FOUNDER EFFECT
IN REINTRODUCED ANATOLIAN MOUFLON
OVIS GMELINII ANATOLICA VALENCIENNES 1856 POPULATIONS

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ABSTRACT

FOUNDER EFFECT
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Reintroduction of Anatolian mouflon population at Bozdağ Protection & Breeding Station to its former habitats (Emremsultan Wildlife Development Area in Ankara-Nallıhan, and Karadağ in Karaman) started in 2004. The magnitude of genetic change among Bozdağ and reintroduced populations was evaluated by 11 microsatellite loci. Study populations revealed close results (±st.dev.) – Bozdağ population: n_k = 2.9091 (±1.1362), A_E = 2.0250 (±0.9537), H_o = 0.3830 (±0.2717), H_e = 0.3956 (±0.2746); Nallıhan population: n_k = 2.9091 (±1.1362), A_E = 2.0592 (±0.9451), H_o = 0.4086 (±0.2977), H_e = 0.4052 (±0.2767); and Karadağ population: n_k = 2.5455 (±1.1282), A_E = 1.8809 (±0.8758), H_o = 0.3388 (±0.2775), H_e = 0.3607 (±0.2716). Population differences for major genetic parameters were not significant ($p > 0.05$) by comparisons with paired *t*-test. Also, temporal change in genetic diversity for Bozdağ population was investigated by comparison with temporal data. Temporal changes in genetic parameters were found to be not significant and possible causes for differences were argued. Additionally, genetic diversity and PI computations for different traps were verified and compared to uncover any potential bias due to the catching method. Comparisons did not reveal significant differences

illustrating the homogeneity among traps. On the other hand, simulations detected the higher sensitivity of allelic diversity (*A*) to founder events than *P* and heterozygosity (H_o & H_e) levels which supports heterozygosity excess method for bottleneck analysis. With the same simulation analysis, observed genetic diversity within reintroduced samples were found to be in the ranges of expectation (99% CI) indicating that translocated individuals were chosen randomly. Bottleneck analysis based on heterozygosity excess method (one-tailed test for heterozygosity excess: p_{SMM} = 0.28515, p_{TPM} = 0.06445, p_{IAM} = 0.02441) and allele frequency distributions method (normal L-shaped) could not detect a recent genetic bottleneck for Bozdağ population. However, simulations determined that these two methods are prone to type II error. Bottleneck detection failure for the study population is probably due to type II error instead of other sources of error like violations of model assumptions.

Keywords: Anatolian mouflon, *Ovis gmelinii anatolica*, reintroduction, conservation genetics, microsatellites, founder effect, population bottleneck

ÖZ

YENİDEN AŞILANAN ANADOLU YABAN KOYUNU
OVIS GMELINII ANATOLICA VALENCIENNES 1856
TOPLUMLARINDA KURUCU ETKİSİ

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Bozdağ Yaban Hayatı Koruma Sahası'nda bulunan Anadolu Yaban Koyunu toplumunu önceki yaşama ortamlarına (Ankara-Nallıhan'da bulunan Emremsultan Yaban Hayatı Geliştirme Sahası ve Karaman'da bulunan Karadağ) yeniden aşılama çalışması 2004 yılında başlamıştır. Bozdağ toplumu ile yeniden aşılanan toplumlar arasındaki genetik farklılığın derecesi 11 mikrosatelit lokusunda araştırılmıştır. Çalışma toplumları yakın sonuçlar vermiştir (±st.sap.) – Bozdağ toplumu: n_k = 2.9091 (±1.1362), A_E = 2.0250 (±0.9537), H_o = 0.3830 (±0.2717), H_e = 0.3956 (±0.2746); Nallıhan toplumu: n_k = 2.9091 (±1.1362), A_E = 2.0592 (±0.9451), H_o = 0.4086 (±0.2977), H_e = 0.4052 (±0.2767); ve Karadağ toplumu: n_k = 2.5455 (±1.1282), A_E = 1.8809 (±0.8758), H_o = 0.3388 (±0.2775), H_e = 0.3607 (±0.2716). Eşleştirilmiş *t*-testi ile yapılan ve temel genetik parametrelere dayanan toplum karşılaştırmaları anlamlı olmayan ($p > 0.05$) farklılıklar vermiştir. Ayrıca, Bozdağ toplumu için genetik çeşitlilik temelindeki zamansal değişim geçmiş verilerle araştırılmış. Genetik parametrelerdeki zamansal değişimin anlamlı olmadığı bulunmuş ve farklılıklara neden olabilecek olasılıklar tartışılmıştır. Ek olarak, yakalama tekniğinden kaynaklanan olası bir istatistiksel yanlılığı tespit etmek için

farklı kapanlara ait genetik çeşitlilik ve PI değerleri belirlenmiş ve karşılaştırılmıştır. Karşılaştırmalar kapanlar arasındaki türdeşliğe gösteren anlamlı olmayan farklılıklar sunmuştur. Öte yandan, simulasyonlar alelik çeşitliğin (*A*) kurucu etkisine, polimorfik lokus oranı (*P*) ve heterizgotluk (H_o & H_e) seviyelerinden daha duyarlı olduğunu tespit etmiş ve toplum darboğazı analizi için kullanılan heterozigotluk fazlası metodu desteklenmiştir. Aynı simulasyon analizi ile yeniden aşılanmış örneklerde gözlemlenen genetik çeşitliliğin beklenti aralığında (%99 GA) olduğu ve bu sayede taşınmış bireylerin rasgele seçildiği gösterilmiştir. Heterozigotluk fazlası metoduna (heterozigotluk fazlası için tek-kuyruklu Wilcoxon testi: p_{SMM} = 0.28515, p_{TPM} = 0.06445, p_{IAM} = 0.02441) ve alel frekansı dağılımları metoduna (normal L dağılım) dayanan toplum darboğazı analizi Bozdağ toplumu için yakın bir genetik darboğaz tespit edememiştir. Fakat, simulasyonlar bu iki metodun tip II hatasına eğilimli olduğunu belirlemiştir. Çalışma toplumu için darboğaz tespit başarısızlığı, modelin varsayımlarına uyumsuzluk gibi bir hata kaynağının yerine büyük olasılıkla tip II hatasından kaynaklanmaktadır.

Anahtar sözcükler: Anadolu yaban koyunu, *Ovis gmelinii anatolica*, yeniden aşılama, koruma genetiği, mikrosatelit, kurucu etkisi, toplum darboğazı

To My Family

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NOMENCLATURE

A	Allelic Diversity *or* Number of Alleles
CE	Complete Enumeration
A_E	Effective Number of Alleles
N_e	Effective Population Size
H_{eq}	Expected Equilibrium Heterozygosity
H_e	Expected Heterozygosity
f	Frequency
GSM	Generalized Stepwise Model
HWE	Hardy-Weinberg Equilibrium
IAM	Infinite Allele Model
KAM	*K*-Allele Model
MC	Markov Chain
n_k	Mean Number of Alleles
n	Number of Gene Copies
H_o	Observed Heterozygosity
PCR	Polymerase Chain Reaction
PI	Probability of Identity
P	Proportion of Polymorphic Loci
N	Sample Size
SMM	Stepwise Mutation Model
TPM	Two Phase Model
UTM	Universal Transverse Mercator

CHAPTER 1

INTRODUCTION

1.1. Current & Historic Range of the Species in Turkey

Anatolian mouflon, *Ovis gmelinii anatolica*, also known as Anatolian wild sheep or as Turkish mouflon, is a subspecies endemic to Turkey. For many reasons, this subspecies came to the edge of extinction in the very recent history. According to TURAN (1967), the population size declined down to 35 to 50 individuals in Bozdağlar region of Konya Province where the last population of Anatolian mouflon was subsisting. Formerly, Anatolian mouflon populations were found in Nallıhan and Polatlı in Ankara, Sivrihisar and Araidbaba in Eskişehir, Emirdağı in Afyon, Karadağ in Karaman, and Ereğli, Karapınar and Bozdağ in Konya. Consequently, the historic range of this subspecies was once covering approximately 50,000km^2 in Central Anatolia (DANFORD & ALSTON, 1877; TURAN, 1984). These populations did not survive, and extirpated between 1940s and 1970s. Among many possible reasons, habitat fragmentation and destruction, predation, hunting, poaching, disease, and food competition with domestic livestock that is caused by heavy grazing seem to be critical ones responsible for the destruction of these populations. Later, a conservation program began in 1966 for the protection of the last survivors in Konya-Bozdağ region and for the recovery of the population. For this purpose, Bozdağ Wildlife Development Area (WDA) was established by Ministry of Agriculture (now Ministry of Environment & Forestry). Therefore, from the time of extirpations to the beginning of reintroduction program in 2004, the only range of this subspecies was Bozdağ.

Reintroduction program currently involves two former habitats of Anatolian mouflon, Nallıhan in Ankara and Karadağ in Karaman, and still ongoing with great efforts. Hence, the current range of this subspecies involves three regions in Turkey; Konya-Bozdağ, Ankara-Nallıhan, and Karaman-Karadağ.

Along with Anatolian mouflon, *Ovis gmelinii gmelinii*, also known as Armenian mouflon, is the second subspecies found in Turkey. Similarly, this subspecies had also faced population losses and severe size declines. Armenian mouflon is listed as vulnerable (VU A2cde, ver.2.3; 1994) in 1996 IUCN Red List of Threatened Animals, but currently needs updating (from IUCN official website). A conservation program was started for Armenian mouflon with the establishment of Van-Özalp Protection & Breeding Area (PBA) in 1971 on a 150,000ha area to the east of Özalp. However, current status of this subspecies in Özalp PBA is known only roughly due to the unsystematic management of the conservation program. The geographic range of this subspecies includes Armenia, Azerbaijan, Iran, Iraq, and Turkey. In Turkey, the distribution covers the region from the south of Mount Ağrı to the north of Mordağlar mountains in Hakkari and from the east of Lake Van to Karadağ (KENCE & TARHAN, 1997; ARIHAN, 2000; ALBAYRAK *et al.*, 2007). Some group of individuals of this subspecies may perform seasonal migrations by moving to Iran in autumn and migrating back to Turkey in spring (KENCE & TARHAN in SHACKLETON, 1997, p.134-138).

1.2. Taxonomy

As given above, there are 2 subspecies of *Ovis gmelinii* (BLYTH, 1840) in Turkey; *Ovis gmelinii anatolica* (VALENCIENNES, 1856) and *Ovis gmelinii gmelinii* (BLYTH, 1841) (ALBAYRAK *et al.*, 2007). However, these two subspecies were firstly classified not as *O. gmelinii* but as *Ovis orientalis* (GMELIN, 1774).

The classification of genus *Ovis* (LINNAEUS, 1758) is problematic and there are usages of various taxon names among different authors (HIENDLEDER *et al.*, 2002). The problem mostly arises from the classification of mouflons and urials. In 1997,

IUCN/SSC Caprinae Specialist Group classified both mouflons and urials in a single species as *O. orientalis* and Anatolian mouflon was classified as *O. o. gmelinii* together with Armenian mouflon (Status Survey and Conservation Action Plan for Caprinae, edt. by David M. Shackleton, 1997). However, currently the classification in 2000 by IUCN/SSC Caprinae Specialist Group is accepted, where mouflons and urials are not identified in a single species as *Ovis orientalis*, but mouflons are classified as *Ovis gmelinii* and urials as *Ovis vignei*. Thus the oldest name, *gmelinii* (BLYTH, 1840), is now applied to mouflons. This is because mouflons have 54 (2n) chromosomes (BUNCH 1998; KIRIKÇI *et al*., 2003), but urials have 58 (2n) chromosomes (BUNCH 1978; SHACKLETON 1997; HIENDLEDER *et al*., 2002), however hybrid forms may have 55 (2n) and 56 (2n) chromosomes (VALDEZ *et al*., 1978). Currently accepted classification of Anatolian mouflon is;

Domain: *Eukaryota* (WHITTAKER & MARGULIS, 1978)
Kingdom: *Animalia* (L., 1758)
Phylum: *Chordata* (BATESON, 1885)
Subphylum: *Vertebrata* (CUVIER, 1812)
Class: *Mammalia* (L., 1758)
Subclass: *Theria* (PARKER & HASWELL, 1897)
Order: *Artiodactyla* (OWEN, 1848)
Suborder: *Ruminantia* (SCOPOLI, 1777)
Family: *Bovidae* (GRAY, 1821)
Subfamily: *Caprinae* (GRAY, 1821)
Genus: *Ovis* (L., 1758)
Species: *gmelinii* (BLYTH, 1840)
Subspecies: *anatolica* (VALENCIENNES, 1856)

Additionally, the number of species in genus *Ovis* (L., 1758) may show variations among the classifications of different authors. WILSON & REEDER (2005) have classified five species in the genus *Ovis* (L., 1758); *O. ammon* (L., 1758), *O. aries* (L., 1758), *O. canadensis* (SHAW, 1804), *O. dalli* (NELSON, 1884) and *O. nivicola* (ESCHSCHOLTZ, 1829), where mouflons are placed in *O.aries* with domestic sheep.

Variously, IUCN/SSC Caprinae Specialist Group has classified seven species of genus *Ovis* (L., 1758); argali sheep which lives in Asia as *O. ammon*, domestic sheep as *O. aries*, bighorn sheep which lives in North America and Siberia (COWAN, 1940) as *O. canadensis*, dall sheep (or thinhorn sheep) which lives in northwest North America as *O. dalli*, mouflon as *O. gmelinii*, snow sheep which lives in Siberia as *O. nivicola*, and urial as *O. vignei*. In this classification, *O. gmelinii* and *O. vignei* each owns six subspecies (Table 1.1).

Table 1.1. Classification of mouflons & urials.

Species	Subspecies	Common Name
Ovis gmelinii		
	gmelinii (BLYTH, 1841)	Armenian mouflon
	anatolica (VALENCIENNES, 1856)	Anatolian mouflon
	laristanica (NASONOV, 1909)	Laristan mouflon
	ophion (BLYTH, 1841)	Cyprian mouflon
	isphanica (NASONOV, 1910)	Esfahan mouflon
	musimon (SCHREBER, 1782)	European mouflon
Ovis vignei		
	arkal (EVERSMANN, 1850)	Transcaspian urial
	bocharensis (NASONOV, 1914)	Bukhara urial
	cycloceros (HUTTON, 1842)	Afghan urial
	punjabiensis (LYDEKKER, 1913)	Punjab urial
	vignei (BLYTH, 1841)	Ladakh urial *or* Shapu
	*blandfordi******	Blandford urial

Source: IUCN/SSC Caprinae Specialist Group.
***** Uncertain, can be an ecotype.

1.3. General Characteristics of Anatolian Mouflon

The appearance of Anatolian mouflon is much differentiated from the domestic sheep. It has a look of a very agile animal with hindlegs longer than forelegs, high shoulders and breasts, and also with their longer, slender bodies. These physical characteristics contribute to the lifestyle in the mountainous Konya-Bozdağ region

where the elevation ranges between about 1000 to 1750m asl. Anatolian mouflon individuals are generally seen on wide and smooth hills between 1000m and 1500m, instead of rocky hills where the slopes are very vertical. The body length varies between 105 to 140cm, and the breast height is about 80 to 90cm. The tail is short, ranging between 10 to 12cm and thin (KAYA, 1989). Both the body length and breast height is generally greater in males. Males are also heavier than females by weighing about 45 to 74kg, whereas the females are about 35 to 50kg. Only the males of this subspecies own horns whereas both the males and females (18-20cm) of the eastern subspecies, *O. g. gmelinii*, have permanent horns. However, the horn tips of Central Anatolian subspecies are more distant than the eastern subspecies and the length of their horns can reach up to 75cm. Anatolian mouflon males start to grow horns after 4 months following birth.

The longevity of Anatolian mouflon is 15 to 18 years. There are 2 techniques that are used for age determination in this subspecies. Age can either be determined by counting the annual rings in the horns of males. However, this method is inapplicable to the females to *O. g. anatolica*. The other method, which is determination of age from incisor teeth structure, is not very efficient and generally is used for ewes. By this method, only individuals younger than 3 years old can be classified since incisor teeth development stops after this age.

The fur color of Anatolian mouflon shows seasonal variations by being pale brown in the summer and reddish brown in the winter. Hairs are also shorter and thinner in the summer period. The fur color can well camouflage the individuals within their habitat. They tend to shed their fur in the beginning of summers during May and June. For males, the hair color starts to darken after 2 years and a light saddle occurs after 3-4 years but saddle may not form in some individuals.

Male and female mouflons reach sexual maturity after 2.5 and 1.5 years, respectively. Breeding takes place during November and December, while ewes give birth in May and June after 148 days of gestation period. While young ewes generally give birth to only one infant, older ewes generally give twin births.

Females care their offspring until December. There is a strong sexual selection during the rutting season due to female choice among potential mates, and inter-male competition for access. Generally older rams with bigger horns are preferred by the ewes. During this season, the sexually dominant male forms a group with the ewes and after breeding period, the group separates. Hence, except for the rutting seasons, males do not hold harems.

Anatolian mouflon generally feeds with steppe vegetation. *Gramineae* family constitutes about half of its diet. The remaining part mostly consists of *Leguminosea* and *Umbelliferae* families. Members of *Fabaceae* also constitute an important part of their diet. Anatolian mouflon is resistant to thirst. However, during fall seasons, they eat bulbs of *Erodium spp*. by digging the ground. This contributes to their water requirement besides nourishment (KAYA, 1989; KAYA & AKSOYLAR, 1992). Anatolian mouflon also feed with additional food (*e.g*. alfalfa) supplied by the wardens especially during snowy winters when food is not easily accessible.

1.4. Conservation of Anatolian Mouflon

The first considerable conservation program for Anatolian mouflon was started in 1966 when a 42,000 ha area in Bozdağlar region of Konya province was converted into a protection area by the Ministry of Agriculture. Although this was the first important progress, there had been other conservation actions for this subspecies prior to this management plan. In 1937, Anatolian mouflon was officially taken under protection by legal restrictions (Land Hunting Law No. 3167). By this law, together with wild sheep, hunting wild goat and chamois was also prohibited. However, this was not enough to secure the populations of Anatolian mouflon and the need for a more sophisticated conservation action emerged in later years.

In 1989, a 5,000 ha area in Bozdağ WDA was fenced as a Protection & Breeding Station (PBS) and a captive population within PBS was established from about 40 to 50 individuals (ARIHAN, 2000). The fences were electrified in 1996. All potential predators (*e.g*. wolves, lynx, caracals, dogs…etc.) were evacuated from PBS to

prevent deaths caused by predation. Surrounding fences also prevented food competition with domestic sheep from surrounding villages. However, with the establishment of fences, the population within Bozdağ PBS was totally isolated from the exterior population of Anatolian mouflon and thus, the mouflon population within Bozdağ WDA became subdivided into two subpopulations.

There is still a small population of Anatolian mouflon persisting outside Bozdağ PBS, but recent observations show that the size of the population is not more than 100 and is thought to be declining. However, size estimations for this population are not trustable and imply contradictions. According to ARIHAN (2000), the size is around 50. It is thought that there are more than 20,000 domestic sheep in the nearby villages since most of the local folk earn from stockbreeding. This leads the exterior population to an extensive competition with domestic stock, in addition to predation and hunting. Thus their number did not show a very positive change over time. Whereas the size of the population within Bozdağ PBS increased fairly well until years 2000 and 2001 when there were about 1000 (data by Department of National Parks, DNP) and 1400 (DNP) inhabitants, respectively (Figure 1.1). However, this number decreased since then and currently, the population size is estimated to be about 600 (Figure 1.1). Since Bozdağ PBS has a limited carrying capacity, the reason for this decline can be assigned as over-dominance because population size rised up to 1400 in 2001. Additionally, since the beginning of 2005, there is an increase in the rate of deaths caused by an unknown disease. In 2007, this desease was identified as paratuberculosis. According to the predictions, paratuberculosis spread faster with overdominance as the physical closeness among individuals has increased. Recent observations show that many individuals in Bozdağ PBS are carrying this disease now. However, appointing the consequences of the disease as the major reason for this decline in population size is still questionable. For a more reliable inference, in addition to the ongoing radio-telemetry studies for demographic analyses, paratuberculosis needs also to be investigated with genetic analyses and urgent action plans must be started accordingly. Furthermore, diseased individuals should be detected and not be chosen for reintroductions.

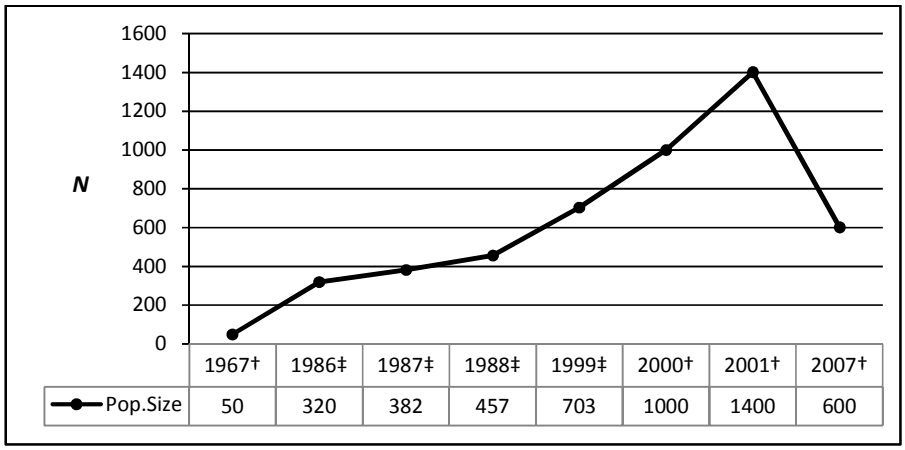

Figure 1.1. Temporal change in Bozdağ population size.
† Data by DNP.
‡ Data by academic researchers. 1986, 1987, and 1988 data from KAYA (1989); 1999 data from ARIHAN (2000).

1.4.1. Reintroduction

1.4.1.1. A General Overview of Reintroduction Programs

Reintroduction is repopulating a suitable area within the historic range of a species or subspecies by the translocation of individuals that were either held in captivity or free-ranging. The aim behind this scenario is to establish a self-sustainable, viable population to increase the chance of survival of the taxa. For a reintroduction study, the site where the individuals are repatriated should not own any conspecific population of any size, however if this criterion is not met, the study can be termed as restocking (KLEIMAN, 1989).

For the success of a reintroduction program, there are many precautions to be taken. Defining the reasons behind the extirpation of former natural populations is of great importance for the success of the reintroduction program. In addition, feasibility studies on the species that is being reintroduced are important and a wide knowledge on the behavior and ecology of the reintroduced species or subspecies may greatly

contribute to the choice of reintroduction sites (KLEIMAN, 1989). Reintroduction site must have suitable carrying capacity in order to support the increasing size of reintroduced population (BRAMBELL, 1977).

Generally, newly reintroduced individuals tend to show unordinary behavior and a stressful mood due to the changes in their environments. In order to soften these types of effect, a pre-release reintroduction may be necessary to prepare the individuals to the food, climate and other local conditions of the release site which can readily increase the post-release survival (DIETZ *et al*., 1988; KLEIMAN, 1989). Especially this holds for the individuals that are translocated from a captive-breeding site and are captive-born. In addition, post-release training (SCOTT-BROWN *et al*., 1986) may further increase the survival chance of the reintroduced individuals, however not every species may need such training conditionings.

The monitoring of reintroduced individuals (*e.g*. radio-telemetric monitoring) after release is crucial for the assessment of causes of death and survival in the reintroduction site (SCOTT & CARPENTER, 1987). Long-term monitoring is especially useful in perceiving the final situation by acquiring information on the demographic parameters and viability of the entire population and thus, for taking new decisions and precautions for the management of the reintroduction program. Long-term monitoring may also serve to conceive better or more efficient strategies (*e.g*. new release strategies) for the current and future reintroduction efforts.

Moreover, for their close relation with experimental studies, reintroduction actions may serve to other purposes of rather theoretical researches in ecology, evolutionary and behavioral biology. By being expensive and demanding studies relative to laboratory experiments, also due to logistical and technical difficulties faced in the field, reintroduction experiments are generally impractical especially in the case of large mammals. For this reason, conservation programs can also be used for these kinds of experiments and may serve helpful opportunities for *real-scale hypothetico-deductive experiments* and meta-analysis for biologists from various disciplines (SARRAZIN & BARBAULT, 1996).

1.4.1.2. Reintroduction of Anatolian Mouflon

Reintroduction of Anatolian mouflon to its former habitats started in 2004 and is still in progress. Emremsultan WDA in Nallıhan (Ankara) and Karadağ (Karaman) had been specified as reintroduction sites for the source population in Bozdağ by DNP and Game-Wildlife Department (GWP). Both Emremsultan and Karadağ reintroduction sites own a fenced area for soft release reintroduction. However the fenced area in Emremsultan WDA is called as Sarıyar PBS whereas the one in Karadağ is not a PBS nor Karadağ itself is a WDA. Unlike a hard release reintroduction - that is releasing individuals directly without any pre- or post-release preparations - the newly reintroduced individuals from Bozdağ PBS are subjected to a pre-release period due to reasons listed above but rather for breeding since mouflons can increase in number faster within the fenced areas where they are protected against the harsher conditions (*e.g.* predators, competition) outside. Some of the individuals are also subject to radio-telemetric monitoring after being released outside the fenced area.

