trees constructed based on genetic distances.

The genetic impact of migratory beekeeping was demonstrated for the first time based on a comparison of assignment probabilities of individuals from migratory and stationary colonies to their geographic populations. Another comparison between regions that are either open to migratory beekeeping or closed let us to evaluate the status of isolated regions and showed the importance of establishing such regions. The effects of queen and colony trade were revealed by the presence of introgression from the highly commercial Caucasian bees. Our findings confirm the previously observed high levels geographically structured genetic diversity in honey bees of Turkey and emphasize the need to develop policies to maintain this diversity.

ÖZ

MİKROSATELİT İŞARETLEYİCİLER TEMELİNDE TÜRKİYE BALARISI TOPLUMLARININ GENETİK ÇEŞİTLİLİĞİ: GÖÇER VE SABİT ARILIKLAR İLE YALITILMIŞ BÖLGELER VE GÖÇER ARICILIĞA AÇIK BÖLGELER ARASINDA BİR KARŞILAŞTIRMA

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Balarısı (*Apis mellifera* L.) dünya çapında belirgin ekonomik ve ekolojik önemi bulunan bir türdür. İspanya, İtalya ve Yunanistan'da yapılan son çalışmalar balarısı toplumlarının yoğun bir karışma yaşadığına ve genetik yapının kaybolduğuna işaret ediyor. Bu durum çoğunlukla göçer arıcılığa ve ana arı ile kovan satışlarına bağlanmaktadır. Bu ticari ana arı satışları çoğunlukla yerel olmayan arıların, bir çok zaman da farklı alttürlerin melezlerinin yeni bölgelere taşınmasına yol açıyor. Her iki uygulama da Türkiye'nin belli bölgelerinde oldukça yaygın. 6 milyon kovanın yaklaşık dörtte üçü her mevsim bir bölgeden diğerine taşınıyor.

Mikrosatelit ve RAPD işaretleyiciler, mtDNA, allozim ve geometrik morfometri yöntemleri kullanlarak yürütülen geçmiş çalışmalar Türkiye'de balarısının beş alttürü (*meda*, *syriaca*, *caucasica*, *anatoliaca* ve Karniyol alttür grubundan bir ekotip) bulunduğunu gözler önüne serdi. Burada ise altı bölgeden 2010-2012 yılları arasında toplanan 250 arı örneği üzerinde genetik yapıya dair bir çalışma yürütülmüştür. Dört multipleks tepkimede toplam 29 mikrosatelit işaretleyici kullanılan çalışmanın sonuçlarına göre genetik yapı, kümeler arasında gen akışının arttığına dair veriler olsa da, hala büyük oranda korunmaktadır.

Sabit kovanlar arasında F_{ST} değeri 0,067 olarak hesaplanmıştır. Bu değer göçer arcılara ait kovanlar arasında 0,015 ve tüm örneklerde 0,047 olarak bulunmuştur. Genetik yapı analizinde dört alttürün coğrafi dağılım alanlarına denk düşen dört ayrı küme ortaya çıkmıştır. Kümeler arasındaki farklılaşma PCA ve FCA'nın yanı sıra genetik uzaklıklar temel alınarak oluşturulan soyağaçlarında da gözlemlenmiştir.

Göçer arıcılığın genetik etkileri bu çalışma ile ilk defa gösterilmiştir. Uygulanan yöntem arı bireylerinin kendi bölgelerinin yerel arı kümesine atanma ihtimallerini karşılaştırmak suretiyle göçer ve sabit arıcılara ait kovanların karşılaştırılmasıdır. Bunun yanı sıra göçer arıcılığa açık ve kapalı bölgeler arasındaki bir diğer karşılaştırma yalıtılmış bölgeler oluşturmanın önemine işaret etmektedir. Ayrıca ana arı ve kovan satışlarının etkisi ticari amaçlarla sıkça kullanılan Kafkas arısına ait genlerin yayılması üzerinden gösterilmiştir. Elde edilen sonuçlar Türkiye balarılarında daha evvel gözlenen yüksek seviyedeki coğrafi genetik yapı ve çeşitliliği teyit etmiş ve bu çeşitliliğin korunması için politikalar geliştirme ihtiyacının altını çizmiştir.

To him, died 50 years ago on 3 June 1963 and
For Living,

Living is no joke,
you must live with great seriousness
like a squirrel for example,
I mean, without looking for something beyond and above living,
I mean, living will be your whole occupation.

You must take it seriously,
I mean, so much so, and to such a degree that,
for example, your hands tied behind your back, your back to the wall,
…
you will be able to die for people,
even for people whose faces you've never seen,
even when no one had forced you to it,
even though you know living
is the most beautiful, most real thing.

I mean you must take living so seriously that,
even at your seventy, for example, you shall plant olive trees,
and not to leave it to your children either,
but because although you fear death you don't believe it,
because living, I mean outweighs.

Nazım Hikmet, 1947

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CHAPTER 1

INTRODUCTION

The Western honey bee, *Apis mellifera* L., is a eusocial insect in the family Apidae and subfamily Apinae which includes honey bee genus *Apis* and its relatives like bumblebees, stingless bees and many others, some of them also being eusocial (Cameron and Mardulyn, 2001). It is a species which plays role together with other pollinators in pollination of wild and cultivated plants (Morse 1991, Breeze *et al*., 2011). The species also have significant economic importance in terms of honey and other bee products output (Delaplane and Mayer, 2000). In addition to its ecological and economic importance, it is a model study organism both for evolution of eusociality and sophisticated cognitive abilities; its genome is sequenced by Honey Bee Genome Sequencing Consortium (Weinstock *et al.*, 2006).

1.1. Geographic Distribution and Lineages

Natural distribution of *Apis mellifera* includes Central Asia, Europe, Near East and sub-Saharan Africa but the species was also introduced to East and Southeast Asia, Australia and the Americas mainly on purpose for its economic benefits (Ruttner, 1988). Morphological and molecular studies point to four major lineages of numerous –more than 20- subspecies (Ruttner, 1988, Whitfield *et al.*, 2006). The four widely recognized lineages are A (Africa), M (western and northern Europe), O (Near East and Central Asia) and C (Eastern Europe) lineages. Studies on SNPs in the past decade supported the hypothesis that *Apis mellifera* would have originated in the tropics or subtropics in Africa and colonize its natural range by two main routes: one through Gibraltar and one through Suez and then Bosphorus, ending up with a secondary contact between highly divergent A and C lineages around Alps (Whitfield *et al.*, 2006).

1.2. Genetic Diversity of Honey Bees in Anatolia and Thrace

Anatolia played a role as a refuge during the last glacier. Distribution and differentiation of many species were affected with glaciation and deglaciation events which could also be detected in honey bees too (Hewitt, 1999). Five different subspecies of *A. mellifera* - which are *A. m. meda*, *A. m. syriaca*, *A. m. caucasica*, *A. m. anatoliaca* and an ecotype from Carniolan subspecies group- exist in Turkey (Kandemir *et al.*, 2005). According to Ruttner (1988) and Kandemir *et al*. (2005) *A. m. meda* is found in the southeastern part of

Anatolia, *A. m. syriaca* is found in Hatay and also probably in Urfa (Bodur *et al*. 2007, Tunca *et al*. 2011), *A. m. caucasica* is found near the Georgian border, *A. m. anatoliaca* is distributed across Anatolia from north to south and east to west with locally adapted ecotypes like Muğla, Giresun and Yığılca (Bouga *et al.*, 2011), and finally the "Carniolan type" is found in Thrace. This last subspecies showed complete differentiation from the rest of the bees in Anatolia in terms of morphometry and allozyme frequencies as well as mitochondrial, RAPD and microsatellite markers and was found to be close to honey bees from Austria (Kandemir *et al.*, 2005, Bodur *et al*. 2007, Tunca and Kence 2011, Kandemir *et al.*, 2006, Solorzano, 2009). Taxonomic status of this subspecies still remains to be resolved taking into account the high gene flow within the subspecies of C lineage (De la Rua *et al.*, 2009).

Major subspecies found in and around Anatolia are shown in Figure 1. As can be seen in the figure, Anatolia and Thrace, when considered together, harbors a vast diversity in terms of present honey bee subspecies, at least five out of just more than twenty honey bee subspecies belonging to two different lineages (C and O –even A lineage genetic material is characterized in *A. m. syriaca* population (Bodur *et al.*, 2007, Tunca and Kence, 2011)-) meet, exchange genes and adapt to local conditions determined by diverse climatic, topographical and floristic variations available (Bouga *et al.*, 2011).

Figure 1. Major honey bee subspecies in and around Anatolia.

Beekeeping is intensively practiced in Turkey where there are about 6 million hives distributed all over the country, Muğla, Ordu and Adana provinces leading the others

with around half a million hives in each, and according to Turkish Statistical Institute records, the numbers are increasing yearly reaching a hive density of 7,67 colonies/km^2. Table 1 shows the yearly change in the number of hives and the honey produced through the years 1993-2011 (data for 2012 is not released yet).

Table 1. Colony numbers and honey production in Turkey, 1993-2011. Red colors indicating higher number of colonies while green colors indicate a lower, yellow colors indicate median values.

Year	# of Villages	# of Modern Hives	# of Traditional Hives	Total # of Colonies	Honey Production (in metric tons)
2011	21.131	5.862.312	149.020	**6.011.332**	94.245
2010	20.845	5.465.669	137.000	**5.602.669**	81.115
2009	21.469	5.210.481	128.743	**5.339.224**	82.003
2008	21.093	4.750.998	137.963	**4.888.961**	81.364
2007	21.560	4.690.278	135.318	**4.825.596**	73.935
2006	22.305	4.704.733	146.950	**4.851.683**	83.842
2005	22.550	4.432.954	157.059	**4.590.013**	82.336
2004	22.133	4.237.065	162.660	**4.399.725**	73.929
2003	22.110	4.098.315	190.538	**4.288.853**	69.540
2002	22.423	3.980.660	180.232	**4.160.892**	74.554
2001	22.606	3.931.301	184.052	**4.115.353**	60.190
2000	22.571	4.067.514	199.609	**4.267.123**	61.091
1999	22.447	4.135.781	185.915	**4.321.696**	67.259
1998	22.302	4.005.369	193.982	**4.199.351**	67.490
1997	22.145	3.798.200	204.102	**4.002.302**	63.319
1996	22.329	3.747.578	217.140	**3.964.718**	62.950
1995	21.987	3.701.444	214.594	**3.916.038**	68.620
1994	22.050	3.567.352	219.236	**3.786.588**	54.908
1993	21.975	3.450.755	234.692	**3.685.447**	59.207

1.3 Microsatellites as Honey Bee Genetic Markers

Short tandem repeats of genome mainly consisting of CA and GT dinucleotide repeats are widely considered as neutral markers (Arias *et al.*, 2006) so they are frequently used for analysis of population structure in many species, including *A. mellifera* (Gouichoux *et al.*,

2011, Bouga *et al.*, 2011). Besides this, microsatellite markers are used to track evolutionary history (Shaibi et al., 2009), study the effects of fragmentation (Shaibi and Moritz, 2010), discriminate subspecies (Estoup *et al.*, 1995), differentiate hybrid individuals (Jensen *et al.*, 2005), characterize commercial breeds (Delaney *et al.*, 2009), map genes (Lattorff *et al.*, 2007), determine mating frequencies (Palmer and Oldroyd, 2000), understand social (Chaline *et al.*, 2002) and reproductive (Barron *et al.*, 2001) behavior and of course to assess genetic diversity of wild (Moritz *et al.*, 2007) and managed (Franck *et al.*, 1998) honey bee populations.

Microsatellite markers were utilized in studies concerning honey bee populations of Turkey as well (Bodur *et al.*, 2007, Kence *et al.*, 2009, Tunca, 2009, Yildiz *et al.*, 2010). These studies demonstrated the high genetic structuring among populations in Turkey and confirmed presence of divergent populations pointing to different subspecies. They, all together, drew attention to the genetic diversity present in Anatolia and Thrace and to its conservation through restrictions on bee trade and creating isolated regions away from the reach of migratory colonies, as well as highlighting possible threats like replacement of queens and colonies and migratory beekeeping which can cause loss of this diversity and the potential benefits this diversity provides or would provide.

1.4 Current Threats to Honey Bees, Their Genetic Diversity and Ecosystem Services They Provide

World food crop production is heavily dependent on wild and managed pollinators and extinctions or declines in pollinators may pose a threat for this output (Klein *et al.*, 2007). While focusing on crop plants it should not be missed out that wild plants are, too, dependent on insect pollinators (Williams, 1994).

Both the honey bees and wild pollinators are thought to be on decline (locally and/or globally depending on the species and region of concern) due to factors some of them closely related to human activities. Among them, destruction and fragmentation of natural habitats, toxicity caused by pollution and pesticides –like widely used neonicotinoids-, diseases and their spreading getting easier, invasive species are leading the way (Meffe, 1998, Brown and Paxton, 2009, Blacquiere *et al*., 2012, Van Egelsdorp and Meixner, 2010). Honey bees also, especially wild populations that are not managed by beekeepers (including the feral populations), take their share from the situation (Dietemann *et al.*, 2009, Genersch, 2010, Oldroyd, 2007, Van Engelsdorp *et al.*, 2009, Evans and Schwarz, 2011).

Besides negative consequences created by such human activities; the genetic admixture of honey bee populations due to queen and colony trade, including complete replacement of local bees with non-natives and beekeeping practices involving movement of colonies

from one region to the other impose another kind of pressure on the species: the loss and/or swamping of locally adapted gene combinations and local or global extinctions of native honey bees, not only of *Apis mellifera* subspecies, races or ecotypes but of other species in the genus *Apis* –namely *Apis cerena*, *Apis florea*, *Apis dorsata* and other native bees of Asia (De la Rua *et al.,* 2009).

De la Rua *et al.* (2009) discuss current threats to European honey bees identifying the majors as: pollution ending up in water and soil contamination and also in global warming; introduction of foreign predators, parasites and pathogens; intensive land use and genetically modified crops causing loss of alternative floral resources and nesting habitats for wild counterparts especially; large scale queen breeding resulting in heavy selection and loss of diversity; reduction in the number of colonies managed due to economic reasons and they identify the lack of plans and protective policies across Europe as worsening the situation.