Totally 192 individuals - 131 individuals to Emremsultan WDA, 61 individuals to Karadağ - were reintroduced and 161 of them were released outside the fenced areas. According to estimations in May 2008, there are about 70-80 individuals inhabiting in Emremsultan WDA. Also, due to the last survey results of Karaman National Parks and Department of Nature Preservation in December 2007, the estimated population size is less than 30 in Karadağ. Both populations are being followed with radio-telemetric monitoring by a group of researchers from Middle East Technical University (METU) and major demographic parameters (birth, death and annual survival rates) as well as habitat selection of wild sheep are being determined. Individuals are followed by radio-collars of appropriate size and weight. However monitoring in Emremsultan WDA is more efficient than in Karadağ since only 6 individuals were radio-collared in the latter whereas this number is 40 in the former area. Studies indicate low annual survival rates both for Nallıhan and Karadağ populations; annual survival rates are 0.346 and 0.420, respectively (DENİZ ÖZÜT, unpublished data). Paratuberculosis has also been detected in both reintroduction

sites and thought to be as one of the major contributors to the low annual survival rates. By detecting and reintroducing individuals that are not carrying the disease, annual survival rates will definitely increase for these populations.

One additional note is that, in 2005, a new PBS was established for wild sheep, wild goat, and gazelle on 80ha area in Malatya-Hekimhan by DNP. Only in 2007, 7 individuals, and in total 10 indiviuals were translocated to Hekimhan PBS from Bozdağ PBS.

1.5. Conservation genetics

It is claimed that human intervention to nature especially in the last two hundred years had greatly increased the rate of extinction causing the loss of threatened species at the first place. Many authorities name it as the sixth mass extinction (LEAKEY & LEWIN, 1995; VINES, 1999) in the history of life and probably more powerful than any of the antecedents. According to 2007 IUCN Red List, the number of threatened species in 2006 is 16,118 that are either endangered, critically endangered, or vulnerable. However, this number was 15,503 in 2004, 12,259 in 2003, 11,167 in 2002, 11,046 in 2000, and 10,533 in 1996/1998. Accordingly, the number of species in the list nearly doubled within less than a decade and vertebrates are at the first place in the list. To give more details, 20% of all mammal, 12% of all bird, 4% of all reptile, 31% of all amphibian, and 4% of all fish species that are described are threatened. This makes 10% of all vertebrates described. Except for 31% of all gymnosperm species also being categorized as threatened, the statistics are more pleasing for remaining taxa (Data from IUCN official website), but still indicating the need for conservation of species and ecosystems as well as genetic resources especially for vertebrates.

In the simplest terms, conservation biology is an interdisciplinary science that aims to protect biodiversity. Three levels of biodiversity are identified by IUCN for this purpose; genetic diversity, species diversity, and ecosystem diversity (MCNEELY *et al.*, 1990). Conservation genetics as a branch of conservation biology is directly

related with the first two of these levels but researchers do not attach much importance to its relation with ecosystem diversity (FRANKHAM *et al*., 2002) however opposing findings are present (*e.g*. REUSCH *et al*., 2005, FRANKHAM, 2005). The aim of conservation genetics is to detect the genetic based factors that contribute to the extinction of species or subspecies or races and serve management plans on this account to minimize the risks of extinction and reduced viability. According to FRANKHAM *et al*., (2002), there are a variety of genetic issues which may directly contribute to extinctions and hence, are within the scope of conservation genetic analyses. These are; (*1*) loss of genetic diversity, (*2*) inbreeding depression, (*3*) outbreeding depression, (*4*) fragmentation of populations and reduction in gene flow, (*5*) accumulation and loss of deleterious alleles, (*6*) genetic drift overriding natural selection, and (*7*) adaptation to captivity. Among these, low genetic diversity and inbreeding depression are most vital ones and nearly inevitable for all endangered species but they can be compensated by suitable management plans (FRANKHAM, 1995, 2003; AMOS & BALMFORD, 2001). Thus, identification of the genetic status of endangered species is very important for conservation programs.

Conservation genetics recognizes many methods for molecular analyses and currently owns usable genetic tools far more than before. Among these, AMPLIFIED-FRAGMENT-LENGTH POLYMORPHISMS (AFLPs), MICROSATELLITES (SSRs), SINGLE-NUCLEOTIDE-POLYMORPHISMS (SNPs), and DNA sequencing are mostly used techniques in conservation genetics. Some of the lesser techniques include MINISATELLITES, RANDOMLY-AMPLIFIED POLYMORPHIC DNA (RAPD), RESTRICTION-FRAGMENT-LENGTH POLYMORPHISMS (RFLPs), DIRECT-AMPLIFIED-LENGTH POLYMORPHISMS (DALPs), SHORT-INTERSPERSED NUCLEAR ELEMENTS (SINEs), and ALLOZYMES (AVISE, 2004; DESALLE & AMATO, 2004).

1.6. Researches for Anatolian Mouflon

Conservation genetics of Anatolian mouflon had not been under fairly consideration since the start of conservation program in 1966. Until a master thesis was submitted in 2001, the genetic diversity and genetic structure of this subspecies were unknown

for the researchers in Turkey. ÖZÜT (2001) collected 48 samples from Bozdağ population - the only extant population at that time - and analyzed at 14 microsatellite loci, but results of 4 microsatellite loci were ignored due to unanalyzable data obtained. The genetic variation of Bozdağ population at 10 microsatellite loci was found to be; P = 1.00, n_k = 2.5 ± 0.7071[1], H_o = 0.3059 ± 0.1784[1], H_e (NEI, 1973) = 0.3310 ± 0.1888[1] (ÖZÜT, 2001). Since 2001, again there had not been any research on population genetics for the source population and reintroduced populations of this subspecies.

Other master studies include ARIHAN (2000) and SEZEN (2000). ARIHAN (2000) investigated the population biology of Anatolian mouflon, whereas Sezen (2000) carried out computer simulated analyses for population viability and the effect of harvesting on the viability of Bozdağ population and prospective reintroductions. And KAYA (1989) submitted a Ph.D. thesis on the general biology of Anatolian mouflon. Additionally, KAYA (1990, 1991) gave information on population density as well as morphology and other general characteristics of Anatolian mouflon. Recently, ÇELİK (2004) investigated the behavioral characteristics of Anatolian mouflon with radiotelemetric monitoring.

DANFORD & ALSTON (1877) made the first study on the distribution of Anatolian mouflon in Turkey. TURAN (1967) prepared a report to the Ministry of Forestry in which he also determined the distribution of this subspecies in Central Anatolia and made the first population size estimation for the subspecies. Recently, KIRIKÇI *et al*., (2003) karyotyped one sample belonging to a female from Konya-Bozdağ with G-banding method and detected 54 diploid chromosomes in which 6 autosomal chromosomes are metacentric, and other chromosomes are acrocentric. Also, KABAKÇI *et al*. (2007) studied on *cystocaulus ocreatus* infection in Anatolian mouflon and dwarf goats.

[1] Standard deviation

1.7. Microsatellites

1.7.1. A General Overview of Microsatellites

Microsatellites (or Simple Sequence Repeats, SSRs) are DNA sequences of simple,-short tandem repeats (ELLEGREN, 2004) found both in coding and non-coding regions of genome and they are one of the most widely used markers (known DNA sequences) in genetics and may be the most. Also named as simple tandem repeats (STRs) (EDWARDS *et al.,* 1991), microsatellites are rare in coding regions and telomeres (HANCOCK, 1999). Microsatellites are co-dominant markers typically involving a base motif of monomer (1bp) to hexamer (6bp) sequences, repeated up to ≈100 times (VOGT, 1990; TAUTZ, 1993; SUBRAMANIAN *et al.,* 2002) and are assumed to be selectively neutral due to their rarity in coding regions. CA/GT dinucleotides are the most common repeat types for humans and other mammals in general (BECKMANN & WEBER 1992). Iterations of longer units form minisatellites which are another class of satellite DNA (ELLEGREN, 2004).

Microsatellites can be genotyped via polymerase chain reaction (PCR) and are useful for genetic analyses. Microsatellites are highly polymorphic loci found abundantly in prokaryote (GUR-ARIE *et al*., 2000) and eukaryote genomes (WEBER & MAY, 1989; TOTH *et al*., 2000), and in the latter, they are present both in nuclear and organellar DNA, and in higher numbers. Albeit no direct correlation between genome size and microsatellite content in natural populations (PRIMMER *et. al*., 1997), generally a positive correlation is observed (ELLEGREN, 2004).

Microsatellites show extremely high mutation rates of 10^{-3} (JEFFREYS *et al*., 1988; KELLY *et al*., 1991; WEBER & WONG, 1993) or 10^{-4} (LEVINSON & GUTMAN, 1987; HENDERSON & PETES, 1992; HANCOCK, 1999) in humans and other specified taxa. Also, co-dominance of microsatellites supports the detection of all genotypes whereas dominant markers such as RAPDs, RFLPs and AFLPs shadow distinguishing between homo- and heterozygote states in diploids or polyploids. In addition, despite their rarity in coding regions, the fairly even distribution and near

ubiquity of microsatellites in the genome (DIETRICH *et al*., 1996), their abundance, and the high levels of polymorphism due to length variability that they possess (BENNETT *et al*., 1998) lead them to be popular markers for various genetic researches as diverse as conservation genetics, genetic mapping (*e.g*. WEISSENBACH *et al*., 1992), molecular forensics, identification of genetic diseases (*e.g*. MURRAY *et al*., 1992) and molecular anthropology (SAINUDIIN *et al*., 2004). The popularity of microsatellites is also due to their low costs and simplicity for genetic analyses.

1.7.2. Mutation Mechanisms

Two mechanisms were put forward for the high rates of mutation in microsatellites; polymerase slippage, or slipped-strand mispairing, (LEVINSON & GUTMAN, 1987; SCHLÖTTERER & TAUTZ, 1992; SIA *et al*., 1997) and recombination processes involving gene conversions and unequal crossing over during meiosis (SMITH, 1976; JEFFREYS *et al*., 1994; HANCOCK, 1999).

High mutation rates of microsatellites are most frequently explained with polymerase slippages which are not always repaired by mismatch repair system (STRAND *et al*., 1993). During slippage, DNA polymerase causes transient dissociation of nascent strand from the template strand by losing its track (LEVINSON & GUTMAN, 1987; ELLEGREN, 2004). This is followed by the introduction of a loop either on the nascent strand or template strand with the former causing an insertion of repeat units, hence to an expansion in nascent strand, while the latter causes a deletion of repeat units leading to a contraction in the length of the nascent strand (ELLEGREN, 2004).

Concordantly, polymerase slippage can take place *in vitro* during PCR amplifications just as well as *in vivo* as explained above (ELLEGREN, 2004). During elongation step, replication slippage can cause minor product peaks called stutter peaks which are 1 to 4bp shorter than the main allele. The percentage of stutter peaks is positively correlated with the length of main allele being amplified in the case of perfect repeat motifs while this correlation is generally invalid when the

repeats are imperfect. Interestingly, stutter peaks may be helpful in some problematic cases during genotyping caused by nonspecific amplifications because nonspecific amplifications do not own stutter peaks and thus, are easily distinguished from specific ones.

Despite the minor changes in the length of replicates caused by slippage, recombination processes can cause more drastic changes with a wider range of novel mutants. During unequal crossover in meiosis, one chromosome obtains more repeats than the other chromosome. However, there is no direct evidence for the contribution of recombination to microsatellite mutations and they are thought to be more effective for minisatellite mutations (ELLEGREN, 2004).

Mutation rates are variable in microsatellites due to the length of the repeats (WEBER, 1990; CHAKRABORTY *et al*., 1997; SIA *et al*., 1997; PRIMMER *et al*., 1998; ELLEGREN, 2004). Longer sequences are more prone to mutation since more loops can occur in the longer strands during replication and consequently, the chance for the failure of mismatch repairing increases. Secondly, the flanking sequences of microsatellites can cause observable differences in mutation rates (GLENN *et al*., 1996; BACHTROG, 2000; ELLEGREN, 2004). In addition, as indicated above, perfect repeat motifs or the purity of microsatellites increases the chance for higher mutation rates whereas interrupted sequences are less likely to form slipped intermediates (KUNST *et al*., 1997; PETES *et al*., 1997). One other factor for the variance in mutation rates for individual loci is sex-biasness that are observed in many organisms where either the males or females show a higher mutation rate (PRIMMER *et al*., 1998; ELLEGREN, 2000; XU *et al*., 2000; BROHEDE *et al*., 2002), however this is not a strict rule since there are also observations for equal rates of mutation among the sexes.

Furthermore, gains being predominant against losses of repeat units lead to heterogeneity in microsatellite mutations (ELLEGREN, 2004). While several studies supported this biased relation with experiments (WEBER & WONG, 1993; AMOS *et al*., 1996; COOPER *et al*., 1999; ELLEGREN, 2000), some researches show a balanced

or near-balanced situation for this case in microsatellite mutations (XU *et al*., 2000; HUANG *et al*., 2002) as a counter idea. This indicates an uncertainty and the need for more researches on the issue.

1.7.3. Mutation Models

Various mutation models have been proposed for microsatellites although a full inference of the mutation processes is still unavailable. A valid mutation model is needed for population genetic analysis since inferences are sensitive to the assumed theoretical model. Two extreme models are *stepwise mutation model* and *infinite allele model* along the continuum of all possible mutation models (CHAKRABORTY & JIN, 1992).

1.7.3.1. Stepwise Mutation Model

In stepwise mutation model (SMM) developed by OHTA & KIMURA (1973), the length of a repetitive array is changed by one repeat unit that is either gained or lost in every mutation process in a constant and unbiased fashion with no allele size constraint (SHRIVER *et al*., 1993; KIMMEL *et al*., 1996; ELLEGREN, 2004). This model also assumes symmetrical distribution of mutations. According to ESTOUP & ANGERS (1998) and SCHLÖTTERER (2000), microsatellites mutate in a stepwise manner with high rates of mutation. It is assumed that alleles that are more closely related in their length have a more recent ancestor. However, in this *ladder model*, any newly mutated allele may not be a novel allele for the gene pool. Thus, homoplasy - alleles are identical in state but not identical by descent - may constitute a problem for this assumption.

SMM is extensively used for genetic data analysis. R_{ST} (SLATKIN, 1995) with D_{SW} (SHRIVER *et al*., 1995) are two statistical measures that are based on SMM. However this model has weaknesses due to its unrealistic assumptions that can lead to discrepancies in statistical analysis. Specifically, SMM cannot explain the multistep mutations (DI RIENZO *et al*., 1994; HUANG *et al*., 2002), upper size limit for

microsatellites (NAUTA & WEISSING, 1996; FELDMAN *et al*., 1997; ELLEGREN, 2004) and biased mutations (KIMMEL & CHAKRABORTY, 1996).

1.7.3.2. Infinite Allele Model

Infinite allele model (IAM) was first proposed by KIMURA & CROW (1964) at the time of protein gel electrophoresis before genetic data could be analyzed. Unlike SMM, it suggests that the change in the length of a repetitive assay can involve any number tandem repeats. However, since polymerase slippage is assumed to cause minor changes in length and be the predominant mechanism for microsatellite mutations, this model is generally thought to be less powerful than SMM. However, still IAM is the other predominant model with SMM in population genetic analyses. Additionally, IAM assumes that every mutation process leads to a novel allele that is not present in the population (ESTOUP *et al*., 1995, 2002). Hence, IAM is known as a nonhomoplasious model (COURNET *et al*., 1999; ESTOUP *et al*., 2002). F_{ST} (Wright) which is a measure of interpopulation differentiation is based on IAM.

1.7.3.3. Alternate Models

DI RIENZO *et al*. (1994) proposed a new model for microsatellite mutations which was named suitably as *two phase model* (TPM) since it owns features both from SMM and IAM. In this model, microsatellite mutations generally involve one repeat unit with a probability P_{SMM}, but can involve higher repeat units with 1 - P_{SMM}. Thus, TPM mostly follows SMM with a limited proprortion of mutations that involve more than a single tandem repeat. Like in SMM, there is an equal probability of contraction and expansion in the length of the nascent strand.

Analogical to TPM, generalized stepwise model (GSM; FU & CHAKRABORTY, 1998) was proposed and this model assumes $P_{SMM} = 0$. This mutation model does not assume any allele-specific mutation rates (*i.e*. like longer sequences being more prone to mutations) or any allele size constraints (GRAHAM *et al*., 2000). There are many studies that assume TPM, or GSM as the most realistic model for

microsatellite mutations (reviewed in ESTOUP & COURNET, 1999). Also, according to ESTOUP *et al*. (2002), GSM with allele size constraints is more realistic than SMM for microsatellites.

CROW & KIMURA (1970) proposed another model, K-allele model (KAM) as an alternative to IAM where there are K possible allelic states. In this model, there is equal probability of mutating towards the remaining K – 1 alleles for every allele. Thus, if K becomes infinitely many, KAM will be the same with IAM. However, due to allele size constraints, KAM is probably more realistic than IAM (ESTOUP *et al*., 2002).

1.7.4. Homoplasy & Limitations of Microsatellites

Homoplasy results when two fragments of the same length are not identical by descent due to the possibility of returning to the ancestral state after population divergences. For this reason, homoplasy at microsatellites is called as *size homoplasy* (ESTOUP *et al*., 2002). Homoplasy is associated with SMM, TPM, GSM (simplified version of TPM), and KAM. However for loci following strict IAM (KIMURA & CROW, 1964), homoplasy is not expected (ESTOUP *et al*., 2002). Thus, homoplasy obviously depends on the kind of mutation model and also, to mutation rate, effective population size, population drift, and the time of divergence between populations (ESTOUP *et al*., 2002). Theoretically, there is a positive correlation between the level of homoplasy and mutation rate in the loci. As mutation rate increases, the chance of gaining the ancestral state also increases. Concordantly, as the time of divergence increases, homoplasy also expected to increase due to the accumulation of ancestral states in the populations. The same positive relation also holds for effective population size (ESTOUP *et al*., 2002). According to GOODMANN (1998), homoplasy can be determined by analyzing many loci.

There are limitations of microsatellites also during PCR amplifications. Stutter peaks that differ from the main template by multiples of repeat unit can occur due to slippage mechanism (SHINDE *et al*., 2003). As mentioned, stutters can be helpful for

correct genotyping but generally they have the potential for causing genotyping errors due to lack of discrimination between homozygotes and heterozygotes (OOSTERHOUT *et al*., 2004). Furthermore, due to low DNA concentrations, allelic dropout (MILLER & WAITS, 2003) can be seen especially when DNA was isolated from material owning low DNA content (*e.g*. feces) and this may cause some alleles not to be amplified or may lead to large allele dropout which is the more amplification of shorter alleles than longer ones (WATTIER *et al*., 1998). Also, due to the primer-site mutations, failure to amplify certain alleles (*i.e*. null alleles) can cause heterozygotes to be genotyped wrongly as homozygotes (SHAW *et al*., 1999; OOSTERHOUT *et al*., 2004). All these cases; stutter peaks, allelic dropouts, and null alleles can violate Hardy-Weinberg proportions which definitely leads to biases in population genetic analyses (OOSTERHOUT *et al*., 2004). However, there are freely available softwares such as GIMLET (VALIERE, 2002), MICRO-CHECKER (OOSTERHOUT *et al*., 2004), PEDMANAGER (EWEN *et al*., 2000), CERVUS 2.0 (MARSHALL *et al*., 1998) and DROPOUT (MCKELVEY & SCHWARTZ, 2005) for the detection and identification of various genotyping errors.

1.8. Purpose of the Study

The primary purpose of this study was to assess the magnitude of genetic change among Bozdağ population[2] (*i.e*. source population at Bozdağ PBS) and reintroduced populations (at Nallıhan & Karadağ) of Anatolian mouflon, *Ovis gmelinii anatolica* (VALENCIENNES, 1856). Also, temporal change in genetic diversity and allelic frequencies of Bozdağ population was determined by comparisons with former genetic analysis (*i.e*. ÖZÜT, 2001).

For the lesser purposes, catching strategy was tested by comparisons among traps for various genetic parameters. Secondly, founder events and bottlenecks were analyzed via computer simulations and the parametric changes were tracked. This enabled to

[2] The population inhabiting in Bozdağ PBS can be named variously as *source population*, *Bozdağ population*, or *captive population* within this study. Individuals inhabiting outside Bozdağ PBS but within Bozdağ WDA were not analyzed by this study.

compare observed and expected genetic diversity values for reintroduced samples and to draw conclusions on the randomness of translocated individuals. In addition, bottleneck detection sensitivity of heterozygosity excess (CORNUET & LUIKART, 1996) and distortion of allele frequencies distribution (LUIKART *et al*., 1998) methods implemented by BOTTLENECK program (PIRY *et al*., 1999) were tested. Based on simulation results, conclusions underlying the bottleneck detection failure for Bozdağ population were drawn.

CHAPTER 2

MATERIALS AND METHODS

2.1. Study Areas

The study areas of the conservation program are; Bozdağ Wildlife Development Area (WDA) in Konya-Bozdağ, Emremsultan WDA in Ankara-Nallıhan, and Karadağ in Karaman. The first area is where the source population is located. The second and third areas are where individuals were first reintroduced and restocking is still in progress.

2.1.1. Bozdağ WDA

Bozdağ WDA is located at 50thkm of Konya-Aksaray highway. This area includes a breeding station surrounded with electroshock fences. The protection area totally covers 42,000 ha whereas the breeding station covers 5,000 ha (Figure 2.1). For this reason, it is possible to say that there are two subpopulations of Anatolian mouflon in the protection area; one subpopulation is within the PBS and one subpopulation is within protection area exclusive of PBS. The elevation of this area ranges between 1000m to 1750m asl. The highest peak is at Hodulbaba mountain (elev. 1746m).

Protection area is generally stony and rocky due to erosion by wind, and soil is generally seen only on the plane regions or the ground valleys (KAYA, 1989). Steppe is typical vegetation which also constitutes most of the diet of mouflon. However, vegetation outside the fenced area is commonly swept by heavy grazing due to

high number of villages around protection area where local folk commonly earns from stock-breeding and agriculture. There are about 350-400 species of plants in the area, but members of *Graminea* family dominates including *Festuca* spp., *Poa* spp., *Dactylis* spp., *Echinaria* spp., *Koeleria* spp., *Phleum* spp., *Stipa* spp., and *Bromus* spp. Members of *Labiatae*, *Rosacea*, *Asteraceae*, and *Umbelliferae* families are also found in the area and consumed by mouflons (DURAL, 1985; KAYA & AKSOYLAR, 1992; ARIHAN, 2000). Shrubs and other woody species are very rare. Typically, *Rhamnus thymifolius*, *Amygdalus korshinskyi*, *Amygdalus balansae*, *Pistacia terebinthus*, and *Rhus coriera* are found in the area and grow not higher than 4m (ARIHAN, 2000).

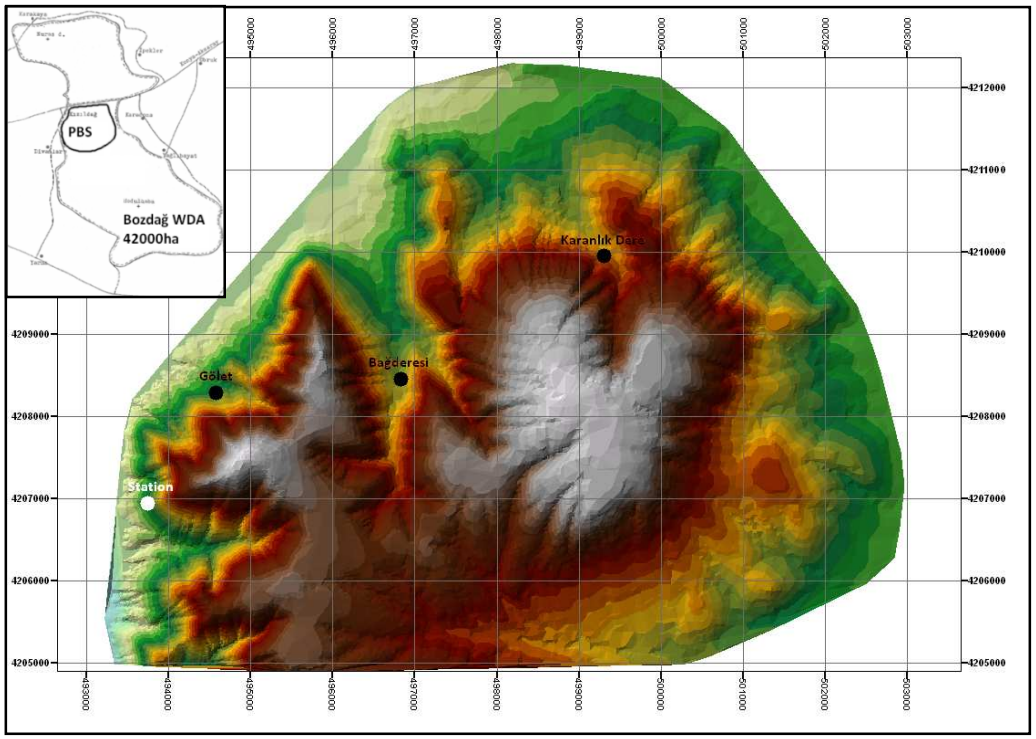

Figure 2.1. Map of Bozdağ PBS with *Universal Transverse Mercator* (UTM) projection.
<Labels/> *White point*, station of wardens; *Black points*, location of traps: Gölet, Bağderesi, and Karanlık Dere.
Geographic coordinates of Bozdağ PBS; 38°01′18″ N 32°58′57″ E.

According to Mineral Research and Exploration Institute, Bozdağ WDA is mainly composed of metamorphic marble and sedimentary conglomerates (KAYA, 1989). In this region of Turkey, continental climate is dominant where summers are dry and hot, winters are cold and snowy. Protection area is covered with snow especially during December and January. Mean annual temperature is 11.4°C; highest in July (23.2°C) and lowest in January (-0.3°C). Annual precipitation is 326.9mm; highest in January (40.9mm) and lowest in September (4.4mm).

2.1.2. Reintroduction Sites

2.1.2.1. Emremsultan WDA

Emremsultan WDA is located in Nallıhan region at the western part of Ankara province. Specifically, Emremsultan WDA is located at the southernmost part of Nallıhan and is bounded from west by Emremsultan and Sarıyar villages, from east by Davutoğlan bridge, from north by Nallıhan-Ankara road and from south by Sarıyar Dam Lake (part of Sakarya river) which also draws the boundary between Ankara and Eskişehir provinces. The distance between Sarıyar and Ankara city center is about 165km.

This protection area was established not only for the protection of Anatolian mouflon but also for partridge and rabbits. The area includes Sarıyar PBS, a fenced area established in 1981, where Anatolian mouflon breed. The total area of protection area is 18,284ha whereas Sarıyar PBS covers only 4ha (data from DNP database). The elevation of this region is lower than the Bozdağ and Karadağ and changes between 430m to 800m (Pazarcıkçı, 1998). Beeline distance from Bozdağ WDA is about 200km whereas road distance is about 350km.

This area mostly has the typical characteristics of Iranian-Turanian phytogeographic region. *Asteraceae*, *Fabaceae*, *Poaceae*, *Brassicaceae*, and *Lamiaceae* families (descending sort) own about 50% of all species and subspecies. The same family order and percentage also holds for genera. Additionally, the highest number of

species and subspecies are found in *Astragalus*, *Centaurea*, and *Bromus* genera in descending sort. Vegetation consists of forest, steppe, and grass vegetations and also rock plants. *Pinus nigra*, *Juniperus oxycedrus*, *Juniperus excels*, *Quercus pubescens*, *Amygdalus webbii*, *Populus tremula* are the mostly found species of forest vegetation in this area. Among steppe vegetation, *Astragalus* spp. are predominant and mainly includes *A. strictifolius*, *A. lydius*, *A. angustifolius*, *A. karamasicus*, *A. wiedemanniiau*. Predominant species for grass vegetation are *Ranunculus repens*, *Melilotus officinalis*, *Veronica anagalis-aquatica*, *Plantago major*, *Selix alba*, *Muscari comosum*, *Colchicum osovitsii*. Typical rock plants are *Acantholimon acerosum*, *Onobrychis armena*, *Sedum sartorianum* (PAZARCIKÇI, 1998).

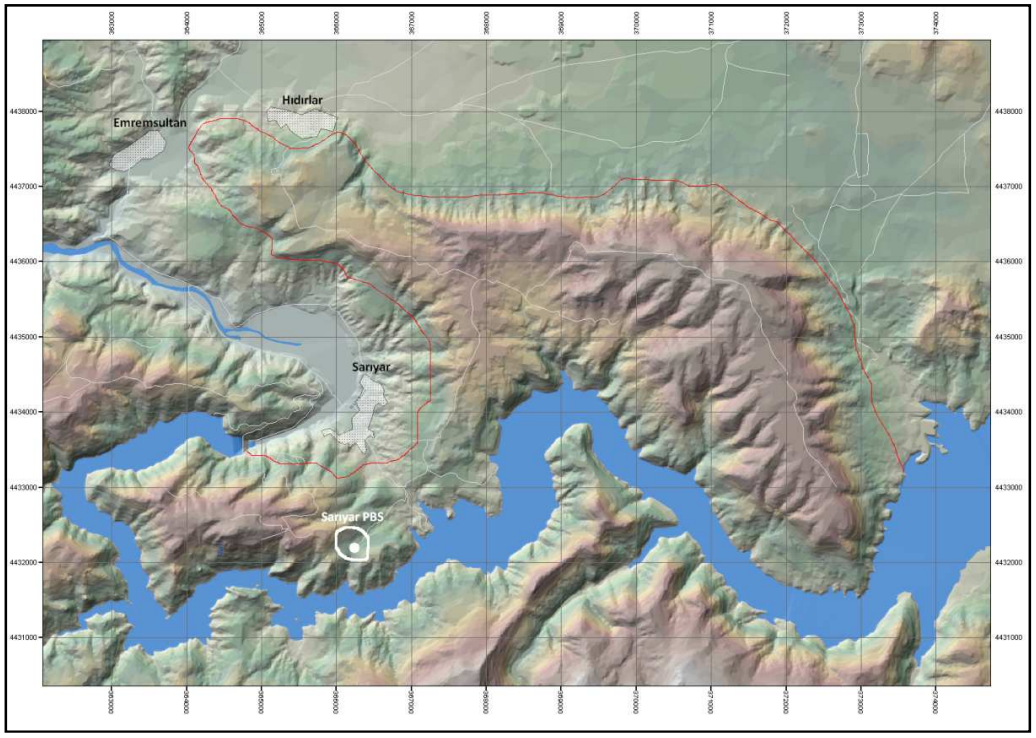

Figure 2.2. Map of Emremsultan WDA with UTM projection.
<Labels/> *Red line*, boundary of protection area; *White label*, Sarıyar PBS; *White point*, station of wardens; *Gray regions*, small towns; *Gray lines*, roads.
Geographic coordinates of Sarıyar PBS: 40°01′39″ N 31°25′57″ E.

Geological characteristics of the region were investigated by Mineral Research and Exploration Institute. The oldest formations are chalks belonging to Paleozoic era, Jurassic and Cretaceous periods, and to Eocene epoch. New formations include clays, sands, and conglomerates belonging to Tertiary (Neogene epoch) and Quaternary periods (PAZARCIKÇI, 1998).

Mediterranean climate is dominant in Nallıhan; summers are hot and dry and precipitations occur in winter and spring. According to the archives of Ankara Meteorology Directorate, annual mean temperature is 14.6°C. In summer seasons, mean temperature rises to about 24°C, where as in winters, it declines to about 4.5°C. Annual precipitation for this area is 380.4mm. December shows the highest precipitation (55.8mm) among all months, and winter seasons show the highest mean precipitation (about 49mm) (PAZARCIKÇI, 1998).

2.1.2.2. Karadağ

Karadağ is located in Karaman province. This reintroduction site is an extinct volcano located at 35 km to the north of Karaman city center very close to the Konya-Karaman boundary. This place is higher than Bozdağ with the highest peak at Mihaliç Tepe (elev., 2271m). Again there is a fenced area in Karadağ established by DNP for pre-release reintroductions but this fenced area is not a PBS for Anatolian mouflon and Karadağ is not a WDA like Bozdağ and Emremsultan sites. There are many small towns and villages on the mountain foot but access to this area is still problematic. This can be inferred from the ratio of beeline and road distance which are about 60 and 150km from Bozdağ WDA, respectively. Transportation problems greatly reduce the efficiency of reintroductions and radio-telemetric monitoring. Consequently, these studies were mostly performed at Emremsultan WDA.

There are 471 different species of plants in Karadağ and more than 60 of them are endemic (*e.g. Astragalus albertshoferi*). Forest vegetation was mostly destroyed but remains of this formation is still found at the part of Karadağ that is between south slopes and the northern slopes of Göztepe at the north (1502m) typically above

1300m and 1150m, respectively. The dominant plants of these remains are oaks (*Quercus* spp.). Also, maple (*Acer* spp.), mountain ash (*Sorbus* spp.), hawthown (*Crataegus* spp.) can be seen. Grass formation is widespread in Karadağ and *Cousinia birandiana* and *Koelpinia linearis* are predominant. Members of *Astragalus*, *Verbascum*, *Tulipa*, *Gagea*, *Thymus*, *Festuca*, *Dianthus*, *Artemisia*, *Tanecetum* genera are also found in the area. This region is under the influence of continental climate. Annual mean temperature for Karaman is 11.7°C. The hottest month is July (23.3°C) and coldest month is January (0.2°C). The annual precipitation for Karaman is 336.4mm. In December, precipitation maximizes to 44.2mm. Lowest precipitation is 4mm and belongs to August. Precipitation is highest in winter (38.4%) and spring seasons (34%). (AVCI, 2004).

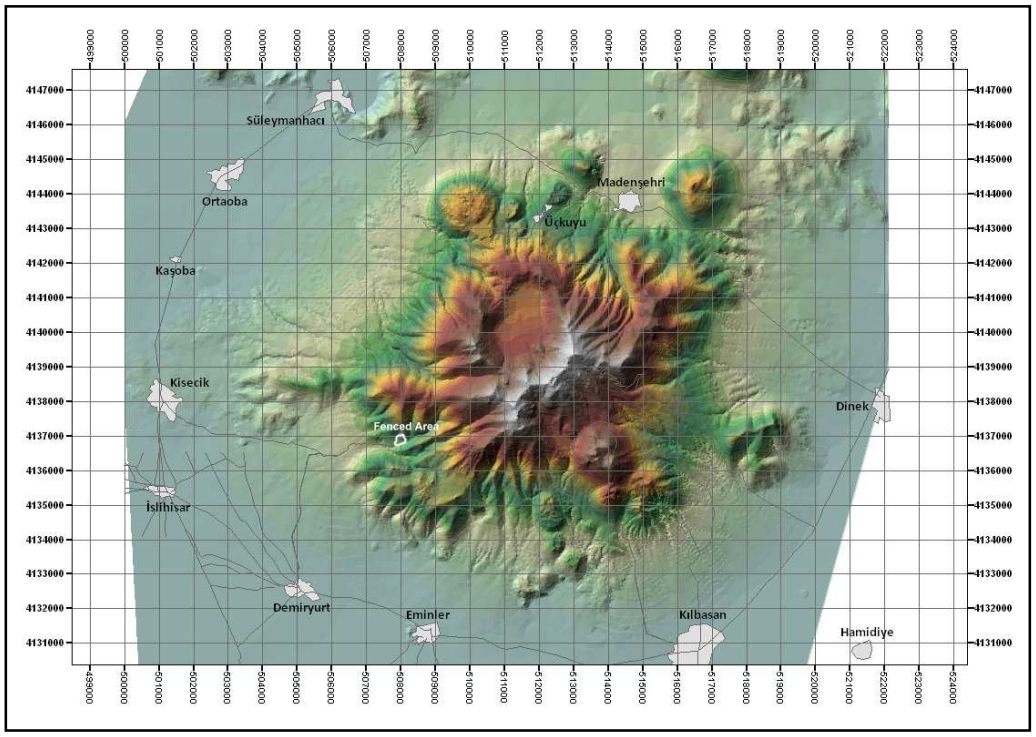

Figure 2.3. Map of Karadağ with UTM projection.
<Labels/> *White label*, location of fenced area; *Gray regions*, villages or small towns; *Gray lines*, roads.
Geographic coordinates of Fenced Area: 37°22′36″ N 33°05′30″ E.

2.2. Capturing & Sampling

All samples were collected at Bozdağ PBS. Totally 172 samples were collected with non-destructive sampling method by cutting a small piece (≈0.2cm^3) from the ear tips with a metal pincer. The samples were preserved with 96% EtOH in 1.5 ml sterile tubes. During sampling, the metal pincer was sterilized by heat to prevent contaminations between steps. Samplings were done with employees from Department of National Parks (DNP) and Game-Wildlife Department (GWD). Nearly all (167) sampled individuals were captured by 3 large traps built by DNP at different places within Bozdağ PBS; Gölet, Bağderesi, and Karanlık Dere (see Figure 2.1). Respectively, 25, 26, and 116 individuals were captured via trapping. This makes a total of 167 individuals. The remaining 5 individuals were shot with anesthetic gun and sampled thereafter which is another way of non-destructive sampling. These individuals were generally rams older than 4 years old who are more sure-footed against the traps than younger mouflons and may wait near the traps instead of just entering. The traps each cover about 180-200 m^2 and are enclosed by 3m high wooden planks. Each trap has one entrance opening to only one direction that allow the animals to interior part but do not permit escaping out. Within the traps there are drinking basins and supplementary food for the captured animals since they may spend some time inside before they are sampled. In order to represent the area homogenously, traps were built at particular positions and more than one trap were used. Otherwise one part of the area could be sampled much more than the remaining parts which will lead to unintended statistical bias for all types of related data.

Following samplings, while some of the individuals were released back to the Bozdağ PBS, the others were translocated either to Emremsultan WDA or to Karadağ. However, not every captured or translocated individual was sampled. Among 131 individuals translocated to Emremsultan WDA, 81 individuals and among 61 individuals translocated to Karadağ only 22 of them were sampled (see Table 2.1). Briefly, microsatellite analyses include totally 172 individuals and 103 of them are translocated individuals.

2.3. Reintroduction

Seven translocations were done since 2004; 4 translocations to Sarıyar WDA and 3 translocations to Karadağ. The individuals were translocated within wooden crates overland. In total, 131 individuals were translocated to Sarıyar PBS and 104 of these individuals were released into Emremsultan WDA. For Karadağ, the numbers are 61 and 57, respectively. Hence, in total 192 individuals were translocated and 161 were released. Table 2.1 summarizes the details and Figure 2.4 represents the age groups for reintroduced and released individuals.

Table 2.1. Recordings of reintroduction studies.

Capture Site	Trans. Site	Trans. Date	Release Date	# Trans. Ind.	# Sampled Ind.	# Released Ind.
Bozdağ	Nallıhan	Sept 2004	Oct 2005	**55** (21F, 6M, 16f, 12m)	**5** (*ni*)	**52** (26F, 18M, 4f, 4m)
Bozdağ	Nallıhan	Febr 2005	Oct 2005	**14** (8F, 5M, 1m)	**14** (8F, 4M, 2m)	
Bozdağ	Nallıhan	Nov 2006	Dec 2006	**15** (5F, 4f, 6m)	**15** (5F, 4f, 6m)	**14** (4F, 4f, 6m)
Bozdağ	Nallıhan	Aug 2007	Oct 2007	**47** (17F, 13M, 9f, 8m)	**47** (14F, 2M, 12f, 17f)	**38** (13F, 13M, 7f, 5m)
Subtotal	-	-	-	**131**	**81**	**104**
Bozdağ	Karadağ	Oct 2004	Oct 2005	**40** (14F, 7M, 10f, 9m)	**4** (*ni*)	**57** (25F, 15M, 8f, 9m)
Bozdağ	Karadağ	June 2005	Oct 2005	**3** (3M)	-	
Bozdağ	Karadağ	Febr 2005	Oct 2005	**18** (7F, 1M, 6f, 4m)	**18** (11F, 1M, 2f, 4m)	
Subtotal	-	-	-	**61**	**22**	**57**
TOTAL	-	-	-	**192**	**103**	**161**

<Abbr/> *Trans*., Translocation; *Ind*., Individuals; *F*, adult female; *M*, adult male; *f*, female lamb; *m*, male lamb; *ni*, no information.

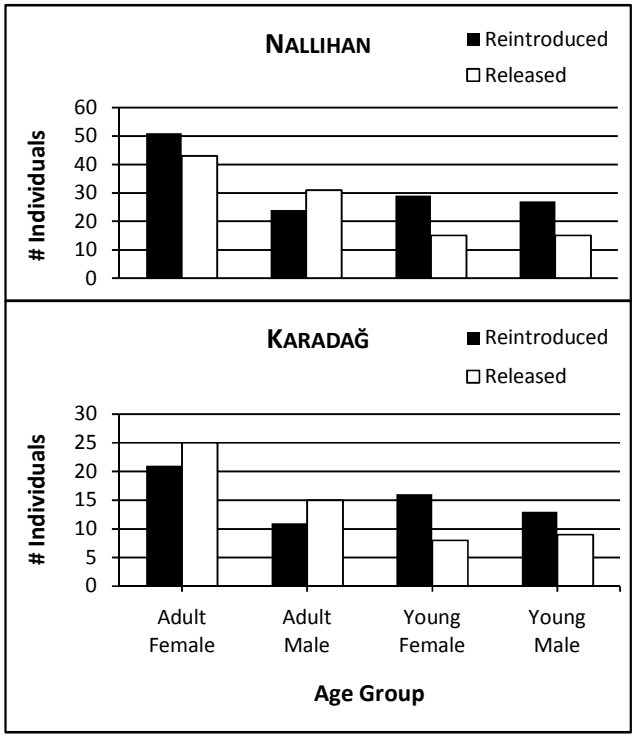

Figure 2.4. Age groups of reintroduced and released individuals.

2.4. DNA Isolation

DNA isolation was performed only with tissue samples. For their very small sizes (about 0.3cm^3), tissue samples were used very carefully. For all DNA isolations, only 20% of each sample was used and the remaining parts are preserved in 99.6% EtOH. The two most important factors for a successful DNA isolation are (*1*) DNA purity and (*2*) DNA yield. A satisfactory DNA purity for Polymerase Chain Reaction (PCR) experiments can be derived even from minute pieces of sample with a suitable protocol. However, for DNA yield largely correlated with the sample size, a more efficient protocol should be adopted. For the current study, DNeasy®Tissue Kit (QIAGEN GmbH, Germany) was used for DNA isolations and the protocol is summarized in Table 2.2. Generally, tissue samples were very fresh, thus 400µl of

AE were used during DNA extraction. This amount is well enough for analyzing 11 microsatellite loci. One of the failures that require cautiousness is to reduce usable DNA content by freezing and defrosting DNA isolates regularly. So as to avoid such a mischance, DNA samples under consideration were kept at 4°C during the time of analyses and then transferred to -80°C.

Table 2.2. DNA isolation protocol for tissue samples.

Before incubation

1. Cut a tiny piece of ~25 mg from the tissue sample with a sterile blade on a sterile surface and put it in a 2 ml o-ringed tube containing 180 µl of ATL buffer.
2. Add 20 µl proteinase K and vortex briefly.
3. Incubate at 56ºC with continuous shaking until the tissue is completely lysed in an incubator/shaker for 4-6 hours (or overnight).

After Incubation

4. Before starting the second part, put AE buffer into water bath at 70ºC.
5. After the incubation, centrifuge the tubes at 5000rpm for 15sec.
6. Add 200 µl AL buffer, vortex for 15 sec. and incubate at 70ºC for 10 min in water bath.
7. Add 200 µl absolute ethanol and vortex for 15 sec.
8. Pipet the mixture (including the precipitates except the hairs) into the DNeasy mini column sitting in a new 2-ml collection tube. Centrifuge for 1 min at 8000 rpm, then discard the collection tube (along with the accumulated liquid in it) and place the mini column in a new 2-ml collection tube.
9. Add 500 µl AW1 buffer into the mini column and centrifuge for 1 min at 8000 rpm. Discard the collection tube (along with the accumulated liquid in it) and place the mini column in a new 2 ml sampling tube.
10. Add 500 µl AW2 buffer into the mini column and centrifuge for 1 min at 8000 rpm. Pour the liquid collected in the collection tube and discard the collection tube.
11. Place the mini column in an eppendorf tube and take AE buffer from the water bath and according to the following instructions;
    a. for old skin samples, horn extracts or pieces of bone : 100 µl AE
    b. for skin samples that are not old : 200 µl AE
    c. for very new skin samples : 400 µl AE
12. Incubate at room temperature for 5 min and then centrifuge for 1 min at 8000 rpm for elution.
13. Depending on the volume of DNA solution required, 12th step can be repeated and elutes are mixed; resulting to a larger volume DNA solution but lower concentration of DNA extract.
14. Discard the mini column. Eppendorf tube now contains DNA isolate.

***** All buffers are available within Qiagen DNeasy®Tissue Kit box.

2.5. Amplification of Microsatellite Loci

Genetic diversity and structure analyses of the source population and 2 reintroduced populations of Anatolian mouflon includes 11 polymorphic microsatellite loci; OarCp20 (EDE *et al*., 1995), ADCYAP1 (WOOD & PHUA, 1993), OarFCB128 (BUCHANAN & CRAWFORD, 1993), OarFCB226 (BUCHANAN *et al*., 1994), OarJMP29 (CRAWFORD *et al*., 1995), BM415 (BISHOP *et al*., 1994), MAF214 (BUCHANAN & CRAWFORD, 1992), SRCRSP3 (AREVALO *et al*., 1994), BM1443 (BISHOP *et al*., 1994), SRCRSP8 (BHEBHE *et al*., 1994), and ILSTS011 (BREZINSKY *et al*., 1993; KEMP *et al*., 1995). The loci analyzed change in their origin as ovine (*Ovis aries*), bovine (*Bos taurus*) and caprine (*Capra hircus*).

Table 2.3. The origin and primer sequences of analyzed loci[3].

LOCUS	Primer Sequence (5′-3′)	Origin
OarCP20	*F* GGCATTTCATGGCTTTAGCAGG *R* GATCCCCTGGAGGAGGAAACGG	Ovine
OarJMP29	*F* GTATACACGTGGACACCGCTTTGTAC *R* GAAGTGGCAAGATTCAGAGGGGAAG	Ovine
OarFCB128	*F* ATTAAAGCATCTTCTCTTTATTTCCTCGC *R* CAGCTGAGCAACTAAGACATACATGCG	Ovine
OarFCB226	*F* GTGAGTCCCATAGAGCATAAGCTC *R* GTTTCTTCTATATGTTGCCTTTCCCTTCCTGC	Ovine
MAF214	*F* AATGCAGGAGATCTGAGGCAGGGACG *R* GGGTGATCTTAGGGAGGTTTTGGAG	Ovine
ADCYAP1	*F* CCAGACGCCGACTTCGCCGAGG *R* GCCTGAAGTCCACTGAGAAGAAAGGA	Bovine
BM415	*F* GCTACAGCCCTTCTGGTTTG *R* GAGCTAATCACCAACAGCAAG	Bovine
BM1443	*F* AATAAAGAGACATGGTCACCGG *R* TCGAGGTGTGGGAGGAAG	Bovine
ILSTS011	*F* GCTTGCTACATGGAAAGTGC *R* CTAAAATGCAGAGCCCTACC	Bovine
SRCRSP3	*F* CGGGGATCTGTTCTATGAAC *R* TGATTAGCTGGCTGAATGTCC	Caprine
SRCRSP8	*F* TGCGGTCTGGTTCTGATTTCAC *R* CCTGCATGAGAAAGTCGATGCTTAG	Caprine

<Abbr/> *F*, forward; *R*, reverse.

[3] Further specifications are presented in Appendix A.

DNA extracts were not diluted because of their low DNA contents. In all PCR experiments, 6µl of pure DNA isolate - directly taken from the stock solution containing 10 to 20ng/µl of DNA - was used with 10µl of PCR mix for every tube (Table 2.4). For the dilution of primers, molecular gradient water was used, but nuclease free water was utilized for PCR experiments.

Table 2.4. Final volume and concentration for PCR chemicals[4].

	Volume		***Concentrations***				
LOCUS	**V_{DNA} (µl)**	**V_{total} (µl)**	**$MgCl_2$ (mM)**	**dNTP[†] (mM)**	**Primer (µM)**	**Taq[‡] (U/tube)**	**BSA (µl)**
OarCP20	6	16	2.5*****	0.2	0.5	1.5	0.25
BM415	6	16	2.5*****	0.2	0.5	1.5	0.25
MAF214	6	16	3.0	0.2	0.2*****	1.5	0.25
ADCYAP1	6	16	3.0	0.2	0.5	1.5	0.25
OarFCB128	6	16	3.0	0.2	0.5	1.5	0.25
OarFCB226	6	16	3.0	0.2	0.5	1.5	0.25
OarJMP29	6	16	3.0	0.2	0.5	1.5	0.25
SRCRSP3	6	16	3.0	0.2	0.5	1.5	0.25
BM1443	6	16	3.0	0.2	0.5	1.5	0.25
SRCRSP8	6	16	3.0	0.2	0.5	1.5	0.25
ILSTS011	6	16	3.0	0.2	0.5	1.5	0.25

***** Dissimilar concentrations.

[†] mix dNTP.

[‡] 10X $(NH_4)_2SO_4$ *Taq* buffer was utilized.

Except during the optimization phase, multiplex PCR applications were not practiced which allow the amplification of more than one locus in one reaction by using more than one pair of primers. The number of cycles for the loci ranged between 40 to 50 cycles, because lesser numbers of cycle were not suitable for intended yields of DNA amplifications In addition to cycles, durations for each step (see Table 2.5) were also beyond normal due to the similar reasons (KIDD & RUANO,

[4] Chemicals and equipments presented in Appendix D and E.

1995). Annealing temperatures (T_A) are also varied ranging between 50 to 61°C, however practicing multiplex PCR experiments is still possible (Table 2.5). Denaturation (T_D) and elongation (T_E) temperatures are 95°C and 72°C for all amplified loci. A final extension period - holding 10min at 72°C after all cycles end - was performed for all PCR experiments.

Table 2.5. Annealing temperatures and # PCR cycles for experiments.

LOCUS	T_A (°C)	# Cycles
BM1443	50	50
OarJMP29	50	40
OarFCB128	51	40
SRCRSP8	51	50
ILSTS011	53	50
ADCYAP1	54	50
OarCP20	56	40
BM415	58	45
MAF214	58	40
SRCRSP3	59	50
OarFCB226	61	45

Cycle durations: *denaturation*, 1min; *annealing*, 1min; *elongation*, 2min.

2.6. Analysis of Amplified Loci

2.6.1. Agarose Gel Electrophoresis

Agarose gel electrophoresis[5] is a widespread method to separate DNA or RNA fragments according to their size via an electric field and is a handy application for checking PCR products. Unlike polyacrylamide gel electrophoresis, agarose gel

[5] Composition of solutions presented in Appendix C.

electrophoresis cannot separate small nucleic acids efficiently and has a low resolution. Hence, it can constitute only a part of the whole analysis of PCR products.