Dietemann *et al.* (2009) also emphasize similar threats to honey bees in Africa including a possible invasion of *A.m. scutellata* inhabited regions by parasitic subspecies *A. m. capensis* as well as the ancient practice known as honey hunting which usually results in the death of the exploited colony.

Pathogens (viruses -deformed wing, sacbrood, Israeli acute paralysis, black queen cell and acute bee paralysis-, bacteria -*Paenibacillus larvae* and *Melissococcus plutonius*-, microsporidia -*Nosema apis* and *Nosema ceranae*- and other fungi, amoeba and other protists) and parasites (*Varroa destructor*, *Varroa jacobsoni*, *Acarapis woodi*, *Apocephalus borealis*) comprise an important and a distinct sphere of threats to honey bees interfering with bee products output, colony survival and pollination services (Genersch, 2010, Evans and Schwarz, 2011, Core *et al.*, 2012).

All those factors and their interactions, including genetic and environmental ones, when combined, may have an increased effect on honey bees and can be the reasons behind continuous or discrete events of sudden colony losses with rapid depletion of worker bees while the queen continues to laying eggs accompanied by lack of dead bees in and around the hive; the syndrome called as Colony Collapse Disorder (CCD) or Colony Depopulation Syndrome (CDS) (Van Engelsdorp *et al.*, 2009, Neumann and Carreck, 2010). The syndrome was first realized in the winter of 2006/2007 in USA and then in Europe and Asia the same year. Since that time, high average colony losses were observed ranging from 29% to 36% in USA (Van Engelsdorp *et al.*, 2012) and median losses of 12-20% were observed in Europe (Van der Zee *et al.*, 2012).

In the winter of 2006/2007, in contrast to previous consecutive years' less than 20%, an average colony loss percentage of 25,9 was observed in Turkey –with percentages as high

as 64,9 in East Anatolia, 56,8 in Northeast, 39,4 in East Mediterranean, 34,2 in Central Anatolia- (Giray *et al.*, 2010) followed by an average 17,4% of loss in the winter of 2009/2010 (Van der Zee *et al.*, 2012), and an average probably between 22 and 25% in the following two years according to a news referring to Beekeepers Association of Turkey published on 16 February 2012 in Sabah, a daily newspaper in Turkey. More clear results will be available when the analysis of the seasonally conducted questionnaires by our laboratory are completed.

Resilience of the honey bees in their struggle against all those factors may be lying in the adaptations they accumulated over thousands of years, and in the new potentials resided in their genetic diversity. Since it is highly suspected that a combination of many above mentioned factors/threats are taking their places in the recent declines by weakening the colonies step by step; and since honey bees' resistance and tolerance to these factors may differ greatly between individuals, ecotypes, races and subspecies; and since locally adapted variants may be encountering less stress thus standing more upright, research on honey bee diversity in various levels (genetic, individual, population) is of great importance.

1.5 Aims of the Study

In the recent years' studies conducted on honey bee population structure in European countries it was shown that the past structure was lost or strongly disturbed. Among the anthropogenic effects, mainly queen and colony trade and replacement of native honey bees with non-natives as well as migratory beekeeping were the usual suspects.

In Spain Canovas *et al*. (2011) found a high level of homogenization in Iberian honey bees in the study they conducted with microsatellite markers. In Italy (including samples from Sardinia and without any samples from Sicilia), a study of microsatellite loci by Dall'Olio *et al*. (2007) showed that the population structuring observed in the past would not be detected now and the whole peninsular populations amalgamated into a single population. In Greece, Bouga *et al*. (2011) reports no significant differentiation based on mitochondrial and allozyme data despite the morphological differences between native subspecies of *A. m. adami*, *A. m. macedonica*, *and A. m. cecropia*.

Introgression of non-native DNA was monitored in wild populations and a comparison between areas with and without apiculture were conducted in a study about influence of beekeeping and queen export in Sudan, locations without apiculture showing less levels of gene flow (El-Niweiri and Moritz, 2010).

In Turkey, despite the data shown about the genetic structuring of honey bee populations in the past, it became a necessity to demonstrate and verify it. This is especially a

necessity for supplying the state officials in charge with the outcomes of scientific research and encouraging them about taking more concrete steps in conservation of the high genetic variability observed in Anatolia and Thrace, which becomes harder under the influence of ideas floating around about the inevitableness of the complete mixing up of native populations till now, ideas which are heavily promoted by queen breeding enterprises, queen exporters and non-governmental organizations mainly backed by investors.

Among the aims of this study were testing the hypotheses about recent heavy/any admixture of populations in Turkey by making use of microsatellite loci; looking for any possible population structuring and comparison with previous data; determination of areas that are genetically distinct from the others thus a candidate for conservation implications to be carried out; evaluating the status of isolated regions where migratory beekeeping is prohibited, restricted or very scarce due to lack of preference of migratory beekeepers or attitude of local beekeepers; to acquire and demonstrate the direct genetic outcomes of migratory beekeeping by comparison of migratory and stationary colonies; showing the effects of unregulated queen and colony trade by figuring out the origin of introgression between populations.

Besides these, traces of recent population bottlenecks due to heavy losses of the past years in apiaries belonging to different categories -of region (isolated or not), subspecies and beekeeping practice (migratory vs. stationary)- were seeked. Also microsatellite markers were evaluated about their efficiency in discriminating honey bee populations in Turkey.

CHAPTER 2

METHODS

2.1 Sampling and Storage

A total of 250 honey bees each from different colonies were sampled from 18 provinces during the period March 2010 and August 2012. Of those 250 honey bees 174 were from apiaries that were stationary and 76 were from migratory ones.

Sampling sites can be seen in Figure 2. There were more than 8 samples in nine of those eighteen provinces which were Kırklareli, Muğla, Eskişehir, Düzce, Ankara, Hatay, Bitlis, Ardahan and Artvin. From the other nine provinces (Edirne, Tekirdağ, Kütahya, Bilecik, Zonguldak, Bolu, Ordu, Elazığ, Erzurum) only few samples were present.

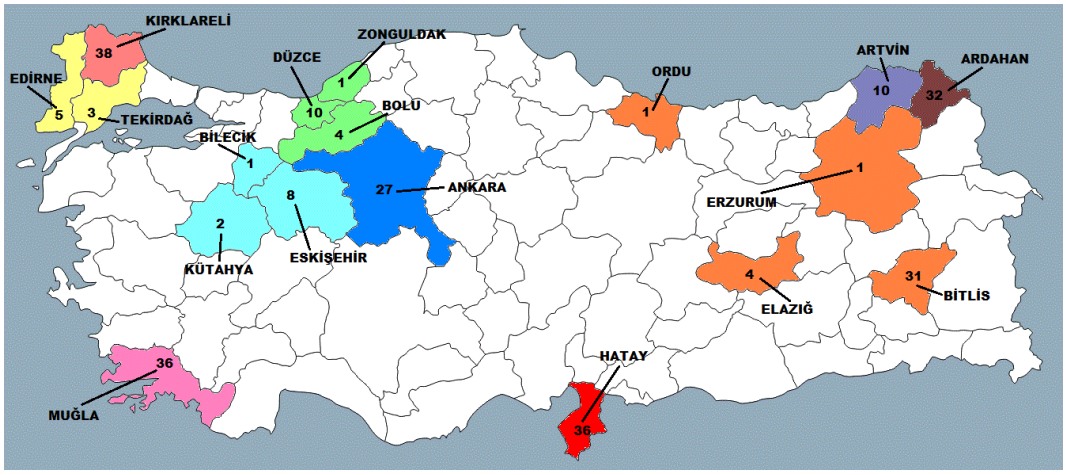

Figure 2. Sampling sites. Same colors indicate grouped samples.

A summary and a schematic representation of sampling sites and sample sizes can be seen in Table 2. Samples from provinces with a small sample size were grouped with nearby provinces to form 10 major localities. “Edirne+” locality brought together Edirne and Tekirdağ samples from Thrace. “Düzce+” locality brought together samples from

Düzce, Bolu and Zonguldak of Western Black Sea. “Eskişehir+” locality gathered samples from Eskişehir, Kütahya and Bilecik, provinces at intersect of Inner Aegean, East Marmara and West Central Anatolia. “Bitlis+” locality gathered samples from Bitlis, Elazığ, Ordu and

Table 2. Sampling sites and number of samples.

PROVINCE	LOCALITY	REGION	STATIONARY	MIGRATORY	TOTAL	LAT. (N)	LONG. (E)
	Kırklareli		37	1	38	41,733	27,217
Edirne			5	0	5		
Tekirdağ			2	1	3		
	Edirne+		7	1	8	41,410	26,923
		Thrace	44	2	46		
Düzce			10	0	10		
Bolu			4	0	4		
Zonguldak			1	0	1		
	Düzce+		15	0	15	40,857	31,321
Eskişehir			8	0	8		
Kütahya			2	0	2		
Bilecik			1	0	1		
	Eskişehir+		11	0	11	39,745	30,374
	Ankara		27	9	36	39,921	32,854
		Middle West	53	9	62		
	Muğla		21	15	36	37,215	28,364
		West	21	15	36		
	Ardahan		32	0	32	41,110	42,702
	Artvin		10	0	10	41,183	41,817
		North East	42	0	42		
Bitlis			0	31	31		
Elazığ			0	4	4		
Erzurum			0	1	1		
Ordu			0	1	1		
	Bitlis+		0	37	37	38,541	41,667
		East	0	37	37		
	Hatay		23	13	36	36,200	36,176
		South	23	13	36		

Erzurum from eastern half of Anatolia. The other localities are individual provinces with relatively large samples. Special attention was paid to represent as diverse parts as possible during sampling of the sites within a locality.

The localities too are combined in 6 regions: “North East” of provinces Ardahan and Artvin near Georgian border; “East” including only “Bitlis+”; “Middle West” bringing together “Eskişehir+”, Ankara and “Düzce+”; “Thrace” combining localities of Thracian provinces Edirne, Tekirdağ and Kırklareli; “West” including only Muğla province at the Aegean coast; “South” from Hatay. The table also shows the latitudes and longitudes of localities, a column of average weighted coordinates used in calculation of geographic distances between localities when nearby samples were combined into a single locality. These new coordinates assigned were done so with considering the relative sample sizes within a locality.

Whenever an apiary was visited for sampling beekeeper contact information, province, district, and village name were recorded together with the date, time, beekeeping practice (migratory vs. stationary), status of frames with brood, honey and bees, as well as flight activity of foragers (number of bees returning from foraging activity in a minute), flora, chemicals used against pests. If the beekeeping practice was migratory then the visited regions were also recorded.

In addition to these, COLOSS (Prevention of Honey Bee Colony Losses Network, an international collaboration founded after 2007 CCD occurences) questionnaires were conducted for monitoring of any CCD like symptoms and colony losses in general.

Randomly chosen six colonies were sampled from each apiary and around 50 honey bees were taken from inner frames of each hive and put into small transparent boxes with small holes for ventilation. The boxes contained fondant and bees were supplied with water by injection through the holes if necessary, so that the bees were kept alive during the field trip and then transferred to laboratory where they were paralyzed by keeping in -80 ^{o}C for two minutes. Then the bees were taken out and 10 bees from each colony were put into 70% alcohol for geometric morphometrics analysis and rest of them were divided into three groups for protein, RNA and DNA based studies and kept in -80 ^{o}C until that time.

2.2 DNA Isolation and Quantification

DNA was isolated from bee heads. QIAGEN DNeasy Blood and Tissue Kit was used for the isolation of DNA following the procedure of the producer for insect samples with slight modifications.

Briefly, for each individual, the head was grounded in 1,5 mL microcentrifuge tube containing 200 µL phosphate buffered saline solution. Protein content was digested with proteinase K treatment at 56 ^{o}C for 2 hours. Using 75% of the advised amounts of kit buffers and reagents was found to be adequate for isolation of enough DNA for downstream applications. Silica based spin columns supplied with the kit were used for binding DNA. After washing steps for removing out other molecules elution of the DNA was finally achieved through sequential centrifugation steps. The elution buffer of the kit is a Tris-EDTA based one providing long term stable storage of DNA. Isolated DNA was quantified using NanoDrop 2000 UV-Visible spectrophotometer.

2.3 PCR Amplification of Microsatellite Loci

2.3.1 Microsatellite loci and primers

A set of 30 microsatellite loci were chosen which included the loci used by Bodur *et al.* (2007) and Tunca (2009) for a possible future co-analysis of data obtained previously by these authors during studies conducted in our laboratory. Other loci were chosen among the widely applied sets in honey bee microsatellite studies (Estoup *et al.*, 1995, Solignac *et al.* 2003, Shaibi *et al.*, 2008).

These 30 loci were grouped into four clusters (for two 7-plex and two 8-plex reactions) to be used in a single PCR (Polymerase Chain Reaction). A software program, Multiplex Manager 1.2 (Holleley and Geerts, 2009), was used for constructing the multiplex groups.

When the microsatellite sequence data and possible allele sizes are input, the program tries to construct groups both by reducing the number of matches in primers that will be used in the same reaction and by bringing together the primers with similar melting points. A maximum nucleotide match threshold of 6 between the primers was set with at least 35 base pairs safety space between two primers labeled with the same dye and the program was let carry out 5 million iterations before giving the optimal combinations.

Only the forward primers were labeled with fluorescent dyes for detection of microsatellite allele sizes in capillary electrophoresis. 6-FAM, VIC, NED and PET commercial dyes from Applied Biosystems (ABI) compatible with ABI 3730XL sequencing machines were used. Availability of the four dye system lets one to increase the number of primers grouped in a single multiplex reaction. HPLC purity primers were kept as 100 µM stock solutions. Microsatellite loci, multiplex groups, forward and reverse (F/R) primers and fluorescent labels are given in Table 3. 6-FAM is a blue colored dye, VIC is a green dye, NED is a yellow dye and PET is a red dye.

Table 3. Microsatellite loci and multiplex primer groups.