During agarose gel electrophoresis, ethidium bromide (EtBr) - the most common dye for nucleic acid staining - was not added to the gel directly due to its increased mutagenic and carcinogenic effects when inhaled after boiling with agarose. Instead, the gel was soaked into EtBr solution after running. All PCR products were electrophoresed on 2% agarose gels and the results were used during genotyping.

2.6.2. Fragment Analysis

Fragment analysis was performed by defined oligonucleotide primers. This requires labeling because detection of fragment lengths is performed by a laser detection system that is capable of assigning a fragment size to all peaks via fluorescent dye-labeled oligonucleotides. There are various fluorescent dyes, but the ones used to label the forward primer sequences (Table 2.3) are: 6-carboxy-fluorescine (6-FAM), hexachloro-6-carboxy-fluorescine (HEX), and tetrachloro-6-carboxy-fluorescine (TET). TET reflects green color, while 6-FAM and HEX reflect blue and yellow colors, respectively. For fragment size assignment, GeneScan™ 350 TAMRA™ Size Standard was used which contains flourescently labelled DNA fragments of known size as reference. Loci are categorized in Table 2.6 with respect to applied fluorescent dyes.

For a more efficient and also cheaper fragment analysis, a single individual's all PCR products were pooled into one capillary injection, however it is critical to ensure that the fragment size range of markers do not overlap when multiple markers of the same dye are pooled. Second critical point is pooling the PCR products in correct ratios to get similar fluorescent intensities across all loci in the pool. The intensity of emitted fluorescence is different for each dye used. Hence, a greater amount is added to the pool from the PCR products labeled with dyes of low emission. It was aimed to get peaks between 1000-10000fu (fluorescent units). The

fragment analysis of products was utilized with ABI PRISM® 310 Genetic Analyzer which is an automated single-capillary analyzer for fragment and sequence analyses.

Table 2.6. Categorization of loci on fluorescent dyes[6].

		LOCUS	Size Range (*bp*)
FLOURESCENT DYE	***6-FAM*** **(blue)**	OarCP20	75 - 100
		OarFCB226	116 - 150
		BM1443	200 - 240
	HEX **(yellow)**	OarFCB128	110 - 130
		BM415	140 - 170
		SRCRSP3	182 - 192
	TET **(green)**	ADCYAP1	95 - 120
		OarJMP29	130 - 150
		MAF214	180 - 190
		SRCRSP8	222 - 251
		ILSTS011	282 - 288

<Abbr/> *bp*, base pair.
*All oligos are 5'-end labeled and purified by HPLC[7].

2.6.3. Genotyping

Genotyping follows fragment analysis and is the assignment of alleles to resulting peaks that correspond to the expected fragment size ranges. During genotyping, results from fragment analysis and agarose gel electrophoresis were collated in order to minimize the errors due to misinterpretation, since the resulting peaks may require a comparison with the gel bands for more reliability. Thus, carefully made agarose gels are of great importance during genotyping. The most common encountered problems during genotyping are (*1*) poor or non-specific amplification, (*2*) incomplete 3´ A nucleotide addition, and (*3*) stutter. These problems can be solved with appropriate techniques (refer to GeneScan® Reference Guide for solutions).

[6] Molar concentrations and compositions of oligos are presented in Appendix B.
[7] High Performance Liquid Chromatography.

2.7. Statistical Analysis

For the analyses of genetic diversity and genetic structure, freely available software programs were used; Genepop v.3.4 (RAYMOND & ROUSSET, 2003), Arlequin v.3.11 (EXCOFFIER *et al*., 2007), Popgene v.1.32 (YEH *et al*., 1999), and *F*stat v.2.9.3.2 (GOUDET, 2002). More than a single software program was used for the same data analysis, if possible.

2.7.1. Genetic Diversity Analysis

Measurements of genetic diversity include *allelic frequencies* (p_i), *proportion of polymorphic loci* (*P*; NEI *et al*., 1975), *allelic diversity* (*A*), *mean number of alleles* (n_k), *effective number of alleles* (A_E or n_e; NIELSEN *et al*., 2003), *observed heterozygosity* (H_o), and *expected heterozygosity* (H_e) according to LEVENE (1949) and NEI (1973).

Allelic frequency for the i^{th} allele is calculated by;

$$p_i = P_{ii} + \frac{1}{2 \cdot \sum P_{ij}} \qquad (2.1)$$

where $j \neq i$. P_{ii} and P_{ij} are genotype frequencies for i^{th} allele in homozygote and heterozygote states, respectively. The variance for the frequency of i^{th} allele is calculated by:

$$V(p_i) = \frac{p_i(1-p_i)}{2N} \qquad (2.2)$$

Proportion of polymorphic loci (*P*) is computed by;

$$P = \frac{n_p}{n_{total}} \qquad (2.3)$$

where n_p is the number of polymorphic loci and n_{total} is the total number of loci (either monomorphic or polymorphic).

Mean number of alleles for a single population is calculated by;

$$n_k = (\frac{1}{k}) \sum_{i=1}^{l} n_i \quad (2.4)$$

where l is the total number of loci and n_i is the number of alleles detected for each loci of a single population. Effective number of alleles is the number of alleles that would be expected at a locus and calculated for a particular locus by;

$$A_E = \frac{1}{1-H_e} = \frac{1}{\sum_{i=1}^{k} p_i^2} \quad (2.5)$$

where H_e is expected heterozygosity (inverse of H_e). Effective number of alleles is important for the establishment of collecting strategies. Like in the case of mean number of alleles, *average <effective number of alleles>* is calculated by taking the arithmetic mean of all locus values.

Observed heterozygosity *(H_o)* is simply the ratio of the number of heterozygotes at a locus to the total number of samples surveyed for that locus. H_o and obs. homozygosity sum up to unity for a locus. Expected heterozygosity (H_e) - or Hardy-Weinberg heterozygosity - is calculated according to Hardy-Weinberg proportions using allele frequencies for a locus (likewise, H_e + *exp. Hom.* = 1). Expected heterozygosity at a locus is formulated as;

$$H_e = 1 - \sum_{i=1}^{k} p_i^2 \quad (2.6)$$

where p_i^2 is the frequency of homozygous genotype for the i^{th} allele and k is the number of alleles. This measure is called *gene diversity* (NEI, 1987). The unbiased estimation of H_e and the sampling variance are formulated as;

$$\widehat{H} = \frac{n}{n-1} \cdot (1 - \sum_{i=1}^{k} p_i^2) \quad (2.7)$$

$$V(\widehat{H}) = \frac{2}{n(n-1)} \cdot \{2(n-2)[\sum_{i=1}^{k} p_i^3 - (\sum_{i=1}^{k} p_i^2)^2] + \sum_{i=1}^{k} p_i^2 - (\sum_{i=1}^{k} p_i^2)^2\} \quad (2.8)$$

where n is the number of gene copies (2 x sample size) and n/n(n-1) is the small sample size correction. k is the number of alleles for a particular locus and p_i is the frequency of the i^{th} allele (NEI & ROYCHOUDHURY, 1974). Thus, the standard deviation for heterozygosity is;

$$s.d.(\widehat{H}) = \sqrt{V(\widehat{H})} \quad (2.9)$$

2.7.2. Genetic Structure Analysis

2.7.2.1. Hardy-Weinberg Equilibrium Test

Hardy-Weinberg Equilibrium, HWE (or *Hardy-Weinberg Principle*, HWP), states that by the absence of factors that tend to change the gene frequencies of a population Hardy-Weinberg genotype frequencies will stay constant. These factors are formulized as selection, random genetic drift, mutation, gene flow, and nonrandom mating. If genotype frequencies are changed, they will return to Hardy-Weinberg proportions after one generation of random mating. Likewise if allele frequencies are changed, then the Hardy-Weinberg proportions will change according to the new frequencies of alleles.

HWE test simulations were performed using two software programs; GENEPOP and ARLEQUIN programs. The former utilizes 3 alternate tests with the same null hypothesis (random mating) but different rejection zones. The first test computes *p*-values with the *Exact HW Test* of HALDANE (1954), GUO & THOMPSON (1992), and WEIR (1996). The second and third tests were utilized to detect heterozygote excess

or deficiency for each locus and for all loci pooled (global test). GENEPOP uses two distinct algorithms according to the number of alleles at a particular locus. These are the Complete Enumeration algorithm of LOUIS & DEMPSTER (1987) and Markov Chain (MC) walk algorithm of GUO & THOMPSON (1992), a form of *Metropolis-Hastings* algorithm (METROPOLIS *et al*., 1953; HASTINGS, 1970). Since the complete enumeration algorithm causes an exponential growth of the number of possible samples as the allele number at a locus increases, this algorithm has practical use only for loci having less than 5 alleles (HERNÁNDEZ & WEIR, 1989; GUO & THOMPSON, 1992). Consequently, for loci having more than 4 alleles, a more efficient MC algorithm is applicable for the exact test for HWE. But, MC method is also performable when allele number is less than 5. However, global test only uses MC algorithm and assumes independence among loci.

ARLEQUIN program tests HWE in a similar simulation. However only the MC algorithm of GUO & THOMPSON (1992) is utilized and *p*-values are computed accordingly.

2.7.2.2. Linkage Disequilibrium Test

Linkage disequilibrium (LEWONTIN & KOJIMA, 1960), or *gametic phase disequilibrium* (CROW & KIMURA, 1970), is the nonrandom association of alleles at different loci into gametes (HEDRICK, 2005), thereby causing some combinations of alleles to occur with different frequencies than would be expected by the observed gene frequencies with the assumption of random association. In other words, linkage disequilibrium makes it more probable to predict a genotype at a locus by knowing the genotype at any other locus (FREEMAN & HERRON, 2001). Therefore, if it exists, knowing the degree of linkage disequilibrium is important for better statistical inference of genetic analysis.

Linkage disequilibrium test simulation was performed with GENEPOP program. After creating the contingency tables for all pairs of loci, a probability test (FISHER's Exact Test) with using the *Markov chain* algorithm of RAYMOND & ROUSSET (1995) was

performed. Linkage disequilibrium test ignores monomorphic loci. Computations of GENEPOP were also checked with ARLEQUIN program.

2.7.2.3. Neutrality Test

Neutrality was tested, because it is crucial to know whether the results of microsatellite analysis are consistent with Neutral Theory (KIMURA, 1968), since it assumed that loci under study are selectively neutral. In order to test neutrality, WATTERSON (1978) developed a test where the Hardy-Weinberg homozygosity (f_e) is compared with equilibrium homozygosity (f_{eq}) which is calculated according to the mutation-genetic drift equilibrium under neutral theory (HEDRICK, 2005).

EWENS-WATTERSON Test was performed with POPGENE program. For the distribution of *F*-values, algorithm of MANLY (1985, p.272-282) is used;

$$F = \frac{\sum_{i=1}^{k} n_i^2}{n^2} \qquad (2.10)$$

where n is the number of gene copies and k is number of alleles for a particular locus, and n_i is the allele frequency of i^{th} allele.

2.7.3. Estimation of Effective Population Size

Effective population size (N_e) is one of the leading parameters in evolutionary biology and ecology for understanding evolutionary processes in natural populations and conservation biology for the management of threatened species. It was first formulized by WRIGHT (1931) as *the number of breeding individuals in an idealized population* – in which any parent(s) can be the parent(s) of any progeny with equal probability - *that would show the same amount of dispersion of allele frequencies under random genetic drift or the same amount of inbreeding as the population under consideration*. Thus, N_e is a measure for the rate of genetic drift, and directly

related with the rates of loss of heterozygosity and inbreeding (Rieman & Allendorf, 2001).

Estimating N_e is very difficult due to the stochastic basis of inbreeding and genetic drift (WAPLES, 1989; WANG, 2005). There are different estimations of N_e based on either demographic data or genetic data. But, estimation of N_e from demographic data is often very difficult and generally leads to unreliable estimations (LUIKART & CORNUET, 1999). Estimation of N_e from genetic data is relatively easy and 4 different methods are available; the heterozygote excess method (PUDOVKIN *et al*., 1996; LUIKART & CORNUET, 1999), the loss of genetic variation (based on heterozygosity) method (*e.g*. HARRIS & ALLENDORF, 1989; RIEMAN & ALLENDORF, 2001), the linkage disequilibrium (between pairs of segregating loci) method (HILL, 1981; WAPLES, 1991; BARTLEY *et al*., 1992; ARDREN & KAPUSCINSKI, 2003), and according to the temporal variation in allele frequencies so called the temporal method (KRIMBAS & TSASKAS, 1971; NEI & TAJIMA, 1981; POLLACK, 1983; WAPLES, 1989). Methods are reviewed briefly by WANG (2005).

Since temporal method and loss of heterozygosity methods need temporal samples for the estimation of N_e, they are not performable with the current data. Hence, N_e was calculated with 2 methods; heterozygote excess method and linkage disequilibrium method. For this purpose, NeEstimator v.1.3 (PEEL *et al*., 2004) and *LDNE* v.1.31 (WAPLES & DO, 2008) software programs were used. The former make estimations by utilizing both methods whereas the latter uses only linkage disequilibrium method.

2.7.4. Population Bottleneck Analysis

Population bottlenecks are important processes for the management of conservation strategies because the negative effects of recent bottlenecks on genetic diversity may still persist. Most threatened species are known to have very low levels genetic diversity due to population bottlenecks (TAYLOR *et al*., 1994; GIBBS *et al*., 1998), since reduction in genetic diversity exerts high selective pressure by decreasing

evolutionary potential and may even lead to extinction of species (ALLENDORF & LEARY, 1986). Conservation plans should include strategies that can compensate with these types of negative effect by identifying recent genetic bottlenecks. For this purpose, detecting population bottlenecks via actual genetic variation has become a common method.

Bottleneck analysis was performed with Bottleneck v.1.2.02 software program (PIRY *et al*., 1999). This test simulation detects recent (within past $2N_e - 4N_e$ generations) severe reductions in N_e. Heterozygosity excess method is used and it assumes that in the case of a recent reduction in N_e, allelic diversity (A) at polymorphic loci is expected to decrease at a faster rate than expected heterozygosity (H_e) (NEI *et al*., 1975; DENNISTON, 1978). Since equilibrium heterozygosity (H_{eq}) is computed using A, a recent bottleneck causes H_{eq} to decrease faster than H_e and leads to heterozygosity excess ($H_e > H_{eq}$). Non-bottlenecked populations are expected to be at mutation-drift equilibrium where half of the loci show heterozygosity excess ($H_e > H_{eq}$) and the other half show heterozygosity deficiency ($H_e < H_{eq}$) (CORNUET & LUIKART, 1996; LUIKART & CORNUET, 1998). However, heterozygosity excess that is higher than expectations points to mutation-drift disequilibrium and recent bottlenecks. Thus, BOTTLENECK program tries to detect heterozygosity excess at analyzed loci with respect to different mutation models (PIRY *et al*., 1999). For this purpose, 3 statistical tests are performed with the same null hypothesis (ie. all loci fit mutation-drift equilibrium); sign test, standardized differences test, and Wilcoxon signed-rank test (PIRY *et al*., 1999). Standardized differences test is not reliable when there are less than 20 loci and can be disregarded for this study. Sign test does not supply *p*-values for heterozygosity deficiency or excess specifically, but Wilcoxon signed-rank test gives one-tail *p*-value for heterozygosity excess. Heterozygosity excess method is extended to strict one-step stepwise mutation model (SMM; OHTA & KIMURA, 1973), infinite allele model (IAM; KIMURA & CROW, 1964), and two phase model (TPM; DI RIENZO *et al*., 1994). Heterozygosity excess is less observable for loci that follow strict SMM (CORNUET & LUIKART, 1996). As loci depart from SMM to IAM, observable heterozygosity excess increases (PIRY *et al*., 1999). Thus *p*-values are smaller under IAM. Distortion graph

for alleles that are binned by frequency into 10 allele frequency classes was also demonstrated (LUIKART *et al*., 1998) which gives either L-shaped or a shifted-mode distribution. The latter supports a recent genetic bottleneck. BOTTLENECK program needs the data of at least 4 polymorphic loci and 20 to 30 samples. Monomorphic loci and loci that are not under HWE are omitted to prevent statistical violations.

2.7.5. Comparison Tests[8]

2.7.5.1. Comparisons among Populations

Comparisons of genetic diversity of the source population and reintroduced populations are important for the assessment of the success of reintroduction program. These comparisons were done for 3 genetic diversity measurements; allelic diversity (*A*), observed heterozygosity (H_o), and expected heterozygosity (H_e) including both NEI's (1973) and LEVENE's (1949) expected heterozygosity (H_e) computations separately. For the comparisons, paired *t*-test was performed for its high statistical power when the sample sizes are small (LUIKART & CORNUET, 1998). Comparisons between these 3 populations are based on 11 microsatellite loci.

Genetic diversity of the source population was also compared with the results of ÖZÜT (2001). In this study, 48 samples from Konya-Bozdağ were analyzed at 10 polymorphic microsatellite loci and 9 of these loci are identic with those of the current study. Thus, comparisons between these two populations include 9 loci and again done by paired *t*-test. These comparisons offer the chance to observe temporal change in genetic diversity for Bozdağ population.

2.7.5.2. Comparisons among Traps

Traps located at Karanlıkdere, Bağderesi, and Gölet were analyzed by trap-trap and trap-source population comparisons for genetic diversity and *Probability of Identity* (PI) values. For the former, same parameters (*A*, H_o, H_e) were compared with paired

[8] For all statistical comparisons SPSS v.16.0.1 software package was used.

t-test. By verifying the significance levels of comparisons, it is possible to infer on the consistency of capture method. If a trap has a much higher or lower genetic diversity than the others, the locations for traps may need revising.

PI gives the probability of identical multilocus genotype (*img*) – probability of owning the same genotypes at all loci – for 2 individuals drawn randomly. PI ranges from 0 to 1 (right-closed). Overall PI is computed by multiplying PI value of each locus. Gimlet v.1.3.3 (VALIÈRE, 2002) software program was used to PI computations. PI comparisons are expected to support genetic diversity comparisons since PI is negatively correlated with genetic diversity. A larger PI for a group captured within one trap compared to a group captured within another trap illustrates that genetic diversity within the former group is less. Additionally, a lower genetic diversity, or larger PI, *may* further indicate that the group consists of individuals with closer relationships when compared with other groups. PI computations are;

$$PI_{theoric} = \sum_i p_i^4 + \sum_i \sum_{j \neq i} 2p_i p_j \qquad (2.11)$$

$$PI_{unbiased} = \frac{n^3(2a_2^2 - a_4) - (2n^2(a_3 + 2a_2) + n(9a_2 + 2) - 6)}{(n-1)(n-2)(n-3)} \qquad (2.12)$$

$$PI_{sibs} = \frac{1}{4} + \frac{1}{2}\sum_i p_i^2 + \frac{1}{2}(\sum_i p_i^2)^2 - \frac{1}{4}\sum_i p_i^4 \qquad (2.13)$$

where p_i and p_j are the frequencies of the i^{th} and j^{th} alleles and $a_n = \sum_i p_i^n$.

Equation 2.11 assumes random mating within population (PAETKAU & STROBECK, 1994). Equation 2.12 is an unbiased estimator with small sample size correction (KENDALL & STEWART, 1977). Equation 2.13 is for populations composed of only sisters and brothers (sibs) (EWETT & WEIR, 1998; TABERLET & LUIKART, 1999). Thus, PI_{sibs} is always higher than the other computations. The most informative comparisons are those made according to $PI_{theoric}$, since assumptions of others are not in accordance with Anatolian mouflon population.

2.8. Simulations

Simulation experiments are widespread approaches when experimental designs are not easily performable or impossible at any condition, either in the field or laboratory. Monte Carlo (MORGAN, 1984; CAFLISCH, 1998; BERG, 2004) and Markov Chain (MEYN & TWEEDIE, 1993) methods are two distinct approaches for scientific simulations. Monte Carlo method is a stochastic process based on repeated sampling from a given set of test subjects using statistically random but not truly random (*pseudorandom*) numbers. For the purpose of this study, Monte Carlo simulations[9] were compiled for population bottlenecks and founder events as they are the major processes leading to rapid genetic change within gene pools. Both simulation programs are *discrete-event simulations* since they are structurally dynamic, stochastic, and discrete.

Founder event simulation program is based on two processes; (*1*) random sampling of founder populations with variable sizes from a given set of genotypic data, and (*2*) data analysis on random samples. By this way, correlations between founder population size (N_F) and some common measures of genetic diversity can be assessed. These measures are mean; (*1*) number of alleles (n_k), (*2*) total number of alleles (A_T), (*3*) number of rare alleles (A_R), (*4*) number of common alleles (A_C), (*5*) proportion of polymorphic loci (*P*; NEI *et al*., 1975), (*6*) observed heterozygosity (H_o), and (*7*) NEI's (1973) expected heterozygosity (H_e). Founder events were simulated with different microsatellite data sets to better characterize the fluctuations in parametric outputs for founder populations when the genetic diversity within the source population is changed. Thus, real data set of honeybees supplied from a different study was also evaluated for comparison. This simulation disregards generations since there is no purpose for observing temporal changes within genetic diversity following any founder event.

Bottleneck simulation program structurally differs from previous simulation. Simulation starts with an artificial population at HWE that is created and

[9] Written in *C* programming language and compiled with Microsoft Visual *C*++ v.6.0.

bottlenecked according to designated parametric values and finally microsatellite data[10] is generated. The parameters for starting population are; (*1*) population size (N_S), (*2*) number of loci, and (*3*) A_C and A_R ($f < 0.05$; 5% criterion) at each locus. Bottleneck event parameters are; (*1*) generation elapsed during bottleneck (*bottleneck period*, T_1), (*2*) bottlenecked population size (N_B), (*3*) generation elapsed with bottleneck size (*permanence period*, T_2), (*4*) generation elapsed after bottleneck (*recovery period*, T_3), (*5*) size of recovered population (N_R), and (*6*) sample size (N) drawn from recovered population (Figure 2.5). Bottleneck simulation is adapted to random mating and discrete generations. Mutational processes, selection, and gene flow are underestimated. Only genetic drift acts as an evolutionary force according to the stochastic nature of the simulation. This simulation can test the efficiency of bottleneck detection methods by simulating genetic bottlenecks of different magnitude and structure. Heterozygosity excess method and allele frequency distribution method implemented with BOTTLENECK program (PIRY *et al*., 1999) were tested since these methods were utilized for the detection of a recent genetic bottleneck for the captive Bozdağ population (source population) at Bozdağ PBS.

Three parameters have known values and kept constant for all simulation experiments; $T_3 = 10$[11], $N_R = 600$ (see Figure 1.1), and $N = 172$. Due to lack of data, other parameters are unknown and various values were assigned. Structurally different two bottleneck types were analyzed; (*1*) T_1 - T_3 route bottlenecks (TYPE I) where $T_2 = 0$ and $T_1 = 10, 100, 500, 1000, 2000, 5000, 10000$, and (*2*) T_1 - T_2 - T_3 route bottlenecks (TYPE II) where $T_1 = 1$ and $T_2 = 10, 25, 50, 100, 250, 500, 1000, 2000$. Experiments replicated for $N_B = 2, 5, 10, 25$, and 50. Distortion of allele frequency distributions (LUIKART *et al*., 1998) and *p*-values by Wilcoxon signed-rank test in the expected direction (*i.e*. one-tailed test for heterozygosity excess) were analyzed to detect the possible reason(s) for bottleneck detection failure, if any.

[10] Genepop format

[11] From the first establishment of captive population at Bozdağ PBS by 1988, about 20 years have passed which constitutes to max. 10 generations if every generation is taken as ~2 years.

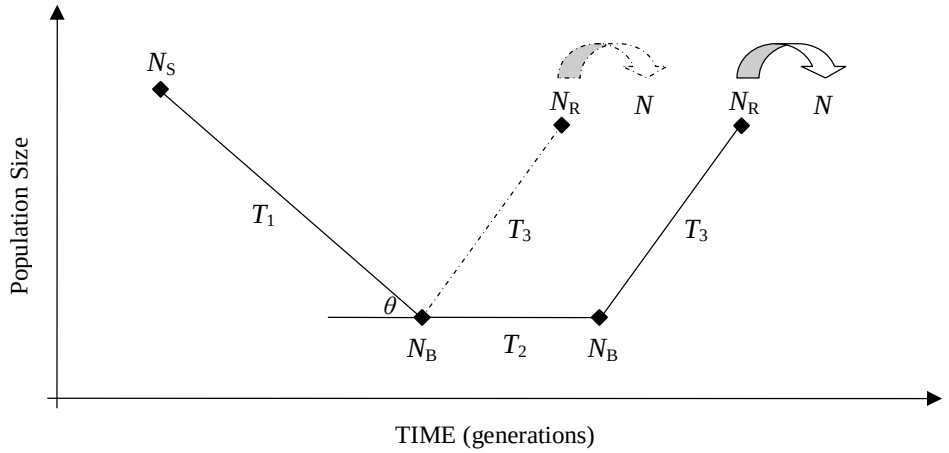

Figure 2.5. Structure of simulated bottleneck events. Simulation follows T_1 - T_3 route (dashed lines; TYPE I), if permanence period is underestimated (T_2 = 0, recovery immediately after bottleneck). Otherwise, T_1 - T_2 - T_3 route (solid lines; TYPE II) is followed. Increased θ indicates increased severity of bottleneck period; (*1*) as time to bottleneck decreases, and/or (*2*) as population size reduction ($N_S - N_B$) increases, θ also increases.