	Locus	F/R	Sequence	Length	Label
	Ap218	F	AGGGATGGAATTCTTCGATT	20	6-FAM
		R	TTGTCACAATTCCGCTTGA	19	
	A113	F	CTCGAATCGTGGCGTCC	17	6-FAM
		R	CCTGTATTTTGCAACCTCGC	20	
	A(B)024	F	CACAAGTTCCAACAATGC	18	VIC
		R	CACATTGAGGATGAGCG	17	
GR 1	Ap249	F	CGCGCGACGACGAAATGT	18	VIC
		R	CAGTCCTTTGATTCGCGCTACC	22	
	A088	F	CGAATTAACCGATTTGTCG	19	NED
		R	GATCGCAATTATTGAAGGAG	20	
	AP001	F	ACACGCGAACAATACAACA	19	NED
		R	ACTAATCGGCACGATGAAG	19	
	Ap043	F	GGCGTGCACAGCTTATTCC	19	PET
		R	CGAAGGTGGTTTCAGGCC	18	
	A079	F	CGAAGGTTGCGGAGTCCTC	19	6-FAM
		R	GTCGTCGGACCGATGCG	17	
	Ac306	F	GAATATGCCGCTGCCACC	18	6-FAM
		R	TTTCGTTGCATCCGAGCG	18	
	Ap226	F	AACGGTGTTCGCGAAACG	18	6-FAM
		R	AGCCAACTCGTGCGGTCA	18	
GR 2	A007	F	CCCTTCCTCTTTCATCTTCC	20	VIC
		R	GTTAGTGCCCTCCTCTTGC	19	
	HB-C16-01	F	AAAATGCGATTCTAATCTGG	20	VIC
		R	TTGCCTAAAATGCTTGCTAT	20	
	Ap068	F	TGTCTGCCCTCCTCTCTGTT	20	NED
		R	CACATCGAGCGAGAAGGC	18	
	A014	F	GTGTCGCAATCGACGTAACC	20	NED
		R	GTCGATTACCGATCGTGACG	20	
	Ap223	F	TCGTACAACGTCGCGCAA	18	PET
		R	GCCGCTCGCCTGTATCTG	18	

Table 3 Cont. Microsatellite loci and multiplex primer groups.

	Locus	F/R	Sequence	Length	Label
	AP019	F	CTCGTTTCTTCCATTGCG	18	6-FAM
		R	CGGTACGCGGTAGAAAGA	18	
	A(B)124	F	GCAACAGGTCGGGTTAGAG	19	6-FAM
		R	CAGGATAGGGTAGGTAAGCAG	21	
	A043	F	CACCGAAACAAGATGCAAG	19	VIC
		R	CCGCTCATTAAGATATCCG	19	
GR 3	A076	F	GCCAATACTCTCGAACAATG	20	VIC
		R	GTCCAATTCACATGTCGACATC	22	
	Ap273	F	GATCTTGTGTTAAACAGCCG	20	NED
		R	GATCTCTGGCAGACGAAGAG	20	
	Ap289	F	AGCTAGGTCTTTCTAAGAGTGTTG	24	NED
		R	TTCGACCGCAATAACATTC	19	
	HB-C16-05	F	ATTTTATGCGCGTTTCGTA	19	PET
		R	CATGGCTCCTCCATTAAATC	20	
	A028	F	GAAGAGCGTTGGTTGCAGG	19	PET
		R	GCCGTTCATGGTTACCACG	19	
	Ap049	F	CCAATAGCGGCGAGTGTG	18	6-FAM
		R	GGGCTTCGTACGTCCACC	18	
	Ap238	F	GTCTCGTGCGTGCGAATG	18	6-FAM
		R	TTCATCATGTTCTCAAATTTCTTTGT	26	
	AC006	F	GATCGTGGAAACCGCGAC	18	VIC
		R	CACGGCCTCGTAACGGTC	18	
GR 4	Ap243	F	AATGTCCGCGAGCATCTG	18	VIC
		R	TGTTTACGAGAATTCGACGGG	21	
	Ap288	F	GTTAGTTCGTCGTCGACCG	19	NED
		R	TCTTAGCTTTATAACGAGCACG	22	
	HB-C16-02	F	TAGTATCGTGCTGTTCATCG	20	NED
		R	ACATACATCTCTTGGCGAGT	20	
	A107	F	CCGTGGGAGGTTTATTGTCG	20	PET
		R	CCTTCGTAACGGATGACACC	20	

2.3.2 PCR

For the PCR, QIAGEN Type-it Microsatellite PCR Kit was used. The guidelines of the producer were followed with minor modifications. 12,5 µL of Master Mix of the kit which contains Taq Polymerase enzyme, dNTPs (deoxyribonucleotides) and the stabilizing buffers as well as Mg^{++} (magnesium), was mixed with 5 µL of Q Solution of the kit, an optional solution that helps to amplify relatively equal amounts of each loci which normally have different primer affinities. Primers were also added to this mixture to obtaine a final concentration of 0,4 µM for each in the complete reaction mixture. Primers were directly added to the mixture from the 100 µM stock to avoid diluting them with TE which contains EDTA and would interfere with downstream applications. 1,5 µL of template DNA of the corresponding sample were also added to 0,2 mL PCR tubes and the reaction mixture was readied by completing the final volume to 25 µL by adding enough molecular biology grade dH_2O (distilled water).

The mixture was vortexed to obtain a homogeneous solution and the bubbles were manually removed by gently hitting the tube and the tube was placed in a TECHNE TC-5000 thermal cycler for the PCR. PCR steps included a 5 min 95 ^oC heat activation of Taq Polymerase, which does not start amplification without this to avoid non-specific products as much as possible. Then a cycle of denaturation-annealing-extension 30 repeats were carried out at 95 ^oC-57 ^oC-72 ^oC and for 30-150-30 seconds respectively. A final extension of 30 minutes at 60 ^oC was included to achieve the amplification of non-complete products. A summary of the PCR conditions can be found in Table 4.

Table 4. PCR conditions.

	STEP	**TIME**	**TEMPERATURE**
Activation		5 minutes	95 ^oC
30 cycles	Denaturation	30 seconds	95 ^oC
	Annealing	150 seconds	57 ^oC
	Extension	30 seconds	72 ^oC
Final extension		30 minutes	60 ^oC

For each multiplex set two negative controls was used without including template DNA in the final reaction mixture to identify any potential contamination with foreign DNA and each of the primers in the set were acting as positive controls to one another.

2.3.3 Agarose gel electrophoresis and visualization

To check if the PCRs were successful in amplifying the target loci, the products were run on agarose gel. LONZA MetaPhor agarose was used for preparation of the gel. Also intactness of the genomic DNA was controlled. The 3% (weight/volume) gel was prepared using 1X TBE (Tris-Borate-EDTA) buffer by applying the instructions of the manufacturer. The steps consisted of consequent boiling and waiting cycles until all the agarose was melted and dissolved. Following the boiling, OLERUP SSP GelRed fluorescent nucleic acid dye was added to the solution. After pouring the gel to the caster one must wait till it cools down to room temperature and then place it in 4 ^{o}C for 30 minutes to obtain optimal resolution.

4 µL of each PCR product were suspended with 1 µL QIAGEN GelPilot Loading Dye and applied to the wells. THERMOSCIENTIFIC pUC19 DNA/MspI (HpaII) ladder was used for sizing. The products were run on gel for 2 hours at 180 Volts. Visualization was carried on with VILBERT LOURMAT UV (ultraviolet) gel imager. Figure 4 is the photo taken of a visualized gel.

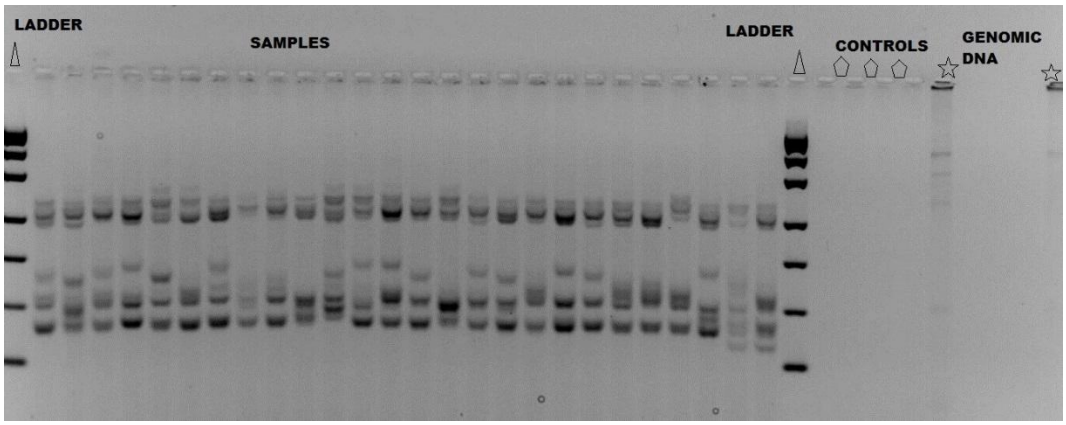

Figure 3. Agarose gel UV visualization.

2.3.4 Purification

To obtain a better resolution in the fragment analysis PCR products were purified by using QIAGEN MinElute PCR Purification Kit. The basic principle of purification is the same with DNA isolation. DNA was bound to silica containing columns and the proteins and other contaminants were washed out with appropriate buffers. DNA was eluted in a 40 µL TE based elution buffer.

2.4 Fragment Analysis and Genotyping

Fragment analysis by capillary electrophoresis -where different alleles were separated from each other according to their size and were recorded according to their fluorescent colors- was carried out by ABI 3730XL capillary electrophoresis of the GENEWIZ Inc. located in New Jersey, USA.

The raw data supplied by the company was analyzed by GeneMapper 5.0, a software for detecting alleles, but the automated detection function were not used due to two reasons. First, dyes used created pull-ups where one obtains multiple signals from different colors for a single allele (obtaining peaks at yellow level whenever there is a peak at green level, red peaks coming with yellow labeled alleles). Secondly, some of the samples were leaked and contaminated while being carried to the company. So the 7500 loci were examined individually and the alleles were determined by eye.

It was possible to differentiate contaminant alleles by a handful of methods developed for identifying DNA belonging to different individuals in crime scene samples which contain DNA from various sources (Budowle *et al.*, 2009). From comparison of relative peak heights to increasing dilutions and adjusting peak detection settings a number of methods were applied to differentiate between contaminants and the original sample.

Capillary electrophoresis gives relative sizes for each allele compared with the peak values of a reference dye which includes DNA fragments of known size, so called the size standard. These relative values rarely correspond to integers. So the alleles were given an integer number checking one by one and comparing each with other values obtained.

As the alleles were determined for each sample, a spreadsheet document was prepared storing sample information about sample number, code name, region, province, beekeeping practice, sampling date and the relative allele size. This spreadsheet document was used as a template for preparing the input of population genetics software used.

2.5 Assessing Genetic Variability and Population Structure

2.5.1 Allelic diversity

Before proceeding any further the thing done after genotyping was to check validity of the data obtained. First, frequency of null alleles –not amplification of the second allele due to mutations in primer binding sites or damage in template DNA- were estimated by FreeNA software using expectation maximization algorithm (Chapuis and Estoup, 2007).

The software also calculates null allele corrected population distance matrix. Secondly, closely related individuals were looked for removal if there were any since they might bias the sample. Kingroup software which calculates relatedness based on shared alleles was used to find out any full-sibs (Konovalov *et al.*, 2004). Allelic diversity observed in the populations were evaluated under two groupings. In the first one the locus based statistics were gathered and in the second one population based statistics were gathered.

Number of alleles and number of effective alleles (a lower bound for actual number of alleles derived from homozygosity) per population and per loci were determined by Popgene software (Yeh *et al.*, 1997). Frequency of each allele was calculated by Convert software which originally converts input files of different population genetics software into each other, private alleles which are unique to certain populations –thus can be helpful for differentiating one population from the other- were found out by this software too. (Glaubitz, 2004). Allelic richness values and unbiased gene diversities -corrected for small sample size- were calculated for each population by Fstat program (Goudet, 1995).

Information index of different microsatellite loci (i.e. their usefulness for differentiating populations in terms of number of alleles and their frequencies) based on Shannon's diversity index were calculated by GenAlEx a Microsoft Office Excel macro developed for population genetics analysis (Peakall and Smouse 2006, Peakall and Smouse 2012).

2.5.2 Heterozygosities and linkage disequalibrium

Proportion of heterozygous individuals were calculated by Genetix software (Belkhir *et al.*, 2004). Alleles were tested for Hardy-Weinberg equilibrium for possible deviations from expected heterozygosities given the number of alleles. Since Hardy-Weinberg (HW) assumes no migrations within or among populations, it is also useful for evaluating the validity of genotyping, by looking at heterozygosity excess. Linkage disequilibrium test between loci (to check if unlinked loci were distributed as if they were linked due to recent admixture of populations) and HW test were carried out by software Arlequin 3.5 (Excoffier *et al.*, 2005).

For population genetics studies the loci used are assumed to be neutral –so that they can freely accumulate differences due to mutations and drift only, thus they can be a measure of relative genetic isolation of populations- but the actual neutrality of the loci are not

known *a priori*. The loci were tested for neutrality with Popgene software by comparing expected and observed homozygosity levels assuming that deleterious alleles would not be widely homozygous.

2.5.3 Bottlenecks

The populations were tested for monitoring any recent bottlenecks by Bottlenecks 1.02 software which tries to capture a pattern in allelic data: it expects loss of allelic diversity faster than loss of heterozygosity, since rare alleles would not affect heterozygosity levels too much but they would easily be lost during bottlenecks (Cornuet and Luikart, 1996). LDNe software was used to estimate effective population sizes which uses linkage disequilibrium and population genetic distances to find out for possible number of breeding individuals (Waples and Do, 2008).

2.5.4 Population distances and phylogenetic trees

Pairwise F_{ST} values that are measures of population differentiation due to genetic structure were calculated by Arlequin. F_{ST} values are indices of fixation which is computed by calculating the probability that two individuals in the population share an identical ancestor and so their alleles are also identical by descent, so the variance in the frequency of an allele in subpopulations and the total population may be used to calculate F_{ST} values.

The pairwise population distances were calculated using the Populations 1.2.32 software (Langella 2011). The software is capable of constructing matrices by different distance measures based on shared alleles, changes arising from mutations and drift together or drift only, populations are then grouped in phylogenetic trees based on these distances. The software can use both Neighbour-Joining Method where first a star shaped tree is produced and then the closest populations are grouped together and added to the tree as a node or can use UPGMA (Unweighted Pair Group Method with Arithmatic Mean) where the two most similar populations or groups are gathered together through nodes first and their values in the matrix are averaged for the next iteration.

Number of migrants between populations were calculated by GenAlEx software by making use of F_{ST} values since they are inversely related, as the number of migrants between populations decrease the less likely two alleles in two populations will be identical by descent.

Population non-differentiation test -which tests the hypothesis that genotypes are randomly distributed to populations thus the populations were "the same"- and Mantel test -for testing the hypothesis that population differentiations were correlated with population geographic distances- were, too, carried out by Arlequin.

2.5.5 Analysis of molecular variance

AMOVA tests were carried out by Arlequin. The test partitions the total variance observed in a group of populations. The partitioning is into individual, population and group based levels so that one can make a comparison between different hypotheses on how to group populations. 30 different AMOVA tests were carried out to test the figures obtained in population trees. Also different groupings of populations corresponding to subspecies distributions were tested to see if they were more homogeneous in between or the variance were cumulated between independent populations. Also tests to check if any of the populations within a subspecies were differentiated from each other were carried out.

2.5.6 Factorial correspondence and principal component analyses

Both factorial correspondence analysis and principle component analysis generate linear combinations of the variables to summarize the covariance so that most of the variation in these variables can be accumulated on gradients.

Genetix software was used for such a visualization of the differentiation of populations by factorial correspondence analysis (FCA). The software demonstrates relationships between individuals based on presence and abundance of each allele. Then uses this matrix to determine a number of axes representing as much as variation present. By plotting individuals on the planes generated by making use of these axes populations are ordinated on three-dimensional space.

PCA-gen software was used to plot populations on a two-dimensional space (Goudet, 1999). Principle component analysis (PCA) works similarly, where different principle components capture a different proportion of variance in the population so they can be used to plot individuals or populations accordingly.

2.5.7 Population structure

Population structure is estimated by using software Structure 2.3.3 (Pritchard *et al.*, 2000). First the program was run to assume the number of distinct populations defined as K. Different K values were tested in terms of their probability. Then the results of these different K values were analyzed by Structure Havester software (Earl and von Holdt, 2012). The program outputs the most probable K values based on examining the variance of the probabilities of these Ks. After estimating the most possible K value the program was run to compute individual membership coefficients, that are probabilities of individuals assigning to different clusters. 150.000 iterations were run for each trial and 26 trials were done for the most possible K.

Clumpp software was used to permute the membership coefficients of individuals so that they were corrected from errors based on values obtained from Structure 2.3.3 (Jakobsson and Rosenberg, 2007). Distruct software was used to visualize the results obtained by Clumpp (Rosenberg, 2004).

These membership coefficients were then used to test hypotheses about beekeeping practices, isolated regions and queen/colony trade. In the first test, assignment probabilities of migratory and stationary colonies were compared. If the migratory colonies acted as a potential vector of foreign alleles then they would have much lower probabilities of being assigned to their own clusters.

First the probabilities were transformed into Arcsine values since further analysis were not compatible for comparing probabilities. Then Mann-Whitney U and t tests were carried out to compare two sets of data coming from migratory and stationary colonies. This was done for colonies in Ankara, Muğla and Hatay, for the three provinces combined and for the total data set.

The second hypothesis was about isolated regions. If the isolated regions were efficient in preserving genetic diversity by preventing gene flow between different clusters then one would expect to see a higher assignment probability for stationary individuals belonging to these regions and lower for individuals that belong to not isolated regions.

Kırklareli is a province where migratory beekeepers rarely visit so it was accepted as an isolated region. Ardahan is legally declared a conservation area for *A. m. caucasica* so migratory beekeepers cannot enter the province and queen import from other subspecies

is forbidden. Artvin is also considered as an isolated area because it is less frequently visited by migratory beekeepers for geographical reasons and beekeepers there do not use non-native queens. These three provinces were compared with the other six (Edirne+, Muğla, Düzce+, Eskişehir+, Ankara and Hatay). Bitlis+ was excluded since all the beekeepers from this locality were migratory beekeepers. Again the same statistical tests were used after the same transformation.

Third set of tests were about the impacts of queen trade. The probabilities of being assigned to a different cluster than the native cluster were compared among individuals of the total data set to find out which cluster contributed most to the other clusters' gene pools. Ardahan and Artvin provinces host the *A. m. caucasica* subspecies which is also widely used for commercial purposes and the caucasica queens and their hybrids are sold all over the country. But these provinces are also isolated places so a possible high introgression of their alleles would mostly be due to queen and colony trade. Same statistical tests with the same transformations were used.

CHAPTER 3

RESULTS

A076 loci was properly genotyped in very few individuals and in the others the amounts were very low when compared to other loci -sometimes hard to differentiate from background noise-, and in most of the samples it wasn't detected at all. So it was excluded from the analysis and the downstream steps were carried out by 29 loci.

3.1 Null Alleles and Closely Related Individuals

There were 4 cases of high null allele frequency observations out of 300 loci-population pairs. These were in loci A(B)024, Ap273 and Ap289 twice, in Artvin, Eskişehir+, Ardahan and Artvin populations respectively. Table 5 shows the null alleles with a high frequency.

Table 5. Null allele frequencies.

LOCUS	POPULATION	FREQUENCY
A(B)024	Artvin	0.20410
Ap273	Eskişehir+	0.21365
Ap289	Ardahan	0.36818
Ap289	Artvin	0.40463

After 1.000.000 simulations the program could not identify any full-siblings. p value was set as 5% and the type II error rate was only 2,6%. So there was no need to remove any samples.

3.2 Allelic Diversity

Private alleles with a frequency higher than 5% were given in Table 6. A total of 110 private alleles out of 467 alleles were observed. 13 of those private alleles had frequencies over 5% in the corresponding population.

Table 6. Private alleles for populations.

POPULATION	LOCUS	ALLELE	FREQUENCY
Kırklareli	AB124	213	0,053
Edirne+	A007	124	0,063
Edirne+	A007	149	0,063
Edirne+	AB124	244	0,063
Edirne+	AP001	252	0,063
Edirne+	AP223	182	0,125
Düzce+	AP238	262	0,067
Muğla	AP289	200	0,056
Artvin	A079	127	0,050
Artvin	A107	185	0,050
Artvin	A113	242	0,050
Artvin	AP249	216	0,050
Hatay	AP243	268	0,056

Table 7 summarizes the loci based information. Significant results are shown in bold for low homozygosity levels, i.e. test of neutrality. Also mean numbers for overall loci were included. Different measures that can be used to evaluate loci in terms of effective discrimination of populations were highlighted with colors for ease of comparison. A red color indicates a high value observed.

Population based allelic richness estimations were given in Table 8 and Table 9. Table 8 contains information about abundance of alleles. Locally common alleles are the ones that are present in a frequency more than 5% in the respective population and only found in 3 or less populations.

Table 7. Loci based allelic diversity.

LOCI	# of ALLELES	# of EFFECT. ALLELES	# of PRIVATE ALLELES	ALLELIC RICHNESS	INFORM. INDEX	HOMOZY. OBS.
A007	46	19,0	11	11,6	3,3	0,05
A014	9	2,2	3	3,2	1,0	0,45
A028	8	1,3	4	2,3	0,5	0,75
A043	13	2,0	5	3,8	1,1	0,50
A079	12	3,9	4	4,9	1,6	0,26
A088	13	2,8	4	3,9	1,3	0,36
A107	25	13,6	2	10,5	2,9	**0,07**
A113	23	9,3	7	8,3	2,4	0,11
A(B)024	8	2,5	2	3,5	1,1	0,39
A(B)124	16	4,8	4	6,4	2,0	0,21
AC006	8	1,2	2	2,2	0,5	0,80
AC306	11	3,5	3	4,3	1,4	0,29
AP001	33	4,7	7	7,1	2,2	0,21
AP019	8	1,6	2	2,9	0,8	0,64
AP043	33	7,6	6	8,5	2,5	0,13
AP049	13	1,9	4	3,9	1,1	0,52
AP068	9	2,8	2	4,5	1,4	0,35
AP218	6	1,1	3	1,8	0,3	0,87
AP223	6	3,1	1	3,9	1,3	0,33
AP226	7	1,3	3	2,4	0,5	0,79
AP238	6	1,8	3	2,3	0,7	0,57
AP243	11	1,2	6	2,4	0,5	0,81
AP249	11	3,6	1	5,2	1,6	0,28
AP273	4	1,8	1	2,1	0,7	0,56
AP288	7	1,7	2	3,2	0,8	0,59
AP289	40	10,7	5	9,9	2,9	0,09
HB-C16-01	40	16,8	2	11,5	3,2	0,06
HB-C16-02	35	3,7	10	7,2	2,2	0,27
HB-C16-05	5	2,8	1	3,1	1,1	0,36
MEAN	16,1	4,6	3,8	5,1	1,5	0,40

Table 8. Abundances of alleles per population.

	# of ALLELES	# of FREQ. ALLELES	# of EFFECT. ALLELES	# of PRIVATE ALLELES	# of LOCAL COMMON ALLELES
KIRKLARELİ	255	115	113	19	31
EDİRNE+	135	135	96	5	16
DÜZCE+	163	103	92	6	17
ESKİŞEHİR+	140	89	85	5	10
ANKARA	236	102	117	11	24
MUĞLA	245	102	123	14	27
ARDAHAN	209	89	101	13	19
ARTVİN	138	138	87	4	15
BİTLİS+	270	103	126	7	35
HATAY	287	101	130	26	32
MEAN	207,8	107,7	106,9	11,0	22,6

Table 9. Allelic diversities per population.

	INFO. INDEX	GENE DIVERSITY	ALLELIC RICHNESS
KIRKLARELİ	1,4	0,62	4,9
EDİRNE+	1,2	0,62	4,7
DÜZCE+	1,2	0,58	4,4
ESKİŞEHİR+	1,1	0,55	4,3
MUĞLA	1,3	0,55	4,6
ANKARA	1,3	0,58	4,8
ARDAHAN	1,1	0,50	4,2
ARTVİN	1,0	0,51	4,3
BİTLİS+	1,4	0,59	4,9
HATAY	1,4	0,60	5,1
MEAN	1,2	0,57	4,6

3.3 HW Test and Linkage Disequilibrium

A total of 10 loci out of 29 showed significant deviation from Hardy-Weinberg Equilibrium (shown as shaded and bold in Table 9). None of these deviations were due to heterozygosity excess, in each case the observed heterozygosity was lower than the expected.

Table 10. Observed and expected heterozygosities for each loci.

Locus	Obs. Het.	Exp.Het.
A007	0,90	0,95
A014	0,46	0,55
A028	0,24	0,25
A043	0,39	0,50
A079	**0,69**	0,74
A088	0,58	0,64
A107	**0,92**	0,93
A113	0,82	0,89
A(B)024	0,56	0,61
A(B)124	**0,73**	0,79
AC006	**0,15**	0,20
AC306	**0,64**	0,71
AP001	0,68	0,79
AP019	0,31	0,36
AP043	0,79	0,87
AP049	0,43	0,48
AP068	0,70	0,65
AP218	**0,06**	0,13
AP223	0,64	0,68
AP226	**0,17**	0,21
AP238	0,43	0,43
AP243	**0,16**	0,19
AP249	0,70	0,72
AP273	0,38	0,44
AP288	0,36	0,41
AP289	**0,66**	0,91
HB-C16-01	0,88	0,94
HB-C16-02	**0,59**	0,73
HB-C16-05	0,62	0,64

Linkage disequilibrium was not widely observed between loci. Only six combination of loci in three populations were significantly in linkage disequilibrium. Kırklareli had 2 pairs, Bitlis+ had 1 and Hatay had 3 pairs of loci in disequilibria. Table 10 shows the loci in linkage disequilibrium.

Table 11. Loci in linkage disequilibrium.

POPULATION	LOCI
KIRKLARELİ	1 & 12
KIRKLARELİ	16 & 22
BİTLİS+	27 & 29
HATAY	7 & 27
HATAY	15 & 20
HATAY	19 & 20

3.4 Bottlenecks and Effective Population Sizes

Only migratory colonies from Thrace seemed to show a significant bottleneck effect but the software gives a signal for a low number of samples and Hatay population was close to significance (p = 0,07) in terms of suffering a recent bottleneck. Effective population sizes for populations were estimated based on clusters. The clusters were Western Anatolia bringing together Middle West and West populations, Thrace, Bitlis+, Hatay and North East. Table 11 shows the estimated effective population sizes for the clusters.

Table 12. Estimated effective population sizes.

POPULATION	EFFECTIVE SIZE
THRACE	1860.3
WEST ANATOLIA	3500.0
NORTH EAST	776.6
BİTLİS+	4655.9
HATAY	665.0

3.5 Population Distances and Phylogenetic Trees

Pairwise F_{ST} values are among the most widely used population differentiation tests. F_{ST} values were calculated by using both the obtained frequencies in this study and by using the null allele corrected frequencies. For the stationary (n = 174) colonies FreeNA software

calculated an overall F_{ST} of 0,065 and an F_{ST} of 0,067 after null allele corrections. For migratory colonies the values were 0,011 and 0,015 respectively and for all the 250 samples the values were 0,046 and 0,047. There wasn't a much difference between the F_{ST} values obtained by the frequencies of the study data and the frequencies after null allele correction.