***** For all simulation experiments; N_S = 10000, # loci = 11, A_C = 10, and A_R = 15.

****** Simulated output data of samples (*N*) were used for analyses.

CHAPTER 3

RESULTS

3.1. DNA Isolation

DNA isolated from a total of 172 individuals using tissue samples and every sampled individual was analyzed for the defined 11 loci. Any kind of sample (*e.g.* feces) other than tissue was not used for DNA isolations. DNA isolates were checked with 2% agarose gel and additionally, with spectrophotometer at wavelengths 260nm and 280nm. However, DNA contents were generally low, ranging between 10 to 20ng/µl, due to very small tissue samples used during isolations. Since about 50ng of DNA is generally recommended for each PCR tube, DNA dilution was not done prior to any of the PCR experiments. Figure 3.1 represents the gel electrophoresis result of DNA isolation for 12 samples. Variations in the amount of DNA between samples were also observed during gel checks in addition to spectrophotometer results. However, the ones that have lower DNA contents also worked fairly well during PCR amplifications. All agarose gel checks included a control group and a marker. The marker used for gel electrophoresis is Lambda DNA/PstI Marker®Fermentas and has a range between 15-11501bp. None of the control groups indicated any sign of contamination during agarose gel electrophoresis.

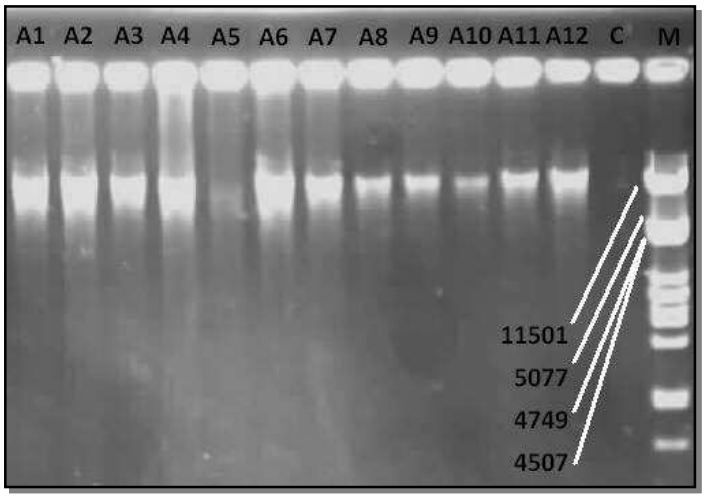

Figure 3.1. Example for agarose gel electrophoresis results. Twelve DNA isolations are shown. "C" stands for the lane of control group and indicates no contamination. "M" stands for the marker (DNA/PstI Marker® Fermentas) and the bands (range between 15-11501) are marked with the corresponding base pair lengths. The DNA band for A5 has a very low intensity but fragment analysis and genotyping was successful for this sample.

3.2. Fragment Analysis & Genotyping

The fragment analysis was successful nearly for all individuals at 11 loci. Fragment analysis utilized with ABI PRISM® 310 Genetic Analyzer and the results were monitored with Genescan® Analysis software program for genotyping. Figure 3.2 exemplifies a typical fragment analysis output and genotyping for one of the samples at 3 loci labeled with the same dye. Out of the total 1892 (11*172) PCR products that had been analyzed, only 24 products could not be genotyped due to poor amplification. Poor amplifications can generate very low peaks that are not usable for genotyping. Generally, peaks lower than 200fu are assumed to be unreliable for correct genotyping and should be ignored. Specifically, 1 individual each for SRCRSP3 / ADCYAP1 / JMP29 loci, 2 individuals for ILSTS011 locus, 4 individuals for SRCRSP8 locus, and 15 individuals for BM1443 locus could not

genotyped. Fragment analysis mostly revealed very clean results with little nonspecific amplifications that do not show stutter bands. Also, amplified loci generally revealed high peaks which lowered the probability for unsuccessful genotyping. The base pair lengths of most alleles were already known, because most of the loci were also analyzed by ÖZÜT (2001). However, new alleles for the shared loci were found (Table 3.1).

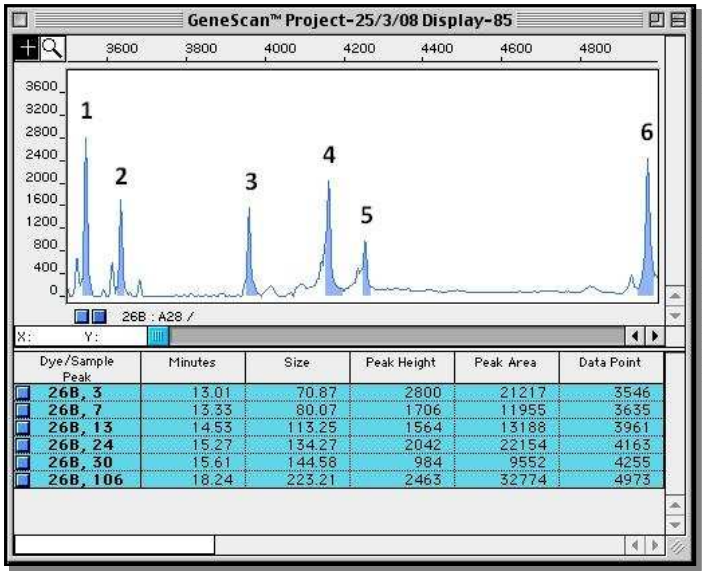

Figure 3.2. Example for Genescan® Analysis outputs. Example shows the fragment analysis result of sample A28 for 3 loci (OarCP20, OarFCB226, BM1443) labeled with 6-FAM. The peaks are labeled from 1 to 6. Peak 1 (70.87bp) and peak 2 (80.07bp) indicates heterozygous state for OarCP20. Peak 4 (134.27bp) and peak 5 (144.58bp) indicate heterozygous state for OarFCB226, whereas peak 6 (223.21bp) indicates homozygous state for BM1443. Peak 3 (113.25bp) is a nonspecific amplification and was not assigned as an allele.

3.3. Statistical Results

3.3.1. Genetic Diversity

Major genetic diversity measures were checked with GENEPOP, POPGENE, ARLEQUIN, and FSTAT programs. All but one locus, BM1443, were polymorphic for Bozdağ (source) population. While Nallıhan population conserves the same status, Karadağ population indicated 2 monomorphic loci, BM1443 and SRCRSP3. Hence, proportions of polymorphic loci (*P*, 99% criterion[12]; NEI *et al*., 1975) are 0.9091, 0.9091, and 0.8182 for Bozdağ (N = 172), Nallıhan (N = 81), and Karadağ (N = 22) populations, respectively. Totally, 32 different alleles for 11 microsatellite loci were identified. The allelic frequencies and related histograms for each population are shown in Table 3.1 and Figure 3.3, respectively. Mean number of alleles (n_k) for Bozdağ and Nallıhan populations is 2.9091, but lower for Karadağ population, 2.5455, since 4 alleles were found to be nonexistent in Karadağ population (Table 3.1). Three of these 4 alleles are actually rare alleles ($f < 0.05$) for Bozdağ and Nallıhan populations. The number of rare alleles (A_R) is found to be 5 (≈16%) both in Bozdağ and Nallıhan populations, but the number is 2 (≈7%) in Karadağ population. Locus-by-locus and average effective (or expected) allele numbers (A_E; KIMURA & CROW, 1964) were also calculated; 2.0250, 2.0592, and 1.8809 for Bozdağ, Nallıhan, and Karadağ populations, respectively. With the same sort, average observed heterozygosities (H_o) are 0.3830, 0.4086, 0.3388. NEI's (1973) expected heterozygosity is 0.3956 for Bozdağ, 0.4052 for Nallıhan, and 0.3607 for Karadağ population. Consequently, the average H_o and H_e computations are highest for Nallıhan population and lowest for Karadağ population. Expected heterozygosity according to the algorithm of LEVENE (1949), which is identical to NEI's (1978) unbiased heterozygosity estimate, was also computed and it points out the same relationship among the populations. Polymorphism Information Content (PIC) computed according to BOTSTEIN *et al*. (1980). Table 3.2 summarizes locus-specific and average values for all populations.

[12] A loci is assumed to be polymorphic when the frequency of the most common allele is smaller than 0.99.

Table 3.1. Allelic frequencies in study populations. Frequencies of 32 alleles at 11 loci for the source population (Bozdağ) and 2 reintroduced populations (Nallıhan & Karadağ) are shown.

LOCUS	allele (*bp*)	BOZDAĞ		NALLIHAN		KARADAĞ	
		n	***f***	***n***	***f***	***n***	***f***
OarJMP29	124	*342*	0.3421	*162*	0.3580	*44*	0.2955
	134		0.4561		0.4321		0.5909
	138		0.2018		0.2099		0.1136
ILSTS011**	281	*340*	0.9206	*158*	0.9304	*44*	0.9091
	289		0.0794		0.0696		0.0909
OarFCB128**	121	*344*	0.0233‡	*162*	0.0185‡	*44*	0.0227‡
	125		0.9767		0.9815		0.9773
SRCRSP8**	216	*336*	0.5804	*154*	0.5649	*44*	0.4773
	230		0.0952		0.1494		0.0682
	234		0.3244		0.2857		0.4545
MAF214**	189	*344*	0.2645	*162*	0.2469	*44*	0.3409
	191		0.1395		0.1049		0.1591
	222		0.3343		0.3457		0.3182
	225*		0.2297		0.2593		0.1591
	230*		0.0320‡		0.0432‡		0.0227‡
ADCYAP1**	104	*342*	0.3246	*160*	0.3375	*44*	0.3182
	106		0.4152		0.3938		0.4545
	110		0.2573		0.2625		0.2273
	118*		0.0029‡		0.0063‡		†
OarCP20**	71	*344*	0.3663	*162*	0.3951	*44*	0.2273
	80		0.6337		0.6049		0.7727
BM415**	106*	*344*	0.1192	*162*	0.1543	*44*	0.1136
	136		0.1105		0.1111		0.0909
	154		0.7703		0.7346		0.7955
SRCRSP3**	179	*342*	0.0526	*160*	0.0688	*44*	†
	189		0.9444		0.9250		1.0000
	191*		0.0029‡		0.0063‡		†
OarFCB226**	134	*344*	0.2558	*162*	0.2222	*44*	0.2727
	140		0.2064		0.2531		0.1591
	144		0.5145		0.5062		0.5682
	152		0.0233‡		0.0185‡		†
BM1443	223	*342*	1.0000	*162*	1.0000	*44*	1.0000

<Abbr/> *bp*, base pair; *n*, number of gene copies; *f*, frequency.

† nonexistent alleles

‡ rare alleles ($f < 0.05$)

* alleles not detected by ÖZÜT (2001).

** loci that were also analyzed by ÖZÜT (2001).

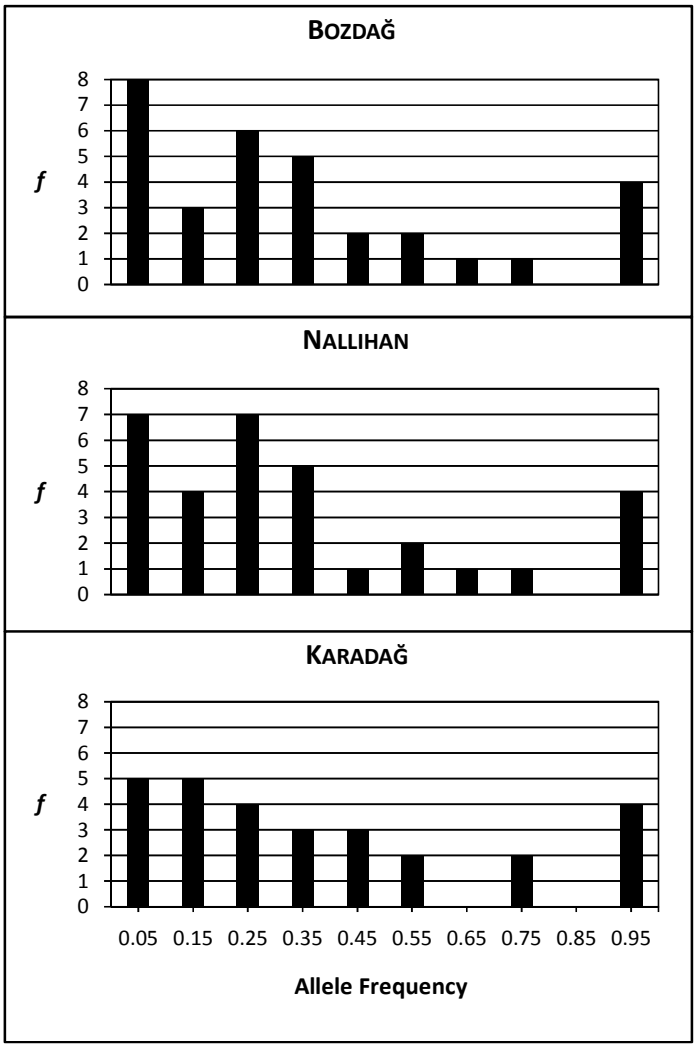

Figure 3.3. Allelic frequency distribution histograms for study populations. Class interval width = 0.1. Total number of alleles (A_T) for the same sort are 32, 32, and 28. Number of common alleles (A_C) are 27, 27, and 26. Number of rare alleles (A_R; 5% criterion) are 5, 5, and 2.

Table 3.2. Genetic diversity within study populations.

LOCUS	*A*			A_E*			H_o			H_e†			H_e‡			
	BOZ.	NAL.	KAR.	BOZ.	NAL.	KAR.	BOZ.	NAL.	KAR.	BOZ.	NAL.	KAR.	BOZ.	NAL.	KAR.	PIC
OarJMP29	3	3	3	2.7337	2.7860	2.2253	0.6667	0.7284	0.5455	0.6361	0.6450	0.5634	0.6362	0.6411	0.5506	0.55911
ILSTS011	2	2	2	1.1712	1.1488	1.1980	0.1471	0.1392	0.1818	0.1466	0.1304	0.1691	0.1462	0.1295	0.1653	0.1355
OarFCB128	2	2	2	1.0476	1.0377	1.0465	0.0349	0.0123	0.0455	0.0456	0.0366	0.0455	0.0454	0.0364	0.0444	0.0445
SRCRSP8	3	3	3	2.2167	2.3636	2.2776	0.5119	0.5195	0.7273	0.5505	0.5807	0.5740	0.5489	0.5769	0.5610	0.4699
MAF214	5	5	5	3.9220	3.8380	3.7231	0.7442	0.8395	0.7273	0.7472	0.7440	0.7484	0.7450	0.7394	0.7314	0.7006
ADCYAP1	4	4	3	2.9074	2.9595	2.7816	0.6023	0.6125	0.5909	0.6580	0.6663	0.6554	0.6560	0.6621	0.6405	0.5829
OarCP20	2	2	2	1.8665	1.9156	1.5414	0.4186	0.4198	0.2727	0.4656	0.4809	0.3594	0.4642	0.4780	0.3512	0.3565
BM415	3	3	3	1.6133	1.7369	1.5292	0.3663	0.4321	0.2273	0.3813	0.4269	0.3541	0.3802	0.4242	0.3461	0.3485
SRCRSP3	3	3	1	1.1176	1.1623	1.0000	0.0994	0.1250	0.0000	0.1056	0.1405	0.0000	0.1052	0.1396	0.0000	0.1002
OarFCB226	4	4	3	2.6786	2.7028	2.3667	0.6221	0.6667	0.4091	0.6285	0.6339	0.5909	0.6267	0.6300	0.5775	0.5635
BM1443	1	1	1	1.0000	1.0000	1.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
mean	2.9091	2.9091	2.5455	2.0250	2.0592	1.8809	0.3830	0.4086	0.3388	0.3968	0.4077	0.3691	0.3956	0.4052	0.3607	0.3510
st.dev.	1.1362	1.1362	1.1282	0.9537	0.9451	0.8758	0.2717	0.2977	0.2775	0.2755	0.2785	0.2779	0.2746	0.2767	0.2716	0.2460

<Abbr/> *A*, allelic diversity; A_E, effective number of alleles; H_o, observed heterozygosity; H_e, expected heterozygosity; *PIC*, Polymorphism Information Content; *st.dev.*, standard deviation; *BOZ.*, Bozdağ; *NAL.*, Nallıhan; *KAR.*, Karadağ.

***** KIMURA & CROW (1964).

† LEVENE's (1949) heterozygosity.

‡ NEI's (1973) heterozygosity.

****** H_o - H_e comparisons by two-tailed paired *t*-test revealed nonsignificant *p*-values for all populations.

******* PIC computed using the whole data set (172 individuals) at **http://www. genomics.liv.ac.uk/animal/Pic1.html** according to BOTSTEIN *et al.* (1980).

3.3.2. Genetic Structure

3.3.2.1. Hardy-Weinberg Equilibrium

Hardy-Weinberg equilibrium (HWE) was tested with GENEPOP and ARLEQUIN programs. For GENEPOP simulations, complete enumeration algorithm of LOUIS & DEMPSTER (1987) was performed for loci that have less than 5 alleles. This was very useful for this study because all but one locus, MAF214, have less than 5 alleles. For loci with more than 4 alleles, Markov chain (MC) algorithm of GUO & THOMPSON (1992) was used. But, ARLEQUIN uses only the latter algorithm. Analyses performed for the whole data (172 samples). Thus, HWE test was not re-performed for Nallıhan and Karadağ populations separately since their data are 2 different subsets of the whole data set.

The results of Hardy-Weinberg exact tests by both methods are nearly identical and confirm each other. BM1443 is a monomorphic locus, hence has been ignored in all computations. All but one locus, SRCRSP8 (GENEPOP, $p = 0.0429$; ARLEQUIN, $p = 0.0441$, s.d. = 0.00063), have indicated HWE. Additionally, *p*-values for heterozygote deficiency and heterozygote excess were computed with GENEPOP program. Similarly, the whole data set was analyzed. SRCRSP8 is again the only locus found to show heterozygote deficiency ($p = 0.0117$). None of the remaining loci has shown any excess or deficiency of heterozygotes. Also, a global test was performed by pooling all loci and it points to strong heterozygote deficiency ($p = 0.0151$, s.e. = 0.0017) for Bozdağ population. The results are detailed in Table 3.3.

Table 3.3. HWE test simulation output.

LOCUS	HW Exact Test		
	CE	***MC***	***MC*** [†]
OarJMP29	0.3484	0.3477	0.3414 (0.00146)
ILSTS011	1.0000	1.0000	1.0000 (0.00000)
OarFCB128	0.0798	0.0799	0.0793 (0.00083)
SRCRSP8*****	0.0423	0.0429	0.0441 (0.00063)
MAF214	0.9828	0.9830	0.9822 (0.00041)
ADCYAP1	0.2403	0.2424	0.2542 (0.00138)
OarCP20	0.1924	0.1913	0.2054 (0.00141)
BM415	0.5697	0.6113	0.6128 (0.00144)
SRCRSP3	0.4107	0.4119	0.3976 (0.00158)
OarFCB226	0.7371	0.7364	0.7346 (0.00124)
BM1443	-	-	-
mean	0.2798	0.2881	-

LOCUS	Het. Excess		Het. Deficiency	
	CE	***MC***	***CE***	***MC***
OarJMP29	0.2154	0.2156	0.7866	0.7876
ILSTS011	0.3260	0.7232	0.6740	0.6739
OarFCB128	0.9982	0.9983	0.0798	0.0801
SRCRSP8	0.9884	0.9885	0.0118	0.0117
MAF214	0.4601	0.4554	0.5383	0.5400
ADCYAP1	0.9135	0.9139	0.0869	0.0874
OarCP20	0.9320	0.9314	0.1222	0.1217
BM415	0.6986	0.7011	0.3014	0.3058
SRCRSP3	0.9186	0.9183	0.3761	0.3763
OarFCB226	0.5641	0.5679	0.4381	0.4384
BM1443	-	-	-	-

GLOBAL TEST	Het. Excess	Het. Deficiency
	MC	***MC***
	0.9850 (0.0019[‡])	0.0151 (0.0017[‡])

<Abbr/> *CE*, Complete Enumeration; *MC*, Markov Chain.

[†] Computed by ARLEQUIN (*dememorization* = 10000; *MC steps*= 100000). Standard deviation in parantheses.

All other computations by GENEPOP (*dememorization* = 1000; *# batches* = 1000; *iterations per batch* = 10000)

[‡] Standard error.

***** Significant departure, $p < 0.05$.

Additionally, global estimates of fixation index (F_{IS}) over alleles and over loci were computed according to WRIGHT (1978) and WEIR & COCKERHAM (1984) as an estimation of heterozygote excess or deficiency. Most of the loci were found to have positive F_{IS} values ($f > 0$), indicating heterozygote deficiency. The methods gave more or less similar results for locus-specific results. According to average values, Bozdağ population ($F_{IS}^{\dagger}$ = 0.0319, $F_{IS}^{\ddagger}$ = 0.035) is found to be heterozygote deficit. Similarly, both methods show that Karadağ population is also heterozygote deficit ($F_{IS}^{\dagger}$ = 0.0607, $F_{IS}^{\ddagger}$ = 0.084), whereas Nallıhan population has indicated mild excess of heterozygotes ($F_{IS}^{\dagger}$ = -0.0085, $F_{IS}^{\ddagger}$ = 0.002). BM1443 was again omitted for all populations, and SRCRSP3 is omitted for Karadağ population for their monomorphic states (Table 3.1). Table 3.4 summarizes the computations in details.

Table 3.4. F_{IS} estimations for study populations.

LOCUS	$F_{IS}^{\dagger}$			$F_{IS}^{\ddagger}$		
	BOZ.	**NAL.**	**KAR.**	**BOZ.**	**NAL.**	**KAR.**
OarJMP29	-0.0512	-0.1362	0.0094	-0.048	-0.130	0.033
ILSTS011	-0.0058	-0.0748	-0.1000	-0.003	-0.068	-0.077
OarFCB128	0.2321	0.6604	-0.0233	0.235	0.664	0.000
SRCRSP8	0.0674	0.0995	-0.2965	0.070	0.106	-0.275
MAF214	0.0011	-0.1353	0.0056	0.004	-0.129	0.029
ADCYAP1	0.0819	0.0749	0.0774	0.085	0.081	0.100
OarCP20	0.0983	0.1218	0.2235	0.101	0.128	0.246
BM415	0.0365	-0.0185	0.3433	0.039	-0.012	0.364
SRCRSP3	0.0554	0.1046	-	0.058	0.111	-
OarFCB226	0.0073	-0.0582	0.2916	0.010	-0.052	0.313
BM1443	-	-	-	-	-	-
mean	0.0319	-0.0085	0.0607	0.035	-0.002	0.084

<Abbr/> *BOZ.*, Bozdağ; *NAL.*, Nallıhan; *KAR.*, Karadağ.

† WRIGHT (1978), based on NEI's (1973) heterozygosity

‡ WEIR & COCKERHAM (1984), based on LEVENE's (1949) heterozygosity

3.3.2.2. Linkage Disequilibrium

Linkaqe disequilibrium was checked with GENEPOP program. Again, the genotypic data for BM1443 was ignored for being monomorphic. A probability test was performed using the Markov chain algorithm of RAYMOND & ROUSSET (1995). The output is given in Table 3.5. Out of the 45 $(10!/(2! \times 8!))$ computable different locus pairs, only BM415-ADCYAP1 pair ($p = 0.013$, s.e. = 0.00103) is found to be under linkaqe disequilibrium ($p < 0.05$).

Table 3.5. Linkage disequilibrium test simulation output.

LOCUS PAIR	X^2	$df^{\dagger}$	p-val	LOCUS PAIR	X^2	df	p-val
CP20 - 226	0.796	2	0.670	128 - MAF	0.805	2	0.669
CP20 - 128	3.501	2	0.174	BM - MAF	0.668	2	0.716
226 - 128	0.406	2	0.816	SP3 - MAF	0.112	2	0.945
CP20 - BM	0.858	2	0.651	ADC - MAF	0.101	2	0.951
226 - BM	2.707	2	0.258	JMP - MAF	2.094	2	0.351
128 - BM	2.573	2	0.276	CP20 - SP8	0.256	2	0.880
CP20 - SP3	1.339	2	0.512	226 - SP8	0.420	2	0.810
226 - SP3	0.980	2	0.613	128 - SP8	0.290	2	0.865
128 - SP3	0.000	2	1.000	BM - SP8	1.664	2	0.435
BM - SP3	1.733	2	0.420	SP3 - SP8	2.423	2	0.298
CP20 - ADC	0.175	2	0.916	YAP1 - SP8	0.230	2	0.892
226 - ADC	2.359	2	0.307	JMP - SP8	3.433	2	0.180
128 - ADC	0.467	2	0.792	MAF - SP8	2.880	2	0.237
BM – ADC*****	8.738	2	0.013	CP20 - ILS	2.827	2	0.243
SP3 - ADC	4.289	2	0.117	226 - ILS	0.155	2	0.925
CP20 - JMP	0.561	2	0.756	128 - ILS	0.806	2	0.668
226 - JMP	1.991	2	0.370	BM - ILS	1.078	2	0.583
128 - JMP	0.957	2	0.620	SP3 - ILS	1.856	2	0.395
BM - JMP	3.017	2	0.221	ADC - ILS	3.131	2	0.209
SP3 - JMP	1.374	2	0.503	JMP - ILS	1.904	2	0.386
ADC - JMP	1.628	2	0.443	MAF - ILS	1.816	2	0.403
CP20 - MAF	1.381	2	0.501	SP8- ILS	0.442	2	0.802
226 - MAF	4.969	2	0.083				

dememorization = 1000; *# batches* = 1000, *iterations per batch* = 1000.
***** $p < 0.05$.
† Degrees of freedom.
Locus names are abbreviated.