Besides the global F_{ST} values, pairwise F_{ST} values were also calculated. Table 12 displays these values for stationary colonies in addition to an estimation of the number of migrants in each generation between the populations whereas Table 13 and Table 14 display migratory and overall data. Shaded boxes are non-significant differentiation results. Rest of the populations were significantly differentiated from each other. No significant results were obtained from non-differentiation tests neither for stationaries nor migratories. But the case was different for isolation by distance test. Genetic distances in stationary colonies showed significant correlation with geographic distance but the genetic distances of migratory colonies were not correlated with geographic distances.

Table 13. Pairwise F_{ST} values between populations (stationary colonies) in the upper diagonal and number of migrants between populations in the lower diagonal

	KIRKLARELİ	EDİRNE+	DÜZCE+	ESKİŞEHİR+	MUĞLA	ANKARA	ARDAHAN	ARTVİN	HATAY
KIRKLARELİ		0,01	0,07	0,05	0,08	0,09	0,11	0,10	0,12
EDİRNE+	22,81		0,04	0,04	0,04	0,06	0,08	0,08	0,09
DÜZCE+	3,37	5,52		0,01	0,02	0,03	0,07	0,07	0,07
ESKİŞEHİR+	4,63	6,84	28,75		0,02	0,02	0,05	0,04	0,07
MUĞLA	2,78	5,42	10,34	15,87		0,02	0,04	0,04	0,05
ANKARA	2,59	3,84	7,30	11,31	16,08		0,03	0,03	0,05
ARDAHAN	2,04	3,01	3,50	5,14	5,91	7,11		0,00	0,09
ARTVİN	2,16	2,72	3,32	5,65	5,52	9,18	137,11		0,08
HATAY	1,83	2,41	3,34	3,13	4,51	4,43	2,53	3,06	

Table 14. Pairwise F_{ST} values between populations (migratory colonies)

	KIRKLARELİ	EDİRNE+	MUĞLA	ANKARA	BİTLİS+	HATAY
KIRKLARELİ	0,00					
EDİRNE+	-0,03	0,00				
MUĞLA	0,07	0,03	0,00			
ANKARA	0,07	0,02	0,02	0,00		
BİTLİS+	0,08	0,01	0,01	0,01	0,00	
HATAY	0,09	0,03	0,01	0,02	0,01	0,00

Table 15. Pairwise F_{ST} values between populations (complete data)

	KIRKLARELİ	EDİRNE+	DÜZCE+	ESKİŞEHİR+	MUĞLA	ANKARA	ARDAHAN	ARTVİN	BİTLİS+	HATAY
KIRKLARELİ	0,00									
EDİRNE+	0,01	0,00								
DÜZCE+	0,07	0,04	0,00							
ESKİŞEHİR+	0,05	0,03	0,01	0,00						
MUĞLA	0,08	0,03	0,02	0,01	0,00					
ANKARA	0,08	0,04	0,02	0,01	0,01	0,00				
ARDAHAN	0,11	0,07	0,07	0,05	0,04	0,04	0,00			
ARTVİN	0,10	0,08	0,07	0,04	0,04	0,03	0,00	0,00		
BİTLİS+	0,08	0,04	0,02	0,02	0,01	0,01	0,04	0,03	0,00	
HATAY	0,10	0,06	0,05	0,05	0,03	0,03	0,07	0,06	0,01	0,00

Population distances were used to construct phylogenetic trees. Both the null allele corrected and uncorrected distances were used. Also two different types of distance calculations were used as well as two different types of tree construction methods. Figures 4-7 display different types of trees constructed.

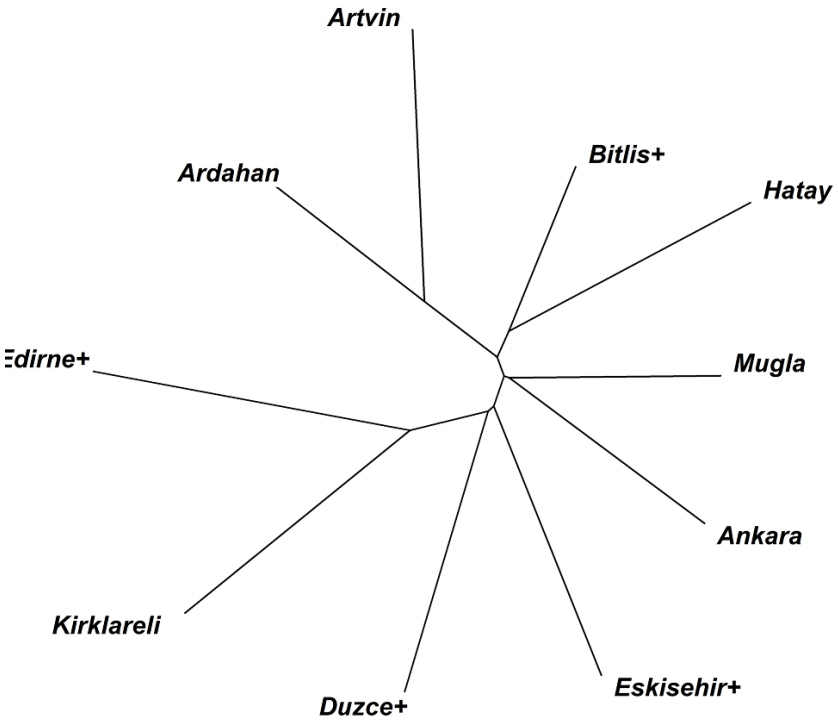

Figure 4. Neighbor Joining tree based on null allele corrected distances and Cavalli-Sforza and Edwards (1967).

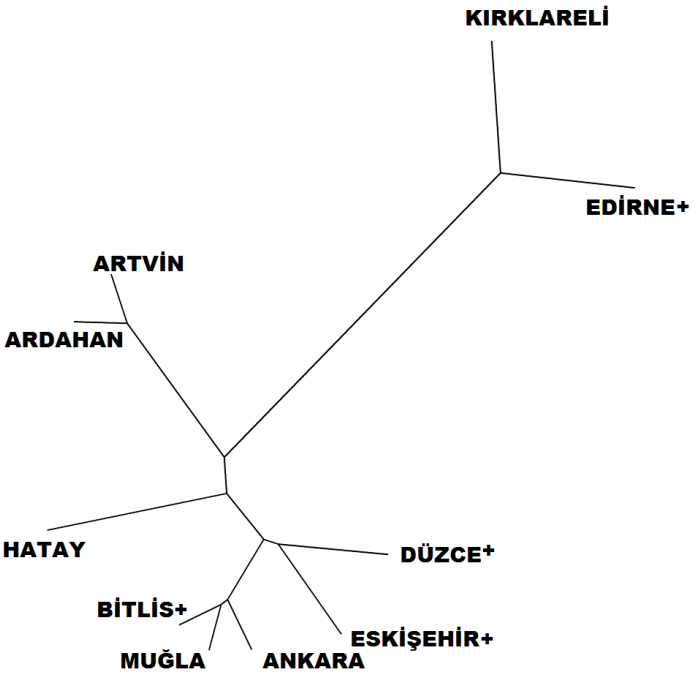

Figure 5. UPGMA tree based on Nei's genetic distance (1978).

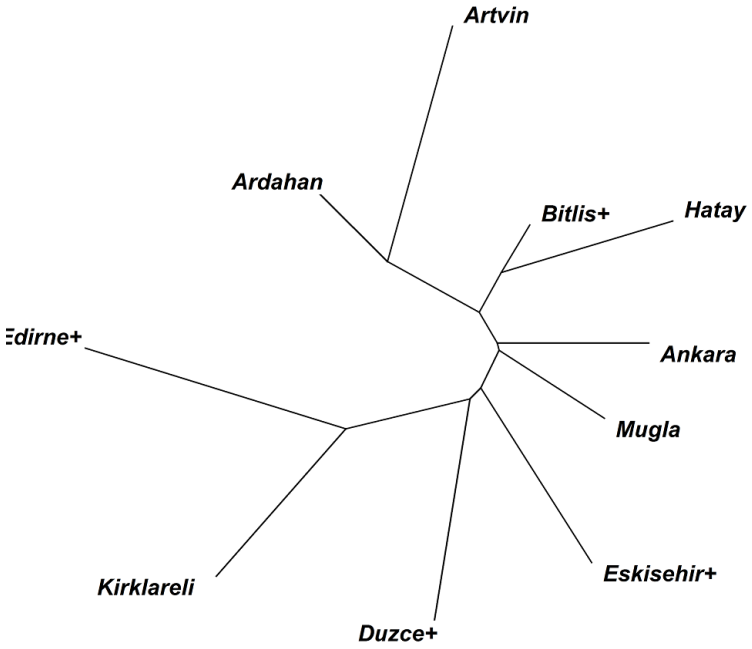

Figure 6. Neighbor Joining tree based on Nei's genetic distance (1983).

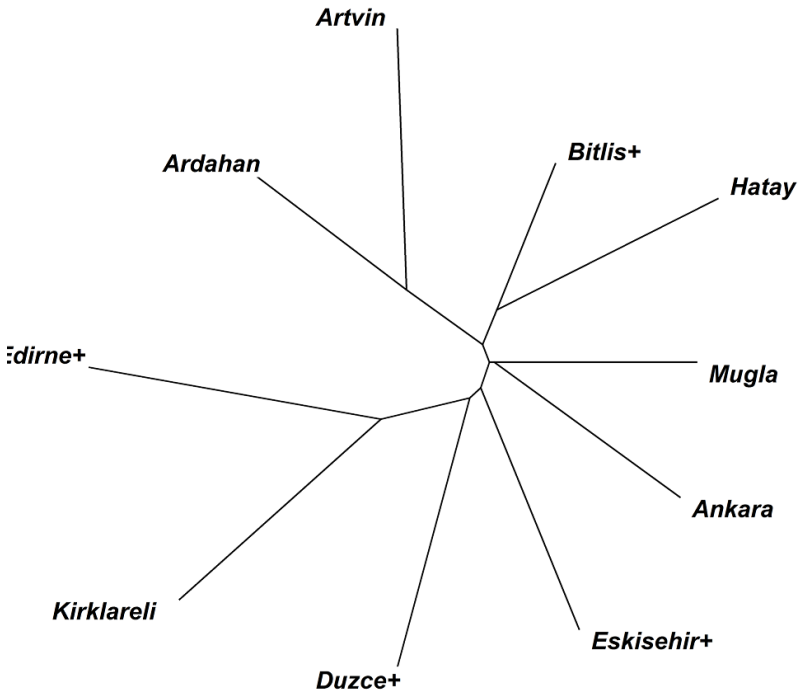

Figure 7. Neighbor Joining tree based on Cavalli-Sforza and Edwards (1967).

3.6 AMOVA

30 population and group level comparisons were made with AMOVA. In some of the comparisons four clusters were created composed of regions. Thrace region was the first cluster, Middle West and West regions were combined in the second cluster, North East region was the third cluster and finally Hatay was the fourth cluster. These clusters were assumed to correspond to distributions of four subspecies of "the Carniolan type", *A. m. anatoliaca*, *A. m. caucasica* and *A. m. syriaca* respectively.

There were no significant or notably high partitioning of variance biased towards the groups formed in many of the comparisons. Among them were the tests conducted to see if any of the West Anatolia populations were distinctly different from each other. No populations from Düzce+, Eskişehir+, Muğla and Ankara group were distinct from the rest. Pairwise comparisons of Thrace, Hatay and North East group with the West Anatolia group also did not produce significant tendency towards a weighted variance to the groups rather than the populations. Some of the notable results are shown in Table 15.

Table 16. AMOVA results. Significant results are indicated with *. Number of * correspond to level of significance.

GROUPING	VARIATION AMONG GROUPS	VARIATION AMONG POULATIONS WITHIN GROUPS
4 DISTINCT GROUPS	6,26***	1,23***
ANATOLIA ALL VS THRACE	5,68*	3,83***
THRACE VS HATAY	10,56	0,89
THRACE VS NORTH EAST	9,94	0,34
HATAY VS NORTH EAST	8,91	-0,25
HATAY+WEST ANATOLIA VS NORTH EAST	2,34***	3,29*
THRACE+DÜZCE+ESKİŞEHİR VS ANATOLIA REST	3,63***	4,39*
THRACE+DÜZCE VS ANATOLIA REST	3,9*	4,37***

3.7 Ordination of Populations

Stationary colonies, migratory colonies and the overall data was plotted on 2D and 3D spaces by carrying out Principle Component and Factorial Correspondence analyses. Figures 8-10 correspond to Principle Component Analysis (PCA) whereas Figures 11-13 correspond to Factorial Correspondence Analysis (FCA).

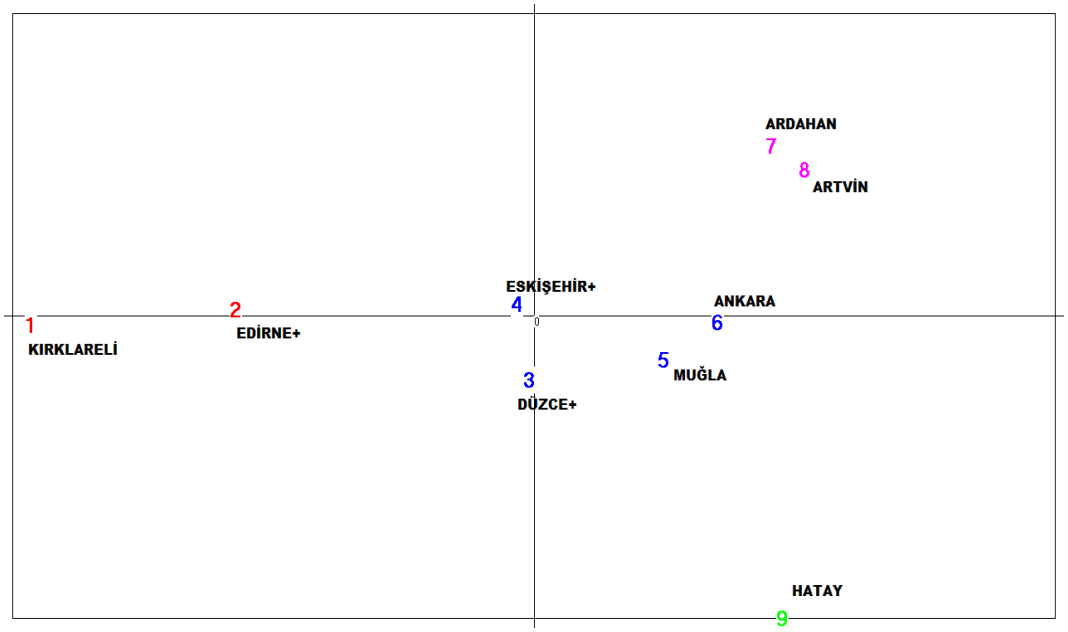

Figure 8. PCA of stationary colonies. Axis 1: 43,19%. Axis 2: 21,87%.

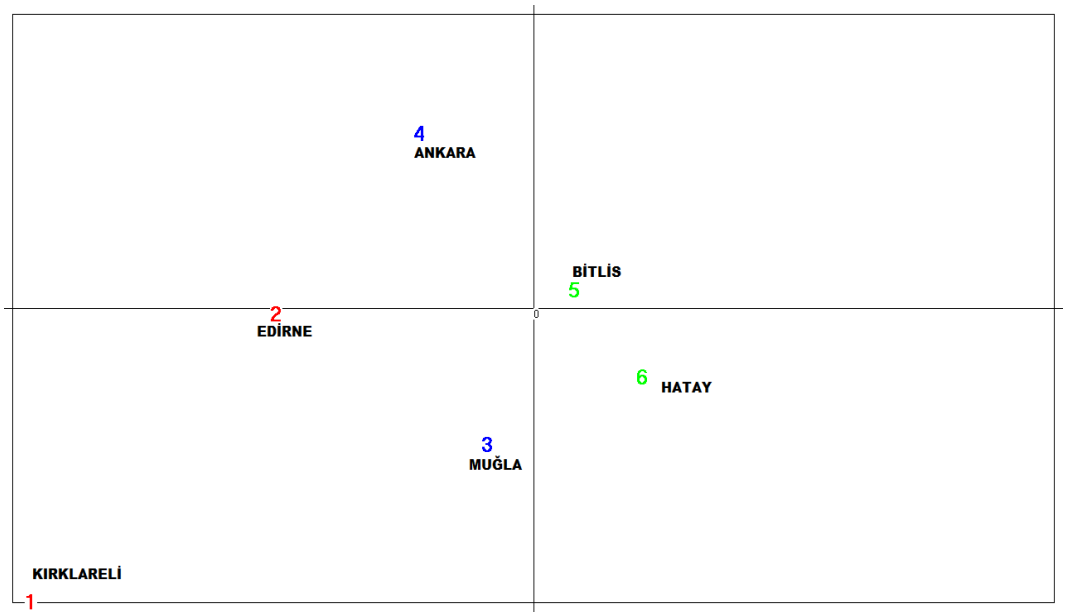

Figure 9. PCA of migratory colonies. Axis 1: 28,21%. Axis 2: 20,98%.

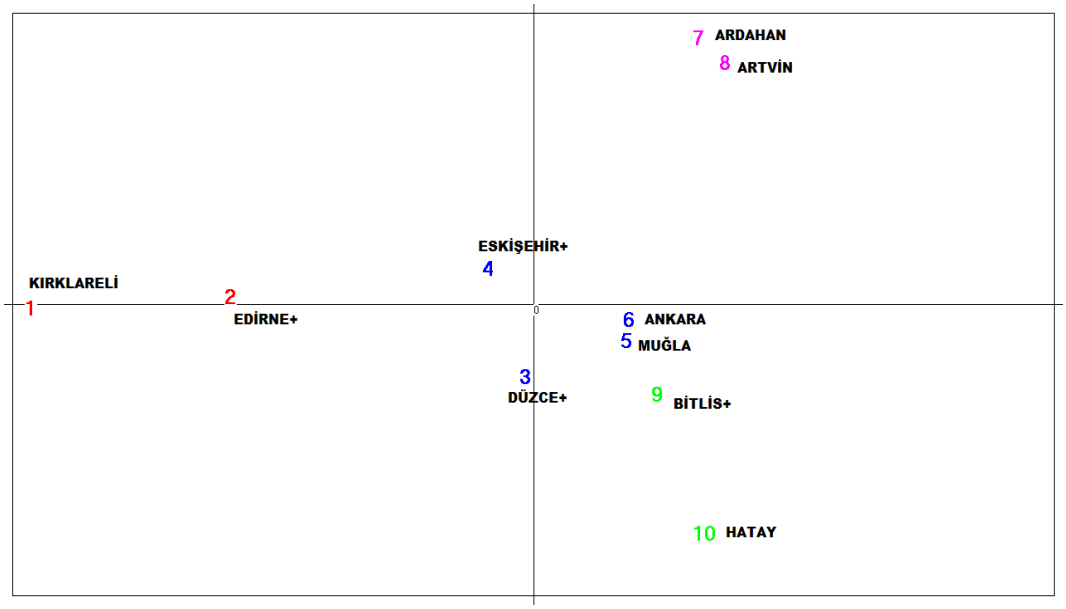

Figure 10. PCA of overall data. Axis 1: 43,16%. Axis 2: 22,12%.

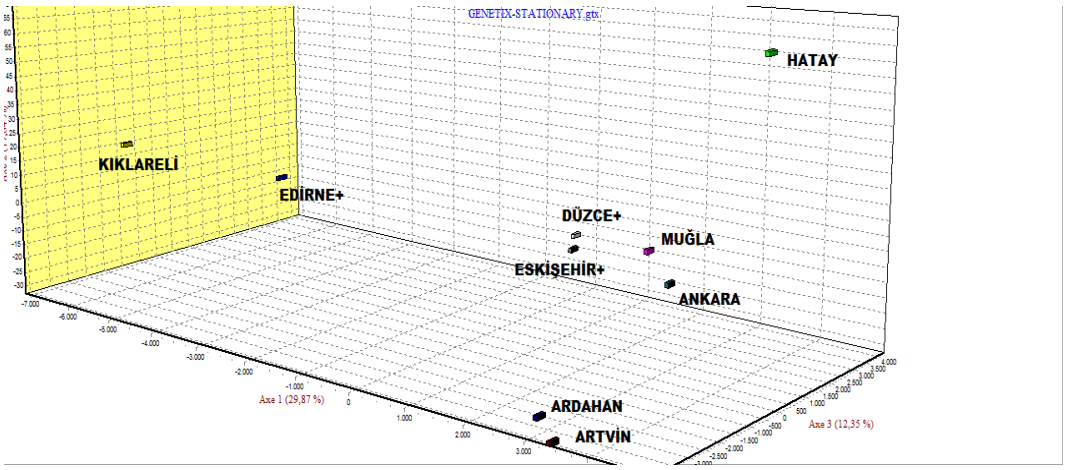

Figure 11. FCA of stationary colonies. Axis 1: 29,87%. Axis 2: 17,84%. Axis 3: 12,35%.

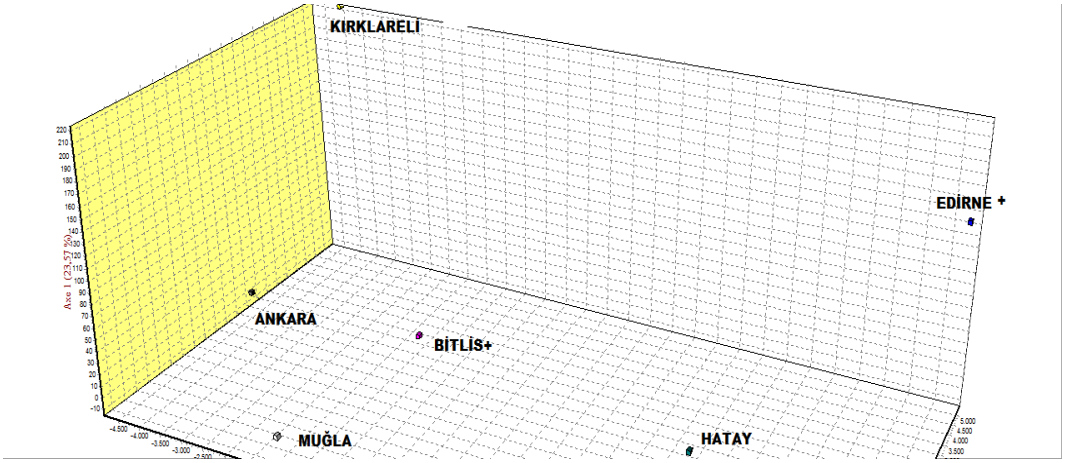

Figure 12. FCA of migratory colonies. Axis 1: 23,57%. Axis 2: 21,82%. Axis 5: 16,15%.

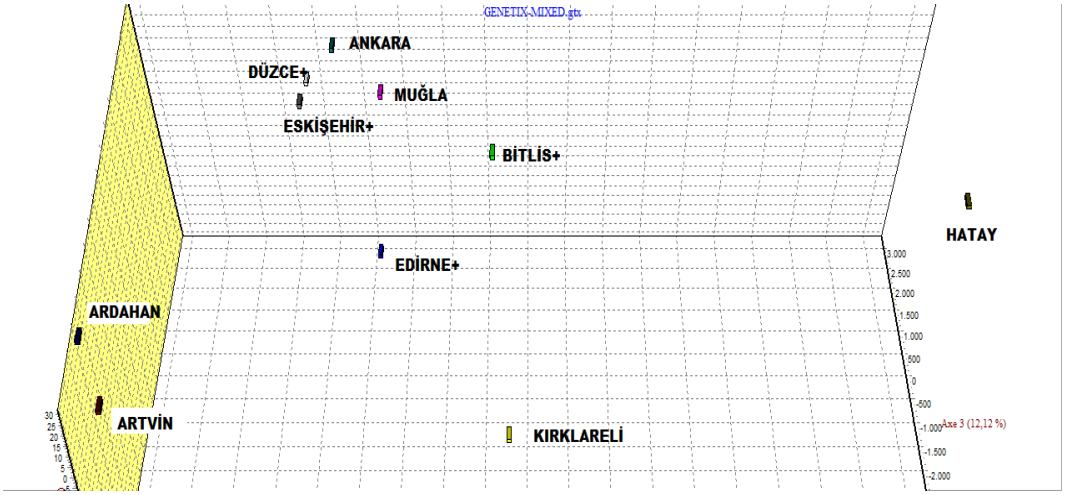

Figure 13. FCA of overall data. Axis 1: 29,93%. Axis 2: 15,68%. Axis 3: 12,12%.

3.8 Population Structure

The last step for the analysis of population structure started with estimating the most probable number of populations. So the Structure program was run through different K values of 1 to 15. These initial runs give an idea about the distribution of K likeliness's. Figures 14 and 15 are the graphical representations of Ks and their standard deviations.

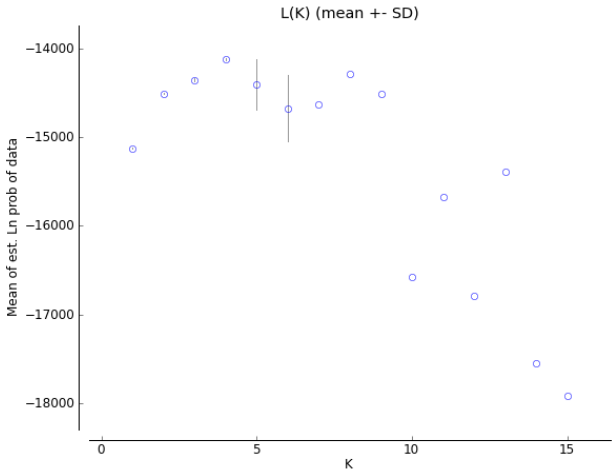

Figure 14. Ln (probabilities) for K values 1-15.

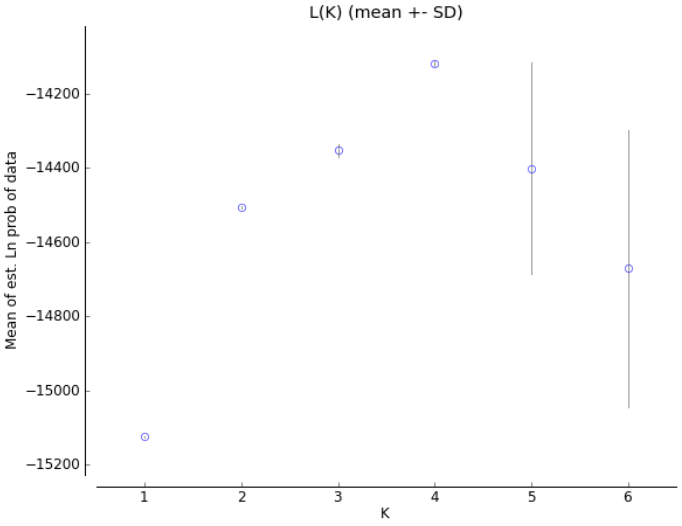

Figure 15. Ln (probabilities) for K values 1-6.

The best K values were then selected by the Structure Harvester program. K = 2 (2 distinct clusters) and K = 4 (4 clusters) were the best K values obtained by the algorithm. Figure 16 and 17 show the sequential steps in the decision process of the algorithm, each of the results from those steps can be an estimator of K.

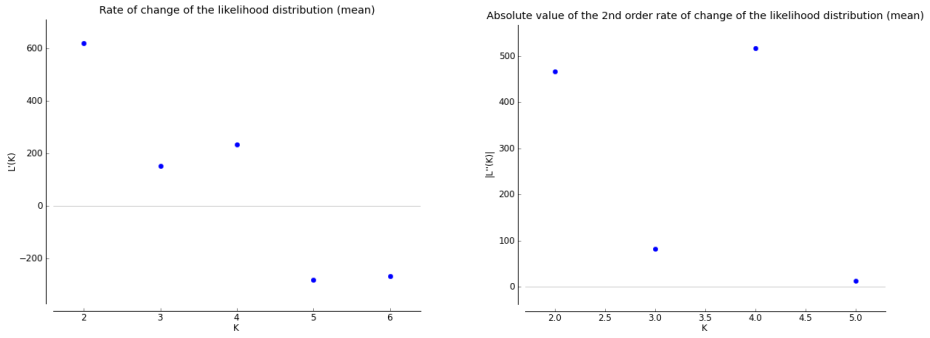

Figure 16. Rates of change in the likelihood of Ks.