3.3.2.3. Neutrality

Neutrality test indicated that most loci are in between their computed lower (L95) and upper (U95) 95% confidence levels. For its monomorphic state, BM1443 was excluded from neutrality test. The loci were checked with POPGENE program which performs *EWENS-WATTERSON Test* for neutrality explained in MANLY (1985, p.272-282). The output data for neutrality test is given in Table 3.6 and related graph is demonstrated in Figure 3.4. For a locus to be assigned as neutral, observed F value calculated by simulated samples should be between the simulated L95 and U95 for that locus. OarJMP29 and MAF214 are found to be slightly non neutral due to their observed F values being smaller than their simulated L95. Remaining loci are in between the confidence levels indicating neutrality (Figure 3.4).

Table 3.6. Neutrality test simulation output.

LOCUS	***A***	**Obs. F**	**Min F**	**Max F**	**Mean***	**s.e.***	**L95***	**U95***
OarJMP29	3	0.3658	0.3333	0.9884	0.7284	0.0359	0.3913	0.9884
ILSTS011	2	0.8538	0.5000	0.9941	0.8412	0.0281	0.5034	0.9941
OarFCB128	2	0.9546	0.5000	0.9942	0.8483	0.0274	0.5029	0.9942
SRCRSP8	3	0.4511	0.3333	0.9882	0.7271	0.0360	0.3844	0.9881
MAF214	5	0.2550	0.2000	0.9770	0.5598	0.0339	0.2836	0.9264
ADCYAP1	4	0.3440	0.2500	0.9826	0.6336	0.0368	0.3317	0.9653
OarCP20	2	0.5358	0.5000	0.9942	0.8428	0.0280	0.5029	0.9942
BM415	3	0.6198	0.3333	0.9884	0.7250	0.0357	0.3877	0.9827
SRCRSP3	3	0.8948	0.3333	0.9884	0.7258	0.0364	0.3837	0.9884
OarFCB226	4	0.3733	0.2500	0.9827	0.6356	0.0367	0.3289	0.9655

<Abbr/> *A*, allelic diversity; *s.e.*, standard error; *L95*, lower 95% confidence level; *U95*, upper 95% confidence level.

***** Computations by 10000 simulated samples.

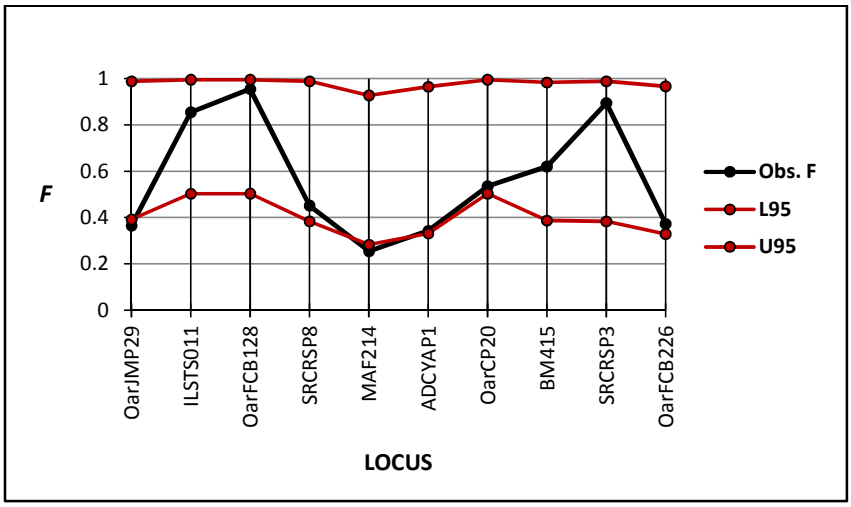

Figure 3.4. Graphical assessment of neutrality test simulation outputs. <Labels/> *Black circles*, obs. *F* value for each locus; *L95*, lower 95% confidence level; *U95*, upper 95% confidence level.

3.3.3. Effective Population Size

NEESTIMATOR and *LDNE* programs were used for effective population size (N_e) estimations. The former uses linkage disequilibrium and heterozygote excess methods, whereas the latter uses only linkage disequilibrium method (Table 3.7). For heterozygote excess method, N_e could not be estimated by NEESTIMATOR due to specific reasons (discussed in Chapter IV).

Table 3.7. N_e estimations for Bozdağ population.

Program	Method	Est. N_e	95% CI
LDNE *	Linkage Disequilibrium	226.9	[108.2, 1250.1]†
NEESTIMATOR		207.7	[122.4, 481.4]
NEESTIMATOR	Heterozygote Excess	infinity	[infinity, infinity]

* Mating model is random. Lowest allele frequency used ≥ 0.00.
† CI according to Jacknife on loci. Parametric 95% CI = [102.4, 1931.6].

3.3.4. Population Bottleneck

Population bottleneck was tested with BOTTLENECK program. All individuals (N = 172) were tested ignoring 2 loci; BM1443 is monomorphic and SRCRSP8 is not in HWE which can cause violations for bottleneck analysis. The test results demonstrate that there had not been a recent population bottleneck in the history of *O. g. anatolica*. The output for the test simulation is given in Table 3.8 which shows Hardy-Weinberg heterozygosity (H_e) according to the algorithm of NEI (1973) and expected equilibrium heterozygosity (H_{eq}) under 3 different mutation models for each locus.

Table 3.8. Bottleneck analysis output.

LOCUS		JMP	ILS	128	MAF	ADC	CP20	BM	SP3	226
OBS.	***n***	342	340	344	344	342	344	344	342	344
	A	3	2	2	5	4	2	3	3	4
	H_e	0.636	0.147	0.046	0.747	0.658	0.466	0.381	0.106	0.629
IAM	H_{eq}	0.267	0.151	0.153	0.430	0.358	0.152	0.266	0.267	0.359
	st.dev.	0.189	0.165	0.166	0.187	0.192	0.165	0.190	0.190	0.191
	DH/sd†	1.955	-0.029	-0.642	1.694	1.566	1.898	0.609	-0.853	1.410
	***p*-val**‡	0.012	0.375	0.437	0.005	0.033	0.081	0.328	0.294	0.066
TPM	H_{eq}	0.334	0.172	0.173	0.538	0.451	0.174	0.336	0.333	0.458
	st.dev.	0.181	0.168	0.169	0.143	0.165	0.168	0.180	0.182	0.162
	DH/sd	1.666	-0.150	-0.076	1.460	1.253	1.729	0.251	-1.248	1.057
	***p*-val**	0.018	0.431	0.366	0.015	0.067	0.095	0.468	0.163	0.135
SMM	H_{eq}	0.429	0.187	0.191	0.647	0.561	0.189	0.427	0.432	0.560
	st.dev.	0.142	0.168	0.170	0.089	0.114	0.168	0.141	0.140	0.115
	DH/sd	1.457	-0.237	-0.860	1.127	0.845	1.641	-0.32	-2.326	0.598
	***p*-val**	0.035	0.478	0.305	0.072	0.197	0.101	0.314	0.030	0.325

<Abbr/> *OBS*, observed; *IAM*, infinite allele model; *TPM*, two phase model; *SMM*, stepwise mutation model; *n*, number of gene copies; *A*, allelic diversity; *st.dev*., standard deviation.
† Standardized difference; (H_e - H_{eq})/st.dev.
‡ *P*-value for H_e
***** Parameters for TPM: variance = 30.00, proportion of SMM = 70%
****** All computations based on 10000 replications.
Locus names are abbreviated.

Accordingly, 3 tests were performed to detect heterozygosity excess ($H_e > H_{eq}$); sign test, standardized differences test, and Wilcoxon signed-rank test. *P*-values are nonsignificant to show a deviation from mutation-drift equilibrium, except for standardized differences test. However, standardized differences test is not reliable when tested for less than 20 polymorphic loci. Especially, Wilcoxon signed-rank test, which has relatively high statistical power (LUIKART & CORNUET, 1998), did not indicate heterozygosity excess except under IAM (Table 3.9). *P*-values are also consistent with normal L-shaped distribution of allelic frequencies illustrating lack of a recent bottleneck (LUIKART *et al.*, 1998), otherwise it would give a shifted-mode distribution. However, the frequency of low frequency (frequency range is (0.000-0.100]) alleles is higher than any other allele frequency class (Figure 3.5).

Table 3.9. Test results for bottleneck analysis.

TEST	IAM	TPM	SMM
Sign test	0.17681	0.26470	0.58815
Std. diff. test	0.00560**	0.03967*	0.26057
Wilcoxon test†	0.02441*	0.06445	0.28515

* $p < 0.05$, ** $p < 0.01$.
† One-tailed test for heterozygosity excess.

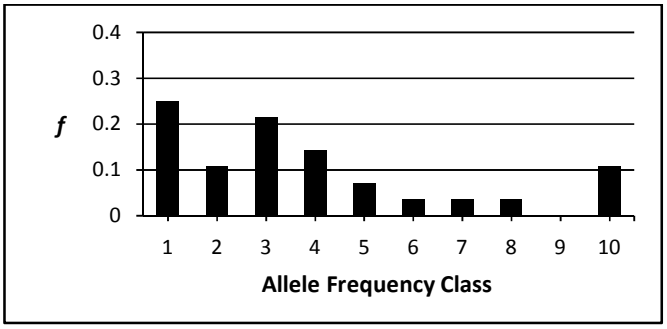

Figure 3.5. Normal L-shaped distribution of allelic frequencies. (Class interval width = 0.1).

3.3.5. Comparison Tests

3.3.5.1. Comparisons among Populations

Comparisons were done among 4 populations; Bozdağ$_1$ (N = 172), Nallıhan (N = 81), Karadağ (N = 22) and Bozdağ$_2$ (N = 48). The results of the first 3 populations belong to the current study. However, Bozdağ$_2$ stands for the Bozdağ population that was analyzed by ÖZÜT (2001) with 48 samples collected from Bozdağ PBS. The results of genetic diversity analysis by ÖZÜT (2001) that were used during comparisons are given in Table 3.10.

Observed (H_o), and expected (H_e) heterozygosity levels, and allelic diversity (A), were compared with paired t-test. Both LEVENE's (1949) and NEI's (1973) expected heterozygosity computations were used during comparisons. B_1 and N have identical alleles at all loci (Table 3.2), consequently A was not compared for this pair. Also, genetic diversity analysis of ÖZÜT (2001) does not involve LEVENE's (1949) H_e (Table 3.10), therefore B_1-B_2 pair also could not be compared for this parameter (Table 3.11).

Table 3.10. Genetic variation within Bozdağ population at 9 loci.

LOCUS	***A***	H_o	H_e
ILSTS011	2	0.1667	0.1544
OarFCB128	2	0.0208	0.0206
SRCRSP8	3	0.4043	0.4001
MAF214	3	0.2917	0.4954
ADCYAP1	3	0.3958	0.4406
OarCP20	2	0.4583	0.5044
BM415	2	0.2174	0.2609
SRCRSP3	2	0.1250	0.1184
OarFCB226	4	0.6250	0.5941

***** From ÖZÜT (2001). OarJMP29 and BM1443 were not analyzed.
<Abbr/> A, allelic diversity; H_o, observed heterozygosity; H_e, NEI's (1973) heterozygosity.

All comparisons for *A* had shown nonsignificant *p*-values ($p > 0.05$), however *p*-value for B_1-B_2 pair is found to be not quite statistically significant ($p = 0.0509$). Only B_1-N pair was found to be significantly different ($p = 0.0428$) for H_o comparisons. For H_e (LEVENE, 1949), none of the pairs revealed a significant value ($p > 0.05$). B_1-K ($p = 0.0329$) and N-K ($p = 0.028$) are found to be quite significantly different for H_e (NEI, 1973) comparison. However, B_1-B_2 pair is not quite significantly different ($p = 0.0508$). Table 3.11 demonstrates computed *p*-values for all comparisons.

Table 3.11. *P*-values for genetic diversity comparisons among study populations.

PAIR	***A***	H_o	H_e[†]	H_e[‡]
B_1-N	[Φ]	0.0428*****	0.0875	0.1315
B_1-K	0.1039	0.2393	0.0814	0.0329*****
N-K	0.1039	0.1193	0.0516	0.0280*****
B_1-B_2	0.0509	0.1179	[Φ]	0.0508

<Abbr/> *A*, allelic diversity; H_o, observed heterozygosity; H_e, expected heterozygosity; B_1 & B_2, Bozdağ; *N*, Nallıhan; *K*, Karadağ.
[†] LEVENE's (1949) heterozygosity.
[‡] NEI's (1973) heterozygosity.
[Φ] Comparison omitted.
***** $p < 0.05$.

3.3.5.2. Comparisons among Traps

3.3.5.2.1. Comparisons among Genetic Diversity Estimations

In total 167 individuals were captured by 3 different traps; Karanlık Dere ($N = 25$), Bağderesi ($N = 26$), and Gölet ($N = 116$). Remaining 5 individuals were captured by using anesthetic guns. Table 3.13 summarizes genetic diversity for each trap.

Similar to population comparisons, traps were also compared for the same 3 parameters (A, H_o, H_e), including LEVENE's (1949) and NEI's (1973) expected heterozygosity computations separately. Again, paired *t*-test was performed for trap comparisons (Table 3.12). Genetic diversity results for trap were also compared with genetic diversity within Bozdağ population (Table 3.2).

Table 3.12. *P*-values for genetic diversity comparisons among traps and Bozdağ population.

PAIR	A	H_o	H_e †	H_e ‡
K-B	0.5884	0.6385	0.3098	0.3030
K-G	0.1669	0.5671	0.7822	0.4909
B-G	0.5884	0.8875	0.3510	0.5244
B_1-K	0.0816	0.5782	0.5600	0.2734
B_1-B	0.1669	0.7971	0.3295	0.5798
B_1-G	0.3409	0.6587	0.4944	0.4070

<Abbr/> A, allelic diversity; H_o, observed heterozygosity; H_e, expected heterozygosity; TRAPS *K.*, Karanlık Dere; *B.*, Bağderesi; *G.*, Gölet; POPULATION B_1, Bozdağ.

† LEVENE's (1949) heterozygosity.

‡ NEI's (1973) heterozygosity.

All except B_1-K ($p = 0.0816$) pair for A comparison revealed high p-values ($p > 0.1$) indicating no significant difference among genetic diversity estimations for groups captured within different traps. On the other hand, it is important to note that the much higher number of individuals captured at Gölet (116 individuals) is not due to any experimental artifact. The other traps are newer than the one at Gölet and since the sampling for this study was started in 2004, more than 60 individuals were captured while there was only one trap in Bozdağ PBS which was at Gölet.

Table 3.13. Summary of genetic diversity within traps.

LOCUS	TRAP														
	KARANLIK DERE					BAĞDERESİ					GÖLET				
	n	***A***	H_o	$H_e^{\dagger}$	$H_e^{\ddagger}$	***n***	***A***	H_o	$H_e^{\dagger}$	$H_e^{\ddagger}$	***n***	***A***	H_o	$H_e^{\dagger}$	$H_e^{\ddagger}$
OarJMP29	50	3	0.7600	0.6424	0.6296	52	3	0.5000	0.6267	0.6146	230	3	0.6783	0.6397	0.6369
ILSTS011	50	2	0.1600	0.1502	0.1472	52	2	0.1538	0.1448	0.1420	228	2	0.1404	0.1461	0.1454
OarFCB128	50	2	0.0400	0.0400	0.0392	52	2	0.0769	0.1448	0.1420	232	2	0.0259	0.0256	0.0255
SRCRSP8	48	3	0.5000	0.5363	0.5252	52	3	0.5385	0.4985	0.4889	226	3	0.5221	0.5611	0.5587
MAF214	50	5	0.8400	0.7894	0.7736	52	4	0.5769	0.7398	0.7256	232	5	0.7500	0.7420	0.7388
ADCYAP1	50	3	0.7200	0.6588	0.6456	52	3	0.5000	0.6606	0.6479	230	4	0.6000	0.6564	0.6536
OarCP20	50	2	0.6400	0.4702	0.4608	52	2	0.3846	0.4827	0.4734	232	2	0.3793	0.4639	0.4620
BM415	50	3	0.3600	0.3747	0.3672	52	3	0.5769	0.4985	0.4889	232	3	0.3103	0.3387	0.3372
SRCRSP3	50	2	0.1200	0.1151	0.1128	52	3	0.0769	0.0762	0.0747	230	2	0.1043	0.1148	0.1143
OarFCB226	50	3	0.3200	0.5069	0.4968	52	4	0.7308	0.6667	0.6538	232	4	0.6638	0.6398	0.6371
BM1443	44	1	0.0000	0.0000	0.0000	50	1	0.000	0.0000	0.0000	210	1	0.0000	0.0000	0.0000
mean	49	2.6364	0.4055	0.3895	0.3816	52	2.7273	0.3741	0.4127	0.4047	229	2.8182	0.3795	0.3935	0.3918
st.dev.	-	1.0269	0.3044	0.2729	0.2674	-	0.9045	0.2517	0.2694	0.2642	-	1.1677	0.2798	0.2787	0.2775

<Abbr/> *n*, number of gene copies; *A*, allelic diversity; H_o, observed heterozygosity; H_e, expected heterozygosity; *st.dev*., standard deviation.

† LEVENE's (1949) heterozygosity.

‡ NEI's (1973) heterozygosity.

3.3.5.2.2. Comparisons among Probability of Identity (PI) Values

Probability of Identity (PI) values were computed by GIMLET program and are shown in Table 3.15. Paired *t*-test was performed for PI comparisons. Trap-trap comparisons did not indicate any significant change for PI ($p > 0.1$). Similar results are also valid for trap-Bozdağ comparisons (Table 3.14).

Table 3.14. *P*-values for PI comparisons.

PAIR	$PI_{theoric}$	$PI_{unbiased}$	PI_{sibs}
K-B	0.3433	0.3889	0.3108
K-G	0.6405	0.3776	0.5325
B-G	0.4904	0.2388	0.5071
B_1-K	0.2792	0.5401	0.2785
B_1-B	0.5581	0.2071	0.5622
B_1-G	0.3287	0.4345	0.3846

<Abbr/> TRAPS *K.*, Karanlık Dere; *B.*, Bağderesi; *G.*, Gölet; POPULATION B_1, Bozdağ.

Both genetic diversity and PI comparisons for trap-trap and trap-population pairs indicate that traps give homogenous data and do not behave selectively and thus, a statistical bias for catching method is unsupported. It is also confirmed that there is statistically nonsignificant difference between the average level of relationship within the groups captured at different traps.

Table 3.15. PI values for traps and Bozdağ population.

LOCUS	POPULATION			TRAP								
	BOZDAĞ			KARANLIK DERE			BAĞDERESİ			GÖLET		
	$PI_{theoric}$	$PI_{unbiased}$	PI_{sibs}	$PI_{theoric}$	$PI_{unbiased}$	PI_{sibs}	$PI_{theoric}$	$PI_{unbiased}$	PI_{sibs}	$PI_{theoric}$	$PI_{unbiased}$	PI_{sibs}
OarJMP29	0.2090	0.2061	0.4851	0.2150	0.1959	0.4890	0.2176	0.1950	0.4971	0.2079	0.2037	0.4835
ILSTS011	0.7396	0.7365	0.8618	0.7381	0.7133	0.8609	0.7462	0.7231	0.8656	0.7409	0.7361	0.8625
OarFCB128	0.9122	0.9110	0.9553	0.9239	0.9155	0.9614	0.7462	0.7231	0.8656	0.9499	0.9488	0.9747
SRCRSP8	0.2824	0.2791	0.5462	0.2928	0.2642	0.5606	0.3488	0.3276	0.5928	0.2763	0.2716	0.5397
MAF214	0.1095	0.1065	0.4049	0.0888	0.0698	0.3854	0.1262	0.1070	0.4187	0.1141	0.1097	0.4091
ADCYAP1	0.1914	0.1887	0.4698	0.2009	0.1822	0.4774	0.1987	0.1807	0.4757	0.1919	0.1877	0.4712
OarCP20	0.3948	0.3926	0.6166	0.3969	0.3815	0.6188	0.3894	0.3766	0.6107	0.3962	0.3929	0.6181
BM415	0.4159	0.4106	0.6639	0.4304	0.3897	0.6740	0.3150	0.2821	0.5843	0.4651	0.4572	0.6977
SRCRSPSP3	0.8055	0.8030	0.8988	0.7935	0.7728	0.8920	0.8575	0.8428	0.9270	0.7909	0.7869	0.8906
OarFCB226	0.2026	0.1990	0.4873	0.3133	0.2813	0.5799	0.1839	0.1625	0.4691	0.1958	0.1908	0.4804
BM1443	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
overall loci	2.24E-5	2.00E-5	7.44E-3	3.25E-5	1.39E-5	9.01E-3	2.05E-5	9.40E-6	6.82E-3	2.53E-5	2.15E-5	7.80E-3

3.4. Simulation Analyses

3.4.1. Founder Event Simulation

Comparative simulation experiments are based on 2 real data sets. One data set belongs to the current study and includes the genotypic data of 172 individuals sampled at Bozdağ PBS for 11 microsatellite loci. The second is the genotypic data of 197 honey bees for 5 highly polymorphic microsatellite loci.

Seven parameters were compared; (*1*) total number of alleles (A_T), (*2*) number of common alleles (A_C), (*3*) number of rare alleles (A_R), (*4*) mean number of alleles (n_k), (*5*) proportion of polymorphic loci (P), (*6*) observed heterozygosity (H_o), and (*7*) NEI's (1973) expected heterozygosity (H_e). A_T, A_C, and A_R were compared separately from other genetic parameters. All experiments start at founder population size (N_F) = 2. Original results for Anatolian mouflon are; A_T = 32, A_C = 27, A_R = 5, n_k = 2.9091, P = 0.9091, H_o = 0.3830, and H_e = 0.3956. For honey bees; A_T = 80, A_C = 22, A_R = 58, n_k = 16.00, P = 1.00, H_o = 0.6536, and H_e = 0.7197.

The purpose for comparing different data sets is to observe the effect of genetic diversity within source population on defined parameters for founder events. The simulation output for 4 parameters (n_k, P, H_o, & H_e) is given in Figure 3.6. For n_k, as N_F increases, the number of alleles carried by founder populations increases continuously. Especially, for $N_F < 25$, n_k increases more rapidly for both data sets. For P, constancy is observed after early stages, but this constancy is delayed for Anatolian mouflon data set. The change in H_o with increasing N_F shows very minor saltations but on the whole there is stability. H_e shows a very close behavior with P and increases sharply at the beginning but there is a very slow continuous increase afterwards. Consequently, n_k is the most sensitive parameter to founder events. Despite its importance for genetic diversity estimations, H_e does not shows a distinctive behavior and has a medium sensitivity to founder events.

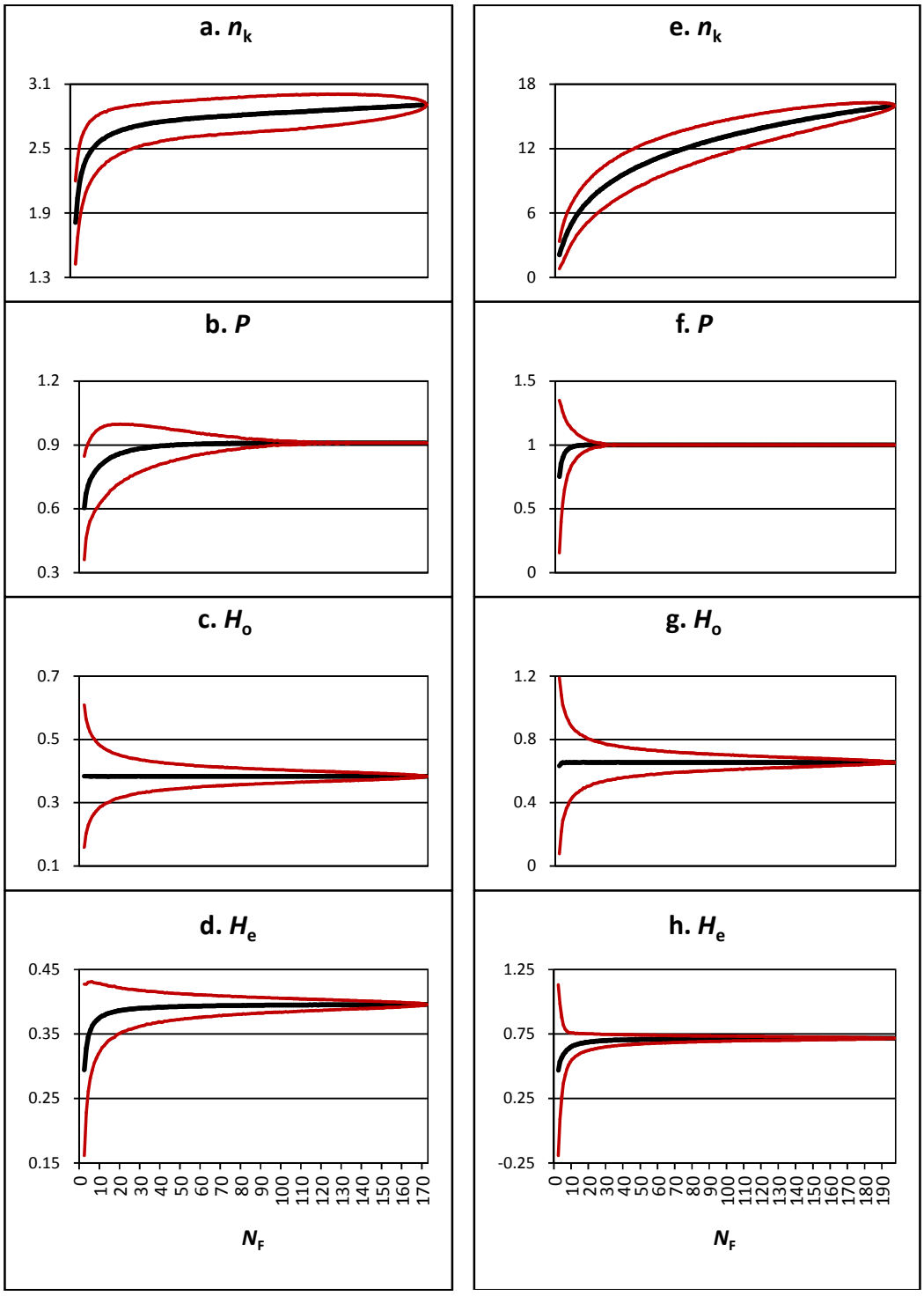

Figure 3.6. Simulation graphs for n_k, P, H_o, & H_e vs. N_F. a, b, c, d) for Anatolian mouflon, and e, f, g, h) for honey bee data. Black line is the expected values for a particular genetic parameter at all possible N_F. Red lines are upper and lower 99% CI for expectations.
***** Data by 10000 iterations.