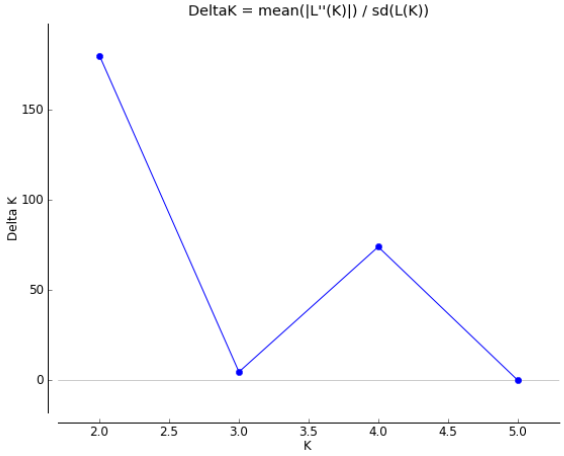

Figure 17. DeltaK values.

After assuming K as 4, the membership coefficients for individuals were determined and then permuted for consistency. Results for stationary and migratory colonies as well as the overall data were presented in Figures 18-20.

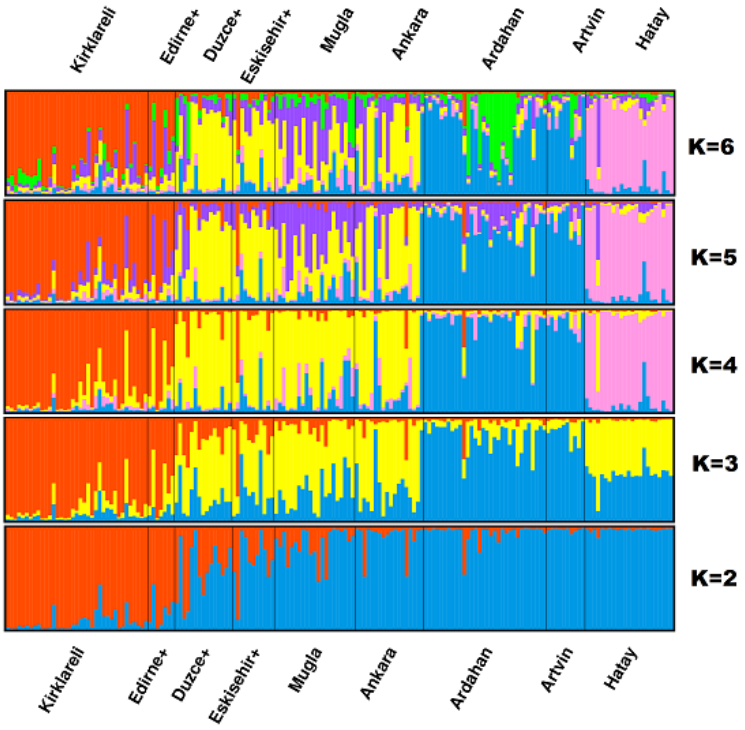

Figure 18. Stationary colonies cluster assignments.

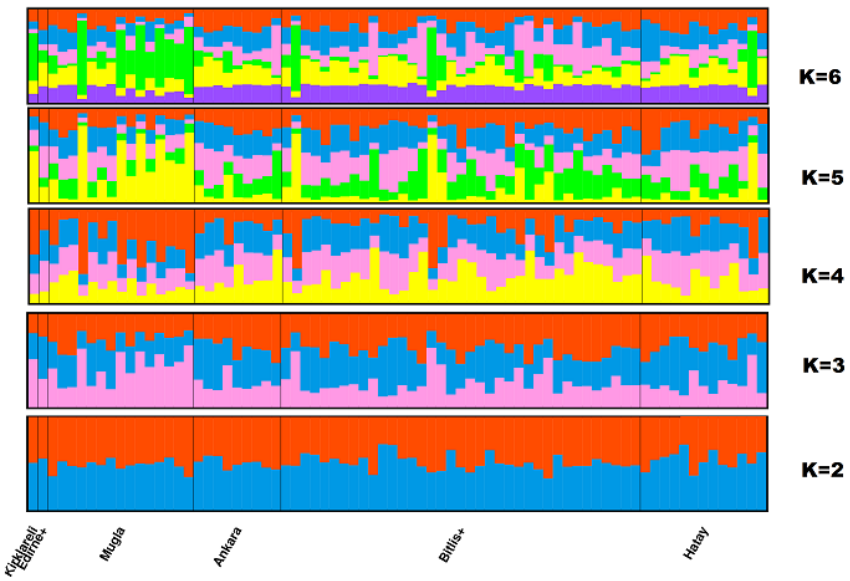

Figure 19. Migratory colonies cluster assignments.

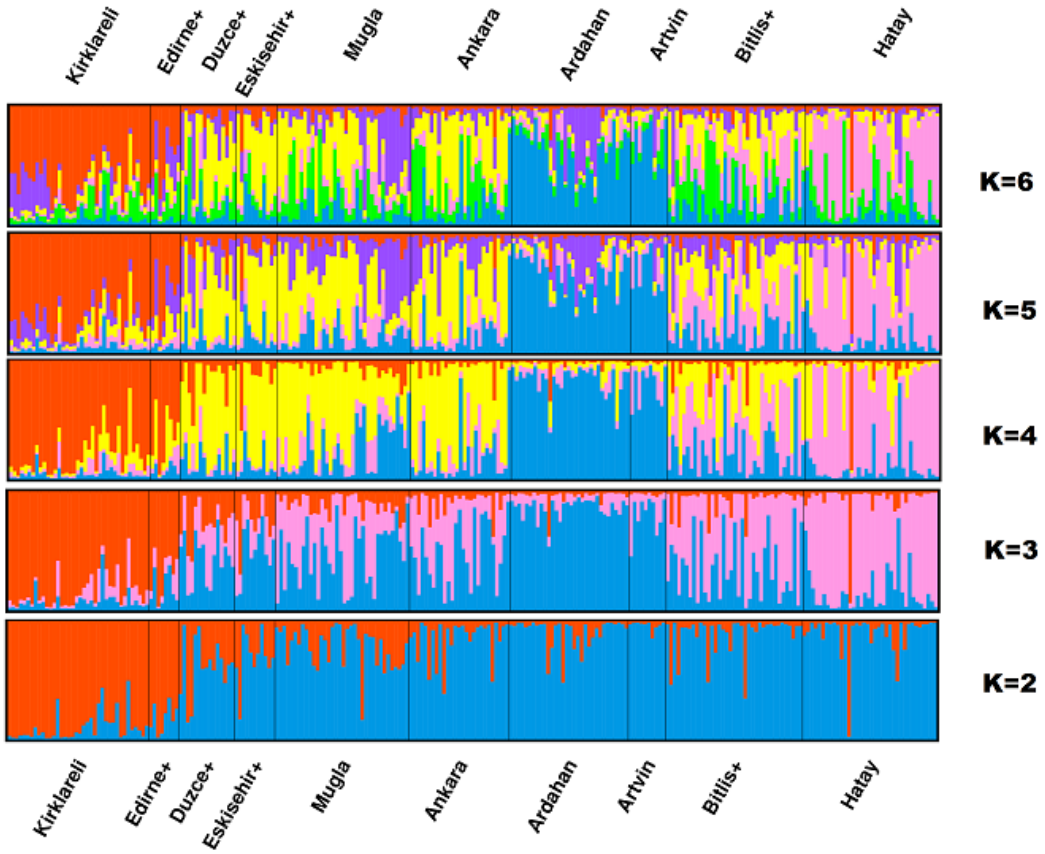

Figure 20. Overall data cluster assignments

3.9 Beekeeping, Isolation, Trade

Four clusters selected by the Structure program and the populations that are majorly assigned to these clusters correspond to the expected geographical distribution of the four subspecies. Without this biological interpretation one can still test the hypotheses about beekeeping, isolation and trade by assuming that these selected clusters are the natural populations of individuals. In other words it can be assumed that a colony from Kırklareli would normally belong to the orange cluster and pink cluster is the native population of a colony from Hatay. If an individual bears more than one color then this means that the individual may be assigned to each of the clusters with a certain probability, actually it can be said that this individual is a hybrid in biological terms.

Based on this assumption individuals from stationary and migratory colonies were compared according to their membership coefficients to their native clusters (or natural populations meaning the same). Stationary colonies from Muğla and Hatay were quite

more likely to be assigned to their own clusters than the migratory colonies from these provinces, the same holds when the data from the three provinces are combined and when the overall migratory and stationary colonies were compared. The situation is reversed in Ankara. Table 16 summarizes the average assignment probabilities for the groups compared and the significance of the contrast.

Table 17. Average membership coefficients to native clusters (beekeeping).

	MIGRATORIES	STATIONARIES	AVERAGE ARCSINE (ASSIGNMENT) MIGRATORIES	AVERAGE ARCSINE (ASSIGNMENT) STATIONARIES	SIGNIFICANCE
MUĞLA	15	21	0,46	0,71	**
ANKARA	9	18	0,95	0,61	**
HATAY	13	23	0,45	1,06	***
COMBINED	37	62	0,58	0,81	**
OVERALL	76	174	0,62	0,88	***

Based on the same assumptions isolated (Kırklareli, Ardahan, Artvin) and not isolated (Edirne+, Muğla, Ankara, Düzce+, Eskişehir+, Hatay) regions were compared in terms of their membership coefficients to native clusters. Table 17 summarizes the results.

Table 18. Average membership coefficients to native clusters (isolation)

	SAMPLE SIZE	AVERAGE ARCSINE (ASSIGNMENT)	SIGNIFICANCE
ISOLATED	79	0,99	***
NOT ISOLATED	95	0,79	

Even if the individuals are assigned with high probability to their own clusters, let's say with a 90% of probability, this means that 10% of their genome still belongs to other clusters. Given there are four clusters it was investigated if any of these clusters were over represented in the genome of individuals. This time "mis"assignment probabilities were compared in between clusters. Table 18 summarizes the results.

Table 19. Average membership coefficients to non-native clusters

	ASSIGNMENT TO ANATOLIAN CLUSTER (A)	ASSIGNMENT TO THRACIAN CLUSTER (T)	ASSIGNMENT TO CAUCASIAN CLUSTER (C)	ASSIGNMENT TO SOUTHERN CLUSTER (S)	TOTAL "MIS"ASSIGNMENT PROBABILITY	COMPARISON BTW C vs A	COMPARISON BTW A vs S
KIRKLARELİ	0,097		0,032	0,030	0,159		
EDİRNE	0,302		0,044	0,035	0,381		
DÜZCE+		0,098	0,078	0,034	0,210		
ESKİŞEHİR+		0,119	0,122	0,040	0,281		
MUĞLA		0,034	0,154	0,102	0,290		
ANKARA		0,038	0,227	0,083	0,348		
ARDAHAN	0,079	0,026		0,041	0,147		
ARTVİN	0,061	0,011		0,068	0,139		
HATAY	0,072	0,012	0,068		0,151		
MEAN	0,0964	0,0415	0,0976	0,0527			
SIGNIFICANCE						ns	*

Genome parts were more frequently “mis”assigned to Caucasian cluster than they were assigned to all others but the difference between it and the frequency observed with the Anatolian cluster was not significantly different. These two clusters (Anatolian and Caucasian) had significantly higher values than Southern and Thracian clusters.

CHAPTER 4

DISCUSSION

Random sampling and transect methods were not used in this study because of mainly three reasons: first this study was conducted as a part of a wider research where the aim is to find out any relation between the genetic diversity and the occurrence of honey bee diseases at five regions in Turkey where there is intense beekeeping and different subspecies, secondly this study did not aim to bring together all the consequences of genetic structuring and diversity present in Turkey; and finally such a sampling strategy would pass beyond the financial limits of the present study.

Considering the high biodiversity harbored in Anatolia and Thrace more detailed studies should be conducted with higher number of samples and higher number of sampling sites. The possible regions falling into *A. m. meda* subspecies' distribution range should be included in the future studies to better monitor the status at the subspecies level. Samples from Bitlis and Elazığ might be considered in or near the distribution of *A. m. med*a but our samples from these regions were not from stationary colonies but from migratory ones, so we couldn't detect a distinct group here but there were some signals like a high number of locally abundant alleles in Bitlis+ samples.

Also geographic positioning of the samples can be added to analyses later. Villages where the samples were taken were recorded so altitudinal and coordinates data might be more efficiently used in further analyses. Implementing geographical data with more advanced techniques like GIS (Geographic Information Systems) is also possible.

The provinces where the samplings too place were sometimes gathered in localities where a few provinces were combined resulting in ten of such localities. These combinations were carried out according to geographical proximity; similarity in terms of climatic, topographic and floral variables; results of previous studies as well as results of preliminary analysis of this study.

Multiplex PCR was chosen as the amplification strategy of this study since it lets one to have higher resolution with a lowered cost. Genotyping is a costly process and it increases efficiency of genotyping when loci are combined. Combining the loci after the reactions were another strategy but it was not favored it increased the time necessary for

carrying out the amplifications and the costs associated with it. Increasing the loci rather than the sample size was also a conscious choice. With a few number of loci one may not detect the structuring due to limited resolution of the loci under study even if the sample size was increased. On the contrary side a high number of loci used was a protection against a small sample size by increasing the discrimination capacity. Pre-optimization for combination of primers were not carried out widely, like testing for necessary amounts of each primer used since fragment analysis for optimization would increase the costs. Rather a certain risk was taken to exclude some of the loci that did not amplify.

The case with A076 seems different. The loci did amplify in trials and controls on agarose gel electrophoresis so it may not be due to null alleles. The reason behind may be related with dilutions. Many times to avoid very strong signals the PCR products were diluted since a strong signal from one dye usually overshadowed signals from other dyes. During these dilutions weakly amplified loci may be lost. So the loci can be kept in the microsatellite data set presented here for further analysis by developing a method for increasing its amplification.