Since allelic diversity is the most sensitive parameter, alleles carried by founder populations were categorized according to their states within the source population either as common or rare (5% criterion). According to the simulation output (Figure 3.7), the steady increase in A_T (and thus for n_k in Figure 3.6) is due to rare alleles, since A_C increases only at the very early stages but A_R is perpetually increasing as N_F increases or in other words rare alleles are lost faster than common alleles. The much sharper increase in A_R for honey bees is due to the much higher number of rare alleles within the source population.

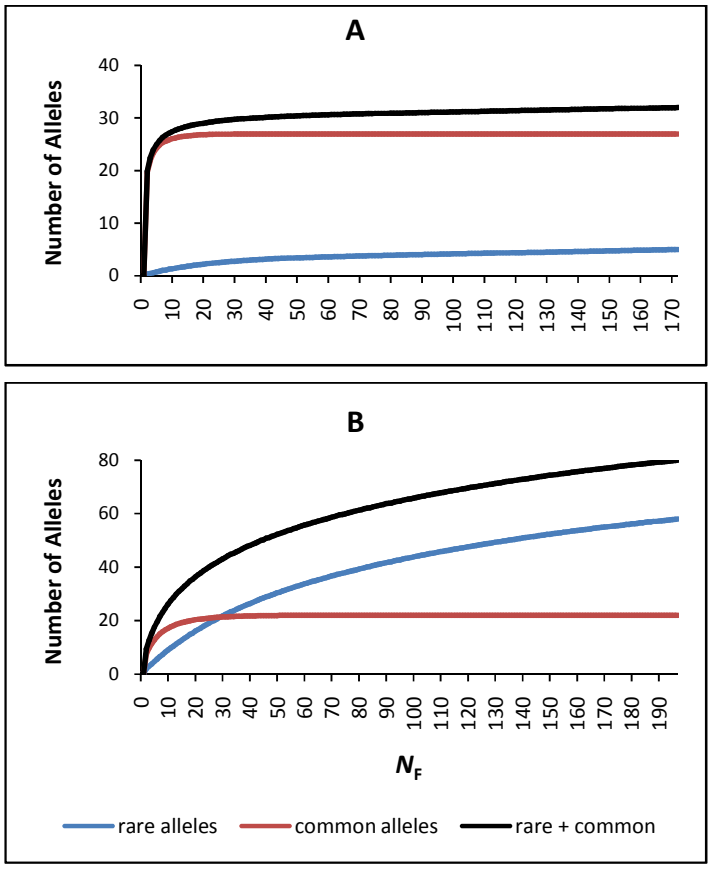

Figure 3.7. Simulation graphs for A_T, A_R, & A_C vs. N_F. A) Anatolian mouflon, and B) honey bee data.
***** Data by 10000 iterations.

Since 22 and 81 individuals were sampled for Nallıhan and Karadağ populations and they are subsets of the whole set of 172 individuals, N_F=22 and N_F=81 according to Figure 3.6 (Graphs a, b, c, & d) should give expected genetic diversity values for reintroduced samples (Table 3.16). Observed results are within the ranges of expectation indicating that reintroduced samples were chosen randomly from the whole set of 172 individuals. On the other hand, if the genetic diversity within 172 individuals is assumed as true population statistics for Bozdağ population (N=600, Figure 1.1), then N_F=131 and N_F=61 should give the expected genetic diversity for reintroduced populations (Table 3.17), since a total of 131 and 61 individuals were translocated to Nallıhan and Karadağ, respectively (Table 2.1).

Table 3.16. Observed and expected genetic diversity for reintroduced samples.

		n_k	P	H_o	H_e
OBS.	**Karadağ**	2.5455	0.8182	0.3388	0.3607
	Nallıhan	2.9091	0.9091	0.4086	0.4052
EXP.	**Karadağ (N_F=22)**	2.6551 (±0.2219)	0.8661 (±0.1306)	0.3828 (±0.0632)	0.3875 (±0.0334)
	Nallıhan (N_F=81)	2.8126 (±0.1674)	0.9082 (±0.0239)	0.3829 (±0.0255)	0.3943 (±0.0131)

<Abbr/> *OBS*., observed; *EXP*., expected.
Observed values were taken from Table 3.2.

Table 3.17. Expected genetic diversity for reintroduced populations.

		n_k	P	H_o	H_e
EXP.	**Karadağ (N_F=61)**	2.7840 (±0.1688)	0.9053 (±0.0489)	0.3831 (±0.0322)	0.3935 (±0.0170)
	Nallıhan (N_F=131)	2.8651 (±0.1409)	0.9092 (±0.0001)	0.3831 (±0.0131)	0.3953 (±0.0067)

<Abbr/> *OBS*., observed; *EXP*., expected.

3.4.2. Bottleneck Simulation

Bottleneck experiments were categorized into TYPE I (T_1 - T_3 route, $T_2 = 0$) and TYPE II (T_1 - T_2 - T_3 route, $T_1 = 1$). For all experiments, (*1*) distortion graphs for allele frequency distributions (LUIKART *et al*., 1998), (*2*) *p*-values by one-tailed Wilcoxon signed-rank test for heterozygosity excess detected at analyzed loci (under 3 mutation models – SMM, IAM, and TPM), and (*3*) genetic diversity (*P*, *A*, & H_e) within analyzed samples were used to characterize the effects of determined parameters. Type II error[13] is assumed, if distortion graph demonstrates a L-shaped distribution of allelic frequencies or p_{IAM}, p_{TPM}, and p_{SMM} is not significant (>0.05) even though population was bottlenecked and fits to model assumptions (discussed in Chapter IV). All experiments were replicated for N_B = 2, 5, 10, 25, and 50. Test results are given in Figure 3.8 & 3.9 for TYPE I and in Figure 3.10 & 3.11 for TYPE II experiments.

According to Figure 3.8 & 3.10, distortion graphs have lower sensitivity to simulated bottlenecks as N_B increases. However, as T_1 and T_2 increases in TYPE I and TYPE II experiments respectively, distortion graphs show shifted-mode distributions and detect bottlenecks. In both types of experiments, heterozygosity excess method under SMM (Figure 3.9 & 3.11) works parallel with distortion of allele frequency distributions method, however both methods starts to be violated after *P* decreases down to 0.4-0.5 (critical point). For example, in TYPE I experiments for N_B = 25 SMM gives significant heterozygosity excess at T_1 = 5000 (Figure 3.9) when distortion graphs (Figure 3.8) for N_B = 25 also shows shifted-mode distribution. Before this duration, neither distortion graphs nor SMM supports bottleneck and result in type II error. However at T_1 = 10000, distortion graph is again slightly L-shaped for N_B = 25 and supportively p_{SMM} = 0.06. The reason is that for N_B = 25, P = 0.432 (constitutes a critical point) at T_1 = 10000 (Figure 3.9).

[13] Type I error: Detecting a bottleneck in an equilibrium population.
Type II error: Failure to detect a bottleneck in a bottlenecked population.

P = 0.4 correspond to about 4 loci (11 loci were simulated) that is consistent with PIRY *et al*. (1999) stating that at least 4 polymorphic loci is necessary for reliable analysis with BOTTLENECK program. Reaching to critical period is earlier in TYPE II experiments, because permanence period (T_2) can more effectively reduce genetic diversity than bottleneck period (T_1). In TYPE I experiments, for N_B = 50, P = 1.00, A = 14.1636, and H_e = 0.8510 at T_1 = 10; P = 0.6454, A = 1.8636, and H_e = 0.2560 at T_1 = 10000. However, in TYPE II experiments, for N_B = 50, P = 1.00, A = 10.0544, and H_e = 0.8090 at T_2 = 5; P = 0, A = 1.00, and H_e = 0 at T_2 = 500 indicating that ~10000 T_1 generations is less effective than ~500 T_2 generations. Thus, p = 1.00 after critical point can be observed under all models for TYPE II experiments and consequently, SMM graph demonstrates a more complete reversed bell-shaped distribution of p-values in TYPE II experiments than in TYPE I experiments. But if generation duration is extented beyond 10000, TYPE I experiments will definitely show the same pattern with TYPE II experiments.

Heterozygosity method is based on the assumption that A decreases faster than H_e. This was shown with founder event simulations (Figure 3.6). Bottleneck simulations also support this assumption. All experiments have the same pre-bottleneck genetic diversity; P = 1.00, A = 25; H_e = 0.95±0.04. If these pre-bottleneck values are compared with the results of TYPE I experiments *e.g.* N_B = 25; P = 1.00, A = 12.5817, and H_e = 0.8368 at T_1=10, while bottleneck sharply decreases A, H_e is still near to the initial value. Same inference is available with TYPE II experiments. Additionally, distortion of allele frequency distributions method assumes that rare alleles are more likely to be lost during bottlenecks. This assumption was also supported with founder event simulation (Figure 3.7).

Bozdağ population was shown to be an equilibrium population by BOTTLENECK program. One-tailed Wilcoxon signed-rank test results were; p_{IAM} = 0.02441, p_{TPM} = 0.06445, p_{SMM} = 0.28515 (Table 3.9) and genetic diversity estimations were; P = 0.9091, A = 2.9091, H_e = 0.3956. If study population had passed through a bottleneck in the recent past which is nearly definite, than Wilcoxon test results constitutes a type II error if all criteria are met and there is no other source of error

(discussed in Chapter IV). Similar situations can be exemplified with different scenarios. *e.g.* in TYPE I experiments for $N_B = 25$ and $T_1 = 2000$; $p_{IAM} = 0.0019$, $p_{TPM} = 0.0192$, $p_{SMM} = 0.1481$ and $P = 0.9727$, $A = 3.5545$, $H_e = 0.5277$ (Figure 3.9). Simulated *p*-values are generally in accordance with the original results. P_{SMM} is nonsignificant and p_{TPM} is very near to original value. However, p_{IAM} is much more significant than original value. Genetic diversity values are also very near to the estimations of study population. Also, if related distortion graph is checked (Figure 3.8, graph 26), similarity with the graph of study population (Figure 3.5) can be observed. Nearly the same comparison is possible for $N_B = 50$ and $T_1 = 2000$ for TYPE I experiments. Additionally, in TYPE II experiments, for $N_B = 25$ and $T_2 = 25$; $p_{IAM} = 0.0214$, $p_{TPM} = 0.0375$, $p_{SMM} = 0.1537$ and $P = 0.9727$, $A = 3.1546$, $H_e = 0.4877$ (Figure 3.11). Simulated *p*-values and genetic diversity values are more similar to original results than the previous examples. Distortion graph (Figure 3.10, graph 12) is also L-shaped and similar to Figure 3.5. Also, for $N_B = 25$ and $T_2 = 50$; $p_{IAM} = 0.0372$, $p_{TPM} = 0.0866$, $p_{SMM} = 0.1486$ and $P = 0.7725$, $A = 2.0728$, $H_e = 0.2884$ (Figure 3.11). Genetic diversity measures are less similar to original values relative to $T_2 = 25$ output, but distortion graph (Figure 3.10, graph 13) is more similar to Figure 3.5. Scenarios can be extended with more precision if TYPE I and TYPE II experiments are collated.

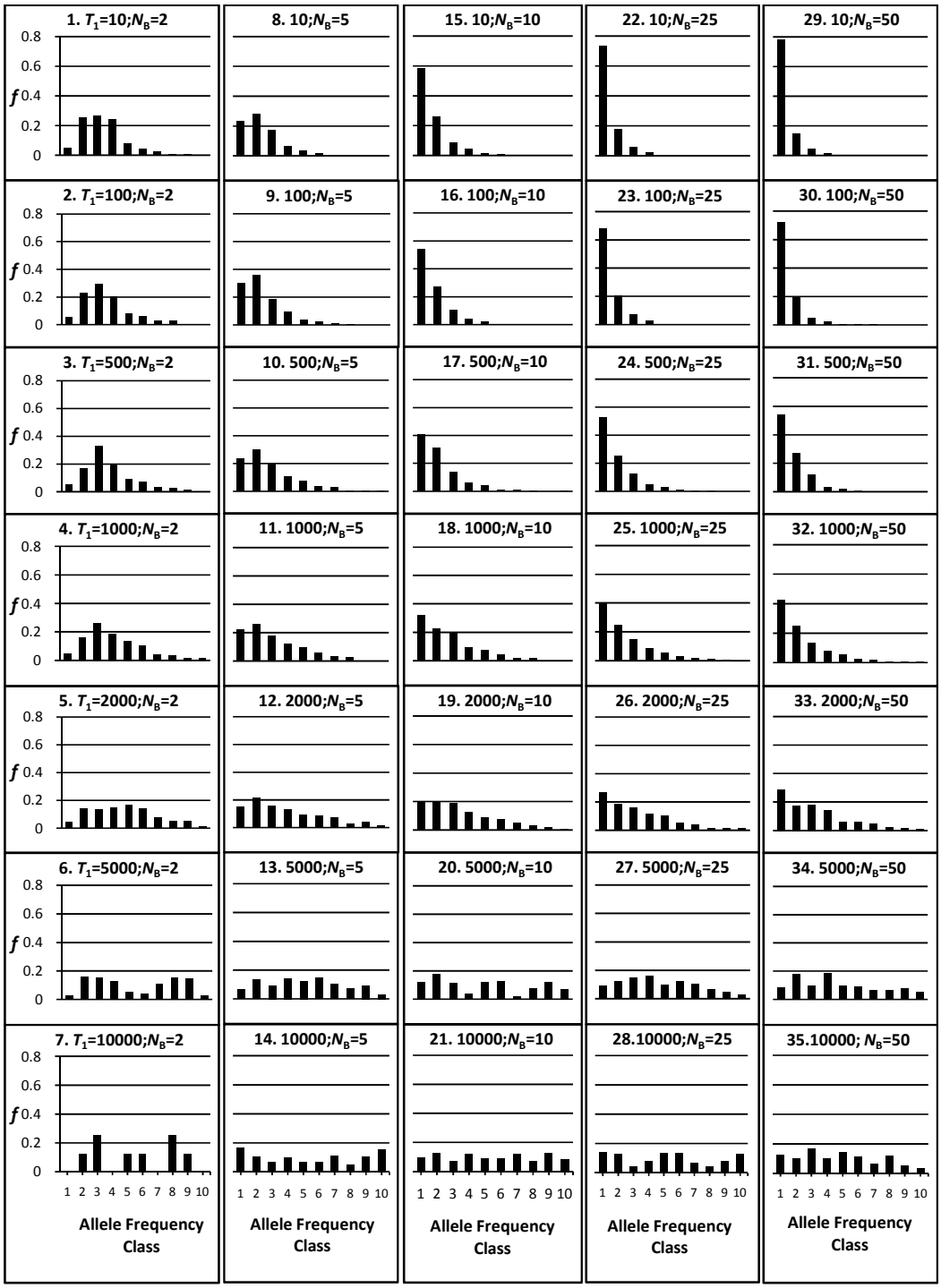

Figure 3.8. Distortion graphs for TYPE I experiments. From left to right, each column belongs to N_B=2, N_B=5, N_B=10, N_B=25, and N_B=50. From top to bottom, each row belongs to T_1=10, T_1=100, T_1=500, T_1=1000, T_1=2000, T_1=5000, and T_1=10000 experiments. As N_B increases, distributions approach to L-shaped, and as T_1 increases distributions approach to shifted-mode. Only T_1=10000 partly violates this pattern, when P is very low (Figure 3.9).

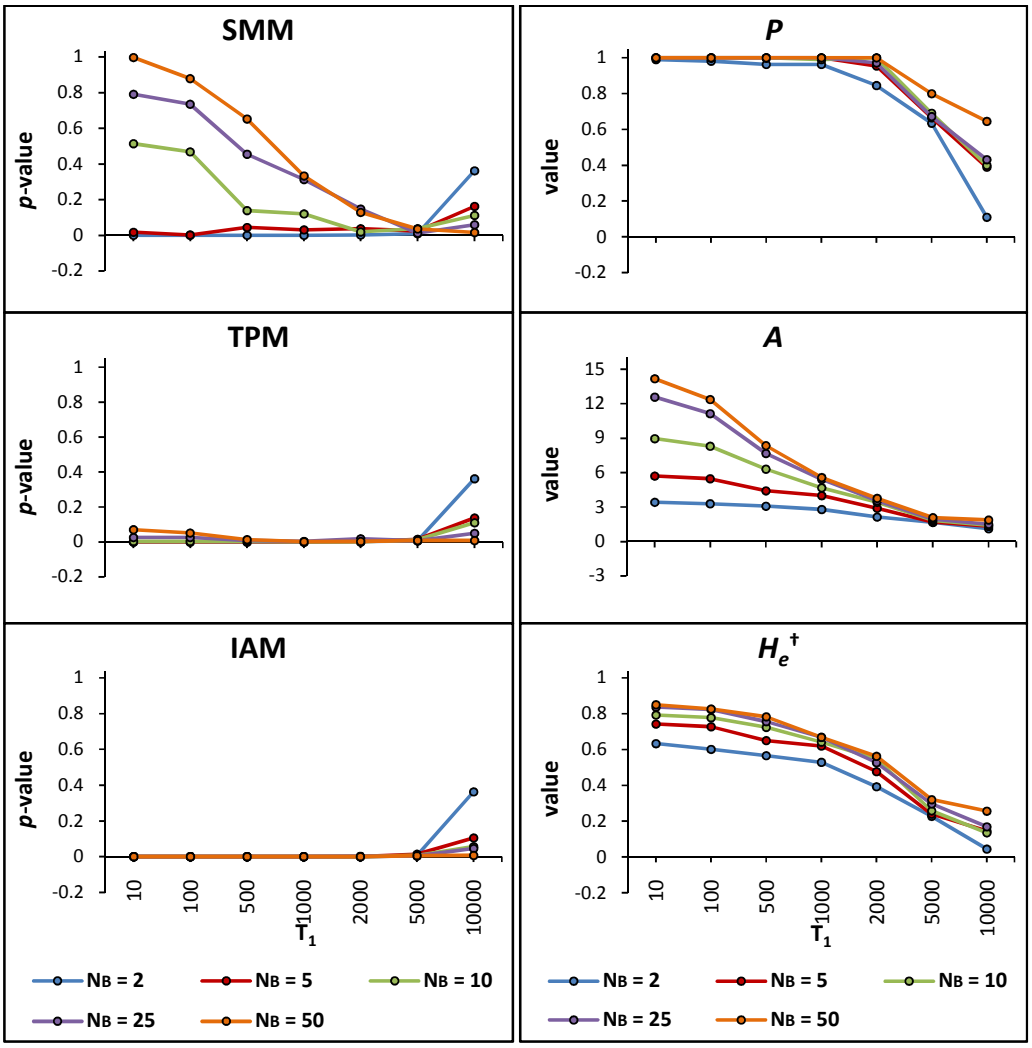

Figure 3.9. *P*-values and genetic diversity for TYPE I samples. SMM has higher type II error relative to TPM and IAM. SMM also has a narrower range (when T_1 > 2000) of detectability for higher N_B (>5) and gives a reverse bell-shaped graph (more obvious in TYPE II experiments). Whereas TPM and IAM shows significant heterozygosity excess even for fast bottlenecks. All models are *P*-senstive and as *P* decreases lower than 0.4-0.5 (critical point), observable heterozygosity excess also decreases. *e.g.* beyond T_1 = 5000, detected heterozygosity excess decreases for lower N_B (<50) since their respective *P* hit critical point between T_1 = 5000 & 10000 (*P* graph). Increase in heterozygosity excess shows a pattern; increase is faster and starts earlier as N_B decreases. This pattern is incomplete because simulations stop at T_1 = 10000. It is more properly observed in TYPE II experiments.
<Abbr/> *SMM*, stepwise mutation model; *TPM*, two phase model; *IAM*, infinite allele model; *P*, proportion of polymorphic loci; *A*, allelic diversity; H_e, expected heterozygosity.
***** Simulations replicated 10 times.
****** Parameters for TPM: variance = 30.00, proportion of SMM = 70%.
† NEI's (1973) heterozygosity.

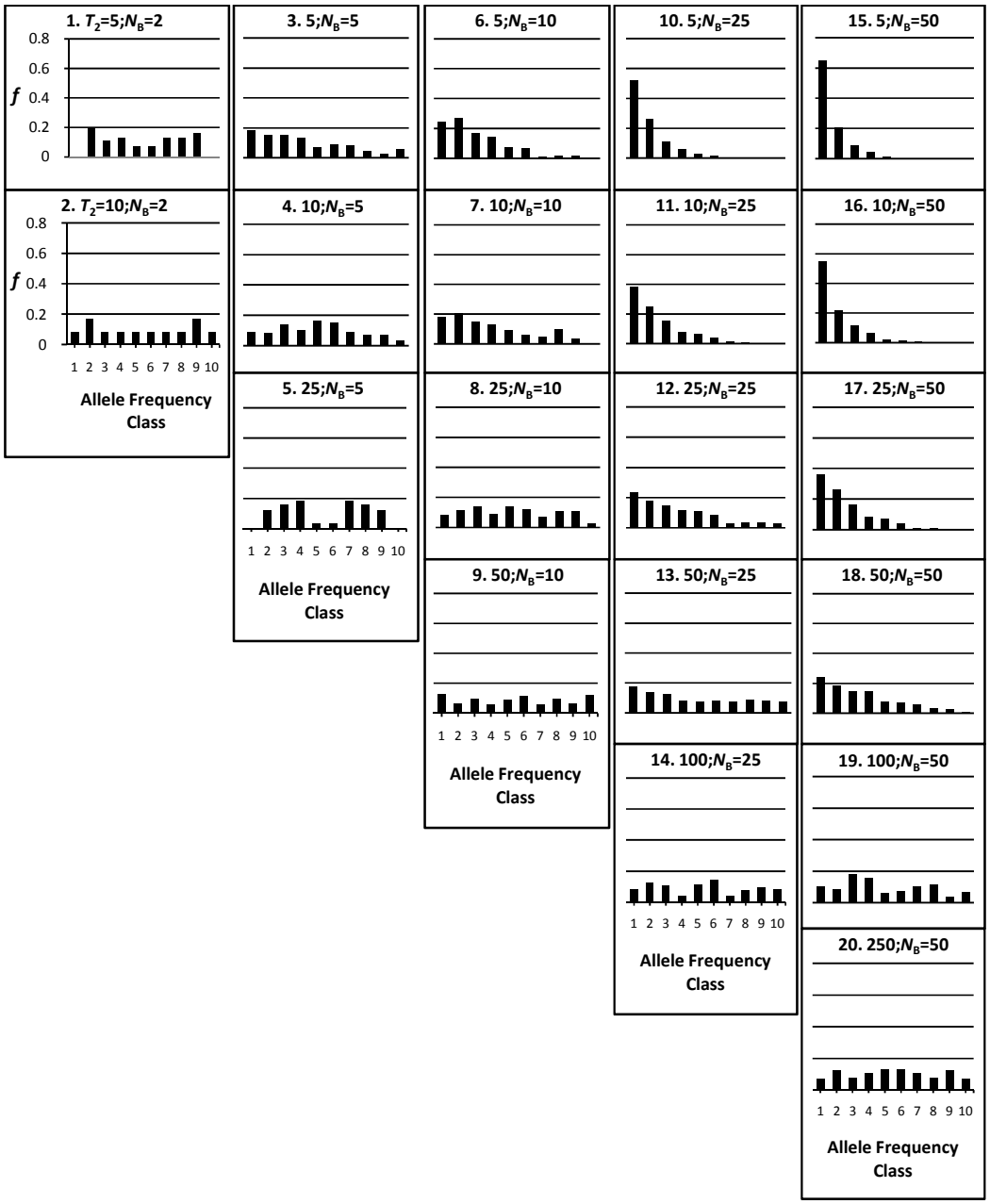

Figure 3.10. Distortion graphs for TYPE II experiments. From left to right, each column belongs to N_B=2, N_B=5, N_B=10, N_B=25, and N_B=50 and from top to bottom, each row belongs to T_2=5, T_2=10, T_2=25, T_2=50, T_2=100, and T_2=250 experiments. Distortion graphs are shown until P = 0 for all N_B, hence the # graphs changes for different N_B. *e.g.* for N_B=2, P=0 at T_2=25 and so p=1 under all models (Figure 3.11). Thereby, only distortion graphs for T_2=5 and T_2=10 are shown for N_B=2. Like TYPE I, as N_B increases, distributions approach to L-shaped, and as T_2 increases distributions approach to shifted-mode. Lowermost graphs may violate this pattern due to low P (Figure 3.11).