An important difficulty that affected the study was the leaking and cross-contamination of some of the samples. Microsatellite loci are used for genotyping individuals from mixed samples. So from comparison of relative peak heights to increasing dilutions and adjusting peak detection settings a number of methods were applied to differentiate between contaminants and the original sample. One can check the validity of the genotyping from many results obtained throughout the study. First of all, the null allele frequencies were generally very high even with the ones as high as 40% as presented in the related table. Also deviations from HW equilibrium were always towards a heterozygosity deficiency not excess. If there was enough “gene flows” from sample to sample then one would expect to see the reverse, more heterozygotes would be obtained than expected.

Also despite lack of a high number significant linkage disequilibria among loci the population structure was very robust, differentiating samples easily according to their origins which would not be the case if there was a high mixing up to affect the results. Phylogenetic trees presented here obtained better resolutions and more consistent results in terms of distribution ranges of subspecies and connectivity of populations than the previous studies’ of Bodur et al. (2007), Tunca (2009) and Yıldız et al. (2010).

Despite very high frequencies in some loci and some populations null alleles did not bother the results a lot. This can be seen from a few cases like comparison of null allele corrected and original data obtained. Corrected and not F_{ST} values show little difference, trees constructed by corrected allele frequencies also created no or very slight modifications. The excess homozygosity may not be actually related with the null alleles but it may be related with the high inbreeding rates of some populations.

During the kinship analysis no closely related individuals were detected, no full-siblings were present, so one can say that such bias were not present in the study. Checking the validity of data was an important part of the study to infer the reliability.

Private alleles with high frequencies (5%<) may not be true for localities with small sample sizes, especially for samples of Edirne+ and Artvin since a single copy would exceed the threshold, but they still need to be considered because a high number of private alleles were detected for both population even with a small sample size.

Some of the loci had very high number of alleles and private alleles as well as locally abundant alleles and a high diversity index, but some of the loci seemed far from explainability failing for all the criteria: low heterozygosity, low number of alleles and low number of private alleles, like Ap273. Some of the loci with low diversity indices had high number of private alleles like loci Ap 218, Ap226, Ap238 and Ap243. Cumulative effect of such alleles might be contributing to high resolution obtained even their individual diversity indices were low.

Kırklareli, Bitlis+ and Hatay populations possessed a very high number of locally abundant alleles. Bitlis+ samples were not differentiated but this high number can be a clue of some unique genes and genotypes present there. A detailed study with stationary colonies should be conducted. Kırklareli and Hatay also had many private alleles when compared to other populations pointing to their divergence and high value as a unique biodiversity resources for Anatolian and Thracian populations. The numbers may be lower in Caucasian samples despite their high differentiation due to bee trade since their alleles might now be scattered the rest of the populations and lost their uniqueness.

No bottlenecks were detected for any populations and beekeeping practice groups. Thracian migratory bottleneck can be an artifact of low sample size (n = 2). A more detailed study with a higher sample size might be needed for checking the near significant results in Hatay samples. The effect of high winter losses in the previous years would be overshadowed by the recent increase of the number of colonies being managed. Effective size estimations may not hold due to problems related with haplodiploidy of the species but may be they can be used as a relative measure of population sizes. No studies were existent with microsatellite data making use of linkage disequilibrium for estimating effective population sizes (SNP data were abundant) so the results should be carefully evaluated to avoid any mistakes.

F_{ST} values obtained were highly significant but they were lower than what Bodur *et al.* (2007) estimated -a global F_{ST} of 0,077 and also higher values for pairwise comparisons among populations- with the samples ten years older than ours. This may indicate a recent

increased gene flow and can be an alarm signal for a trend. Similar studies should be actualized to see if there is such a trend. Number of migrants per generation are very low except Düzce+ & Eskişehir+, Edirne+ & Kırklareli and Ardahan & Artvin population pairs where pairs are adjacent to one other. The high degree of structuring was lost in migratory colonies according to F_{ST} results, meaning they are less differentiated from each other.

NJ trees placed Bitlis+ samples with Hatay but the UPGMA tree grouped them with Middle and Western Anatolia samples. This might be again related with the migratory colonies used, reflecting their status of representing both the local unique combinations – which may also include *A. m. meda* contributions- and general trends found in other populations. Stationary colonies would probably give a different result. Also Bitlis+ locality brought together geographically distant samples -even if the number of such samples were low- and this would have played some role in obtaining such results.

Thracian samples were completely distinct from others pointing to an early division of populations and limited gene flow. This supports the hypothesis for a Carniolan (C-lineage) descent of Thracian bees in Turkey. This hypothesis may also be supported by microsatellite alleleic data if it would be shown that alleles frequently observed in C-lineage were also present in Thracian honey bees. A more detailed data mining is necessary to show such a similarity between allele frequencies observed. Actually making use of samples from the major C-lineage subspecies would confirm the subspecies which these highly differentiated (from Anatolian samples) bees belong. West Anatolian, Hatay and Caucasian populations were also easily discriminated.

AMOVA results confirmed highly significant differentiation of populations studied into 4 clusters. Besides this, variance between groups stayed higher than variance between populations when Thracian samples were grouped together against Anatolian samples pairwise or as a whole. It hasn't possible to evaluate why the results were not significant in pairwise comparisons of North East and Hatay populations with Thrace depite extremely high levels of partitioning of variance between groups. Hatay samples according to AMOVA results seemed to be more closely related to West Anatolian populations then they were to North East populations. Including Düzce+ and Eskişehir+ populations in Thrace group lowered the percentage but did not change the significance status pointing to a considerable level of gene flow to Düzce+ and Eskişehir+ populations from Thrace.

FCA and PCA results also confirmed the 4 different clusters those were inferred from tree topology but also showed the differentiation between migratory colonies were very low when compared to the whole set and stationaries. Bitlis+ was resided with Middle and West Anatolian populations.

The two most possible K values in structure analysis were K = 2 and K = 4, both results supporting the hypotheses of populations belonging to 2 separate lineages and 4 distinct subspecies. Results of migratory beekeepers samples lacked almost any population structuring further clarifying the highly hybridized status of migratory apiaries. Overall data showed that Bitlis+ samples were highly admixed with major contributions from *Apis mellifera syriaca*, *A. m. caucasica* and *A. m. anatoliaca*.

Stationary apiaries, as expected, yielded highly structured groups where all the subspecies could be detected. When K was 2, the structure analysis of two distinct clusters showed that there was a transition zone between Thracian and Anatolian samples around Marmara Sea and Aegean. This may be a hybrid zone between the C and O lineages like the ones identified before between M and C lineages in Alps and Apennine Peninsula and between A and M lineages at the Iberian Peninsula and Mediterranean islands. When K was considered as 4 all the four subspecies were easily differentiated from each other, in accordance with the previous studies. The significance of two distinct clusters (K = 2) was higher than four (K = 4). The differences between the populations belonging to C (Thracian) and O (Anatolian) lineages are more clear-cut than differences between the populations of four different subspecies. *A. m. anatoliaca* samples fell in the middle of the other subspecies in ordinations and phylogenetic trees, being similar to all other populaions according to F_{ST} values and as well as being a distinct cluster in structure analysis which may point to a significant historical contribution to Anatolian populations from the neighboring regions. This was different than what was observed in all-migratory Bitlis+ samples where a mixture of different gene pools was observed instead of a distinct identity.

When the K value was increased to 6 in stationary colonies, further structuring of Muğla and Ardahan bees was observed which can be an important point to investigate the Anatolian honey bee diversity in detail. Muğla bees have different life-history traits compared to other Anatolian bees although they have the same genetic make-up with the rest of the Anatolian bee populations. Since now no special clustering of Muğla bees were shown despite their morphological, behavioral and annual life cycle differences with other bees.

In addition to its potential implications for conservation purposes, this study also contributes to the understanding of phylogenetic relationship between the populations in Anatolia. A better understanding can be developed if populations neighboring Anatolia and Thrace were also sampled. This can be a direction for future research too for shedding light on the complicated taxonomic status within and between the C and O lineages.

Results from many analyses conducted here confirmed the presence of clusters but also they pointed to the status of migratory colonies: they might be acting as a hybrid zone mobile in space and time, being at one region in spring and at others in summer and fall,

becoming vectors of local gene combinations. Statistical results confirmed the significant gene flow towards the migrants from local bees. A significant gene flow towards local bees was also observed in the comparison between isolated and not-isolated regions. This result is also pointing to the vitality of establishing areas away and free from migratory beekeeping for preservation of honey bee genetic diversity.

One interesting point in those was that the trend of the migratory colonies in Ankara. They had a significantly higher probability of being assigned to their own clusters. This may be related with the regions they visit during their migratory cycle or it may be due to queen bee replacement by stationary beekeepers. The low assignment degree of stationary colonies in Ankara may also be related with Kazan apiary of TKV (Development Foundation of Turkey) placed there where hundreds of colonies of Caucasian bees are raised and sold around for more than 30 years. The same practice is also carried out by many queen bee breeders in Kazan region. Gene flow through these apiaries and queen bees distributed locally by trade contribute to such an admixture observed in stationary colonies in Ankara.

Honeybees from stationary colonies were assigned more often to their native clusters but they were also assigned to other clusters with lower probabilities. Honey bees assigned to Caucasian cluster more often than they were assigned to others. This is probably because wide distribution of Caucasian queen bees by trade. Migratory beekeeping is not carried out in Ardahan and Artvin where Caucasian bees are native. This introgression could mainly be attributed to queen bee replacement. It can be seen that this practice is also swamping the gene pool of honey bees in Turkey. Levels of average Caucasian introgression of as high as 22% were observed in Ankara. *A. m. anatoliaca* alleles also showed high introgression especially to Edirne+ of Thrace as high as 30% but also around 5-10% in other regions. These high levels may be related to its geographical proximity to other populations or the wide practicing of migratory beekeeping in Western and Central Anatolia.

Results of the many statistical tests carried out and analysis applied in this study clearly showed that the genetic structure of honeybee populations in Turkey were highly conserved and still maintained. But that doesn't mean that the structure and diversity observed is secure. Rather it should be considered under threat since the anthropogenic factors leading to gene flow are still functioning and mixing the populations as can be seen from the data and figures presented here. What is more interesting was that, the

preservation of structuring was achieved despite a very high number of colonies moved from one location to the other and despite unregulated and frequent queen and colony sales. Future research may also need to focus on how this biodiversity and structuring was preserved.

Importance of establishing isolated regions was highlighted with genetic data. The results of the statistical tests showed a significant difference between the conservation of identity in and out of isolated regions with isolated regions staying purer. Such isolated regions were shown to be effective in conservation of unique diversity present in the populations there. In the light of this study we propose establishing such regions where migratory beekeeping is strictly prohibited as well as replacement of queen bees with non-native ones. However these isolated regions should be wide enough with additional buffer zones for efficient isolation and sufficient effective population size.

Queen bee trade is not currently subjected to any restriction or regulation. Restriction and regulations should be applied to avoid extinction of native races, ecotypes and diversity present in these populations. Genetic similarity of donor and recipient populations should be considered while determining migration routes for migratory beekeepers and determining permissions for queen and colony trade.

Central and western Anatolian populations suffer from significant gene flow from Caucasian populations. Special consideration should be taken for preserving *Apis mellifera anatoliaca* subspecies. The case with Hatay populations can be worse since the migratory beekeeping practice is heavily carried out in the region and queen bee replacement with non-native races is frequent. *A. m. syriaca* colonies can be very limited to a few points and apiaries. A conservation program should be actualized immediately. Thracian populations show a significant differentiation from the rest of the bees in Anatolia but the subspecies which they belong to is not characterized on a strong basis yet and this unique population is not registered officially. Identification and registration should be put into practice as soon as possible. An improvement based on molecular genetic techniques can be applied to the conservation programs going on for the *A. m. caucasica* subspecies. This holds for other subspecies too. More attention should be paid to genetically characterize *A. m. meda* subspecies which can be under threat due to migratory beekeeping and queen bee replacement.

Rather than queen bee replacement locally adapted native bees should be improved for desired characters. Such improved breeds should be used locally and not distributed country-wide. So that local adaptations can be preserved while bees are selected for resistance to pests and pathogens, hygienic behavior, reduced aggressiveness, reduced tendency for swarming, higher winter survival, higher productivity and for increased

pollination. For obtaining better results research concerning the smoothing, development and extension of breeding locally adapted native bees and artificial insemination techniques should be given higher priority.

CHAPTER 5

CONCLUSIONS

1. Genetic structure is observed in stationary colonies while no structure is observed in migratory ones in contrast to the general belief that honey bee populations in Turkey are totally admixed due to anthropogenic factors. Any trend in terms of loss of population structure should be investigated with future research with higher number of samples and a wider cover area which may include neighboring regions to Anatolia and Thrace.

2. Thracian bees are genetically distinct from the Anatolian bees. They may belong to the C lineage whereas Anatolian bees are from O lineage. Western Anatolia and Marmara regions may be a transition zone in between two.

3. Four different subspecies still maintain their identity even if they are under threat and may be limited to a tighter range than the past. Immediate and concrete steps should be taken for registration and preservation of the Thracian, Anatolian and the Hatay populations. Molecular genetic approaches should become a part of current conservation programs for Caucasian bees as well as others. More attention should be paid for genetic characterization of *A. m. meda* subspecies.

4. Substructuring within distribution ranges of subspecies is also observed pointing to local differences within subspecies which can house a vast and important diversity considering the environmental heterogeneity of the whole region under scope.

5. Colony trade, queen replacement and migration routes of migratory colonies should be regulated in order to prevent the gene flow between populations and conserve the genetic structure and diversity present.

6. Wide isolated regions should be established to reduce the effect of migratory beekeeping and sustain a healthy population as large as necessary to conserve as much diversity as possible.

7. Breeding of locally adapted native bees and improving them for desired characters should be paid more attention than selling queen bees from a few sources to the whole country.

8. Research concerning the smoothing, development and extension of breeding locally adapted native bees and artificial insemination techniques should be given higher priority.

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