***** Scale of y-axis is [0.8, 0] for all graphs.

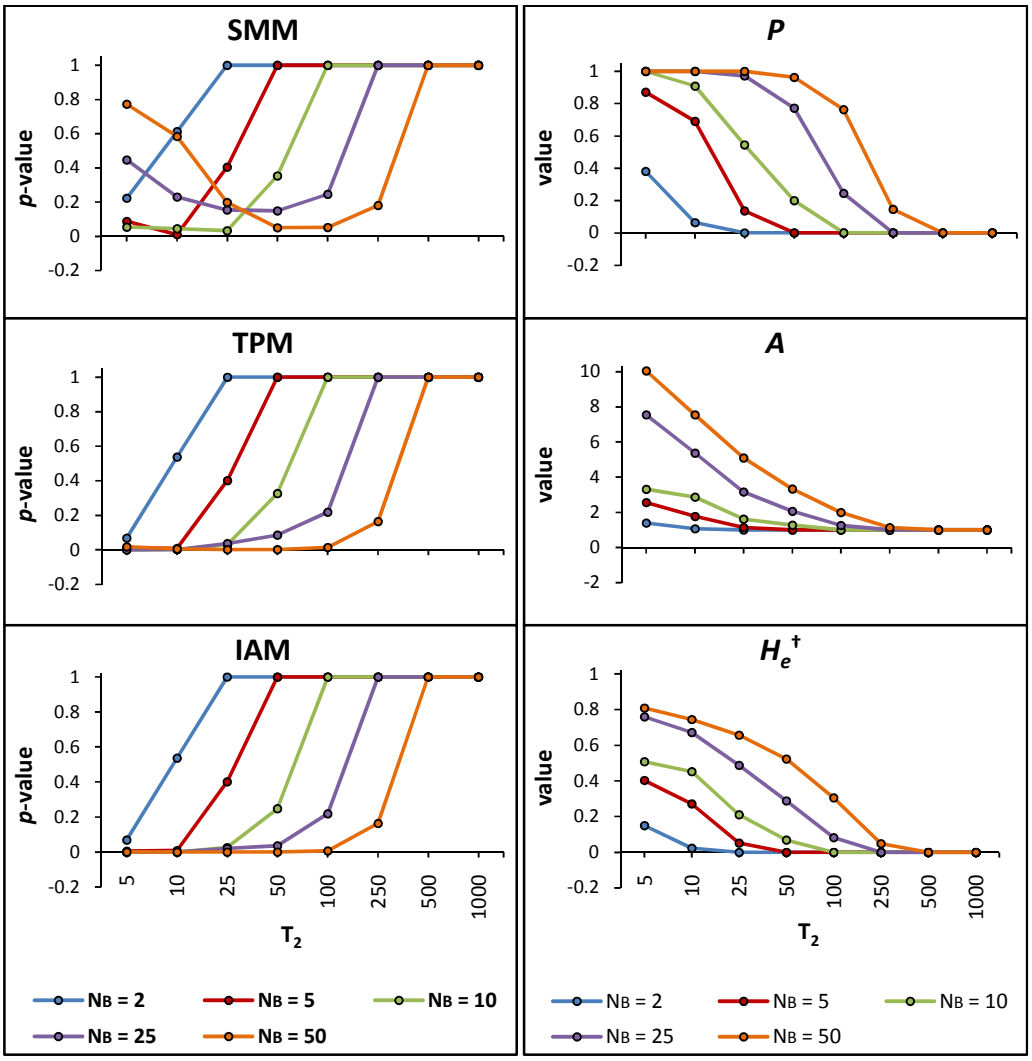

Figure 3.11. *P*-values and genetic diversity for TYPE II samples. As in TYPE I experiments, SMM has a narrower detectability range, hence shows higher type II error. Under SMM, as T_2 increases, obs. het. excess approach to significant *p*-values whereas as T_2 further increases, obs. het. excess again decreases, since *P* hits the critical point (*P* graph). This gives a more complete reversed bell-shaped graph. *e.g.* for N_B=50 and under SMM, *p*-value decreases until T_2=100 and increases afterwards, and according to *P* graph, beyond T_2=100, N_B=50 hits the critical point (*P* = 0.4-0.5) where # polymorphic loci is inadequate to detect het. excess. Whereas for N_B=2, under SMM, het. excess could be not be detected, because *P*=0.4 even at T_2=5. TPM and IAM give significant results for shorter permanence periods, but their behavior is again same with SMM after critical point.

<Abbr/> *SMM*, stepwise mutation model; *TPM*, two phase model; *IAM*, infinite allele model; *P*, proportion of polymorphic loci; *A*, allelic diversity; H_e, expected heterozygosity.

***** Simulations replicated 10 times.

****** Parameters for TPM: variance = 30.00, proportion of SMM = 70%.

† NEI's (1973) heterozygosity.

CHAPTER 4

DISCUSSION

Verification of genetic diversity is important for captive breeding programs since many captive populations are established after only very small number of individuals are left in the wild, e.g., speke's gazelle from only 4 founders (1 male and 3 females), and both Przewalski's horse and European bison. *Bison bonasus* from 13 founders. It is assumed that captive population at Bozdağ Protection & Breeding Station (PBS) was established from a total of 40 to 50 individuals between 1988 – 1995 (ARIHAN, 2000). The genetic diversity of this population was analyzed only once (ÖZÜT, 2001) prior to this study, but for captive breeding programs genetic data should be updated more frequently in order to analyze and assess temporal change in genetic diversity to take precautions. The primary objective of this study was to demonstrate the genetic change for reintroduced populations with respect to the source population. Such a comparison needs revising genetic diversity within source population at Bozdağ PBS. Since, 103 reintroduced individuals (81 to Nallıhan, 22 to Karadağ) were part of the source population prior to translocations, these individuals were collated with the 69 individuals – that were captured and sampled but were not translocated – in order to estimate genetic diversity within source population. Consequently, genetic diversity estimation for the source population was based on 172 samples, while estimations for Nallıhan and Karadağ populations were based on 81 and 22 samples, respectively.

Genetic diversity within source population was found to be low; n_k (mean number of alleles per locus) = 2.9091, H_o = 0.3830, H_e (NEI, 1973) = 0.3956 (Table 3.2). When

compared with the results of ÖZÜT (2001), which were; n_k = 2.5, H_o = 0.3059, H_e = 0.3310, an increase can be observed for all genetic measures (Figure 4.1). Possible reasons for this difference can be; (*1*) effect of temporal change within allelic frequencies, (*2*) effect of different loci analyzed, and (*3*) effect of other reasons including experimental artifacts. If only heterozygosity values were increased, the reason could be attributed only to temporal changes within allelic frequencies, however since n_k is also higher, there should be an effect of at least one of the alternate reasons, because there is only 7 years between the studies and this time interval is unlikely to mutate novel alleles even though microsatellites show extremely high mutation rates (JEFFREYS *et al*., 1988; KELLY *et al*., 1991; WEBER & WONG, 1993). If we first criticize on the second alternate reason, it is possible to say that different loci analyzed are definitely effective for obtaining various results for the two studies but assigning this as a single reason or together with the first alternate reason is not enough since there is also difference among identic loci. In ÖZÜT (2001), 10 loci were analyzed and only OarAE119 was not analyzed by the current study. Similarly, current study analyzed 11 loci and only 2 loci, OarJMP29 and BM1443, were not analyzed by ÖZÜT (2001). Hence, 9 loci are identical for both studies. Regarding only these 9 loci, results for the current study are; n_k = 3.11, H_o = 0.3515, H_e = 0.3688 and by ÖZÜT (2001); n_k = 2.56, H_o = 0.3053, H_e = 0.3321. Again higher values are observed for the current study. Hence, the effect of the first two alternate reasons can not be denied but no matter what their effects are, they can not be attributed either singly or together as the reason for the difference among studies. Briefly, the third alternate reason is supported and at least one other reason should have contributed.

Two situations are considerable for the third alternate reason; (*1*) different sample sizes, and (*2*) genotyping errors. In my opinion, the first situation possibly has a priority. ÖZÜT (2001) made his genetic analyses on 48 samples but 172 samples were utilized by the current study. It is possible to say that rare alleles ($f < 0.05$) were better detected by the current study (Table 3.1), because ÖZÜT (2001) detected 23 alleles for 9 loci (*i.e*. those identical with this study) and 4 of them were rare alleles. However, 2 of these rare alleles were not assigned as rare by this study,

because higher frequencies were detected. In addition, again for the shared loci, this study detected 28 alleles and 5 alleles are rare but 3 of these rare alleles were not detected by ÖZÜT (2001).

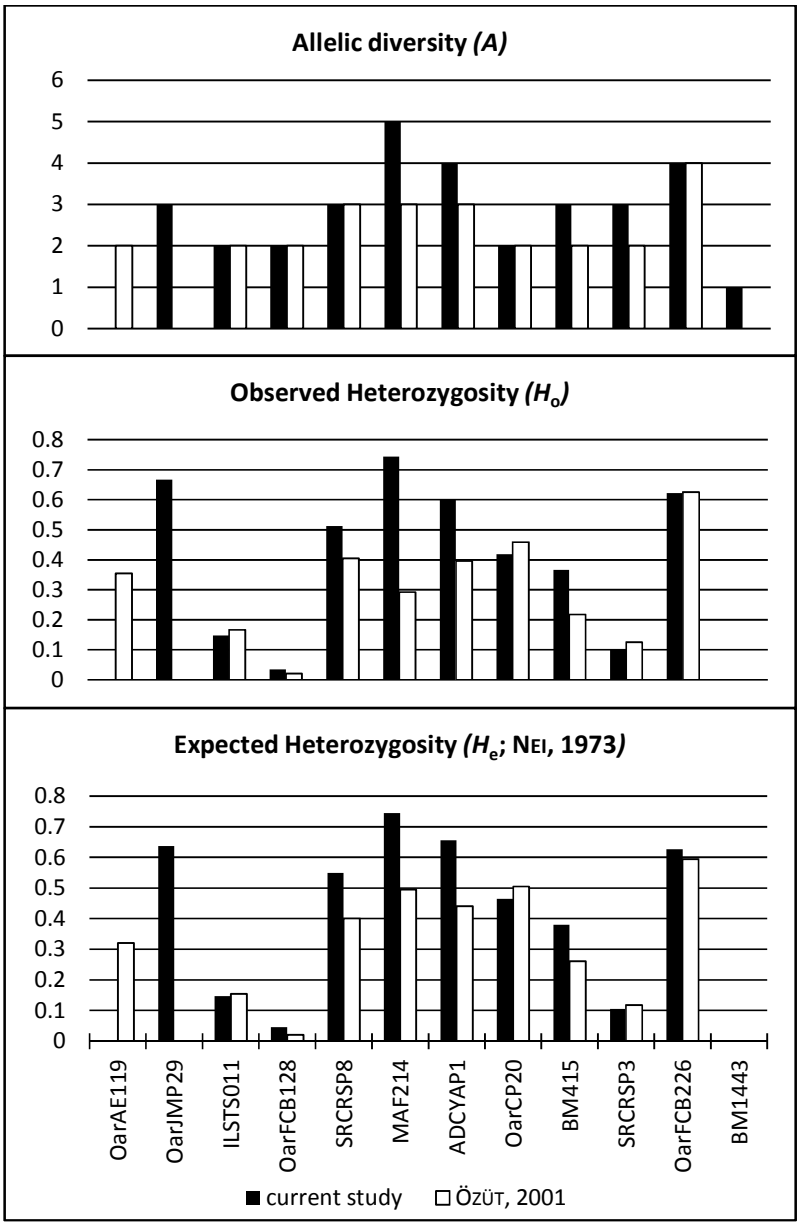

Figure 4.1. Comparison of current genetic diversity with ÖZÜT (2001).

Lesser reason can be different sampling methods used which may lead to genotyping errors. ÖZÜT (2001) had done noninvasive sampling by collecting feces from Bozdağ PBS, but for this study we used only tissue samples collected nondestructively by cutting a tiny piece from ear tips. One of the most important parameters for reliable PCR amplifications is template DNA concentration, and low concentrations may lead to genotyping errors. Allelic dropouts are primer causes of genotyping errors concerning microsatellites. Unlike those studies with tissue samples, studies on feces can experience allelic dropouts with higher probabilities. However, ÖZÜT (2001) performed allelic dropout test and could not detected any. But, despite test evidence, allelic dropout is still possible and alleles could have been lost. Consequently, allelic dropout can be a supplemental reason, but the effect of sample size seems more powerful and undeniable. Briefly, since not only allelic frequencies but also allelic diversity is different among studies, taking the difference only as a temporal change is impossible. The effect of different loci analyzed is not enough to be the only reason, because identical loci also show variations. Hence, at least one other situation is effective, which presumably is the different sample sizes among studies but experimental artifacts due to genotyping errors could have also contributed. Also, differentiation in genetic diversity was not significant as shown with comparisons of 3 genetic parameters by paired *t*-test; *A* (p = 0.0509), H_o (p = 0.1179), and H_e (NEI, 1973; p = 0.0508) (Table 3.11). *P*-values are generally near significance but still leads to the consensus that the change is not considerable.

Genetic diversity estimations for reintroduced populations gave similar results with the source population – Nallıhan population; P = 0.9091 n_k = 2.9091, H_o = 0.4086, H_e (NEI, 1973) = 0.4052; and Karadağ population; P = 0.8182, n_k = 2.5455, H_o = 0.3388, H_e = 0.3607. When compared to the genetic diversity within source population (n_k = 2.9091, H_o = 0.3830, H_e = 0.3956), the results are very favorable for Nallıhan population but not too well for Karadağ population. These results were somewhat expected because the size of reintroduced populations are large especially for Nallıhan where 131 individuals were reintroduced and 81 of them were sampled. For Karadağ, the number of reintroduced individuals is 61 but only 22 of them were sampled and analyzed (Table 2.1). The lower n_k for Karadağ population is most

probably due to small sample size. This is not explicit, but can be inferred from founder event simulation (Figure 3.7). If reintroduced samples are randomly selected, then observed measurements given above should be within the simulated ranges of expectation (99% CI). If not, we can conclude that reintroduced samples are not random. However, all observed measurements are found to be within the ranges of expectation (Table 3.16) indicating random selection of reintroduced samples. It was also confirmed that the low genetic diversity within Karadağ samples is not unexpected, and is due to the small sample size for Karadağ population. On the other hand, by assuming estimations based on 172 samples as the true population statistics for Bozdağ population (N=600, Figure 1.1), expected genetic diversity for the entire Karadağ (N_F=61) and Nallıhan (N_F=131) populations were also computed (Table 3.17). Expected values for reintroduced populations are very near to each other and to the estimations of Bozdağ population, since genetic parameters show low sensitivity to founder events except for n_k (Figure 3.6).

Comparisons among source-reintroduced pairs by paired t-test for A, H_o, and H_e generally did not reveal significant differences (Table 3.11). Only Bozdağ-Nallıhan (p = 0.0428) and Bozdağ-Karadağ (p = 0.0329) pairs showed slight significant difference for H_o and H_e, respectively. On this basis, it is reasonable to state that reintroduced populations are not significantly different from Bozdağ population.

All but SRCRSP8 locus ($p < 0.05$) are found to be under HWE and Bozdağ population was also found to be under Hardy-Weinberg Equilibrium (HWE) overall loci (CE, p = 0.2798; MC, p = 0.2881, Table 3.3) which indicate an equilibrium state within source population. Linkage disequilibrium was tested with MC algorithm (RAYMOND & ROUSSET, 1995) and out of 45 locus pairs for 10 loci - BM1443 was ignored due to monomorphic state - only one pair, BM415-ADCYAP1 (p = 0.013) showed linkage disequilibrium. This constitutes only 2.22% of all locus pairs. This percentage is very low enough to assume that among the loci analyzed there is linkage equilibrium and alleles at one locus do not show any statistical association (*i.e.* nonrandom association into gametes) with alleles at other loci. *EWENS-WATTERSON Test* for neutrality gave supportive results. Since microsatellite loci are

generally in the non-coding region, we expect them to be selectively neutral. Eight out of 10 loci - BM1443 was again ignored - shown to be neutral indicating the consistency of Hardy-Weinberg homozygosity within source population with that expected from mutation-drift equilibrium (*equilibrium* homozygosity) under neutral theory. Remaining 2 loci, OarJMP29 and MAF214, were not neutral due to slightly smaller observed *F* values than their simulated L95% CI (Figure 3.4). Generally speaking, HW disequilibrium, linkage disequilibrium, and non neutrality with low levels may only be due to random processes, because genetic drift can also create such deviations from expected.

Effective population size (N_e) was estimated but our data allowed the use of only two methods: heterozygote excess method and linkage disequilibrium method. Heterozygote excess method assumes that if the number of breeders in a population is small, then by chance effect allelic frequencies among sexes will be different and this leads to heterozygote excess within progeny (LUIKART & CORNUET, 1999). However, heterozygote excess method did not give a numerical estimation (infinity, [infinity, infinity][14]). This is not unexpected when this method is applied for N_e estimations (WANG, 2005). LUIKART & CORNUET (1999) applied this method to 10 empirical and simulated data sets with small parental population sizes, but still infinitely large N_e were found for 5 data sets, even though the precision of this method is negatively correlated with true effective population size (WANG, 2005). This estimation is useful only for very small randomly mating populations and when a substantial amount of loci are analyzed with a large sample (LUIKART & CORNUET, 1999; WANG, 2005). However, Anatolian mouflon population is not a randomly mating population. Another disadvantage of this method is that it only works for species with separate sexes and needs modification of equations for other types of species (LUIKART & CORNUET, 1999). Linkage disequilibrium method can be used to estimate N_e, since linkage disequilibrium is a consequence of genetic drift if neutral loci are assumed to be unlinked with selected loci (HILL, 1981; WANG, 2005). However, this method also has a low precision but still supplied numerical

[14] 95% CI

estimations; NEESTIMATOR: 207.7 [122.4, 481.4][15], *LDNE*: 226.9 [108.2, 1250.1][2,16]. According to recent analyses, the actual population size within Bozdağ PBS is 600 (Figure 1.1). Hence, estimations with linkage disequilibrium method are reasonable. However, estimating N_e with genetic data by several independent N_e estimators and taking their harmonic mean is more promising for reliable analysis (WAPLES, 1992; PUDOVKIN *et al*., 1996, LUIKART & CORNUET, 1999).

One of the lesser objectives of this study was to detect any possible statistical bias caused by the catching method. For this purpose, *A*, H_o, and H_e (LEVENE, 1949 & NEI, 1973), and Probability of Identity (PI) were compared for trap-trap, and Bozdağ-trap pairs by paired *t*-test. Differences among pairs were not significant ($p > 0.05$) for all compared genetic parameters (Table 3.12). H_e (NEI, 1973) for traps at Karanlıkdere, Bağderesi, and Gölet are 0.3816, 0.4047, and 0.3918, respectively and for the source population it is 0.3956 (Table 3.13). Similarly, PI comparisons among pairs also revealed nonsignificant differences (Table 3.14). In my opinion, the more informative are the comparisons among trap-trap pairs. If there had been a significant variation for genetic diversity or PI values among different traps, then this would indicate a selective or heterogenous behavior by traps and the need to revise the locations of the traps or the one that departs from the others. Comparing only the genetic diversity parameters would be enough for our inferences but PI values are also assistive in this context. PI show the probability of owning identical genotypes at all related loci (*img*, identical multilocus genotype) for two randomly chosen individuals from the same group. Thus, it is negatively correlated with genetic diversity and should support comparisons based on genetic diversity. Furthermore, PI can indicate the kinship levels within groups. This is especially important for mouflon population, because they form groups of related individuals and this can depart genetic diversity estimation from true population statistics since the chance of capturing and sampling related individuals is high. If individuals within a group show closer relationships relative to another group, then PI is expected to be higher within the former group. Similar average $PI_{theoric}$ values were

[15] 95% CI
[16] Jacknife method

computed for traps at Karanlıkdere, Bağderesi, and Gölet; 3.25E-05, 2.05E-05, and 2.53E-05, respectively (Table 3.15). Average $PI_{theoric}$ for the source population is 2.24E-05 which is also closer to the computations for traps. The generalization for the relation between genetic diversity and PI is also confirmed when H_e and PI computations for traps and source population are compared. Briefly, traps do not show significant variation and represent the source population with approximate results. However, this does not necessarily mean that traps efficiently represent the actual genetic diversity within the source population. Even though this assumption is not met, it is still possible to find homogeneity among traps. However, an efficient representation of actual genetic diversity is a highly probable expectation with 172 samples as we assume the actual population size to be about 600 (Figure 1.1).

Simulation experiments for founder events were informative since two real data sets were used with dissimilar genetic diversity contents; genotypic data for (*1*) Anatolian mouflon (172 individuals at 11 loci) and (*2*) honey bees (197 individuals at 5 loci). Among the analyzed parameters (n_k, P, H_o, and H_e; NEI, 1973), only n_k showed considerable variability when tested for different N_F (Figure 3.6, Graph a & e). However, the degree of variability for this parameter was different among the data sets. This difference is not structural but proportional that is caused by different ratios of rare and common alleles within the data sets. Specifically, for Anatolian mouflon: A_T = 32, A_C = 27, A_R = 5, and thus the ratio of rare alleles to common alleles (A_R/A_C) is 0.185. Whereas for honey bee data; A_T = 80, A_C = 22, A_R = 58 and A_R/A_C = 2.636. Since ratio between rare and common alleles is much higher for honey bee data, the graph showed a steeper increase for n_k with increasing N_F. So, while common alleles can migrate more efficiently, rare alleles are less efficient (*e.g.* 3 of the 4 nonexistent alleles in Karadağ population were assigned as rare for the source population, Table 3.1) or in other words rare alleles are lost faster than common alleles. This confirms the basic assumption of allele frequency distribution method for bottleneck analysis.

SPENCER *et al.*, (2000) made laboratory experiments on population bottlenecks of different magnitude with mosquitofish, *Gambusia affinis*, and examined 8

microsatellite loci to analyze the change in A, H_o, H_e, and P with respect to the source population. It was found that A shows highest sensitivity between bottlenecked and source populations whereas H_e shows intermediate, and H_o and P show lowest sensitivity. In our simulation experiments, heterozygosity levels and P were also affected less than A during founder events. On the whole, H_o was always constant for both data sets. Hence, the simulation results fit well with the results of SPENCER *et al*., (2000). Simulation results also confirm the basic assumption of heterozygosity excess method of CORNUET & LUIKART (1996) for bottleneck analysis which states that allelic diversity decreases at a faster rate than H_e during population bottlenecks.

Bozdağ population was found to be under mutation-drift equilibrium by BOTTLENECK program (PIRY *et al*., 1999). Except standardized differences test, *p*-values were generally not significant indicating the lack of considerable heterozygosity excess ($H_e > H_{eq}$) (Table 3.9). Non-bottlenecked populations (*i.e*. equilibrium populations) are expected to show slight heterozygosity excess at 50% of all loci analyzed and slight heterozygosity deficiency ($H_e < H_{eq}$) in the other half due to random genetic drift (LUIKART & CORNUET, 1998). However, a bottlenecked population is expected to show heterozygosity excess in substantial amount. Standardized differences test gave significant *p*-values under IAM and TPM for heterozygosity excess, however for less than 20 loci this test is not reliable (PIRY *et al*., 1999). Wilcoxon signed-rank test also gave significant *p*-value (0.02441) under multistep mutations (IAM). But, since single step mutations (SMM) better conform to microsatellite loci (SHRIVER *et al*., 1993; ELLEGREN, 2004), analysis under IAM is also not very reliable and can be neglected. On the other hand, the proportion of single step mutations was 70% (30% multistep mutations) when heterozygosity excess was tested under TPM. If this proportion is decreased, p_{TPM} approaches to p_{IAM} and bottleneck detection rate increases, but as mentioned above analysis becomes less reliable. Specifically, when tested for 60% single step mutations, p_{TPM} was significant (< 0.05). p_{SMM} and p_{TPM} were further supported by normal L-shaped distribution of alleles (Figure 3.5). Since a genetic bottleneck is nearly definite for Bozdağ population, there is obviously a detection failure. Four reasons are possible

for the detection failure; (*1*) incorrect basic assumptions for applied methods, (*2*) violations of model assumptions, (*3*) weakening of heterozygosity excess due to gene flow, or (*4*) type II error due to mutation models.

The basic assumptions of heterozygosity excess method (*i.e*. allelic diversity decreases faster than heterozygosity during a bottleneck) and allele frequency distribution method (*i.e*. rare alleles are lost faster than common alleles during a bottleneck) were shown to be correct with simulations by this study. Furthermore, the faster decrease in allelic diversity was also noted by NEI *et al*. (1975), DENNISTON (1978), MARUYAMA & FUERST (1985), and ALLENDORF (1986). Although there can be exceptions, these assumptions are correct in most cases. Thus, excluding the probability of exceptional cases, first alternate reason is unlikely for a detection failure in bottleneck analysis.

For the second alternate reason, first of all, bottlenecks are assumed to be recent. Recentness is defined as within the last $2N_e$ to $4N_e$ generations which changes according to the severity of bottleneck (θ) and mutation rate at analyzed loci (PIRY *et al*., 1999). Otherwise, a bottleneck event is unlikely to be detected by heterozygosity excess method since the population will again reach to a new mutation-drift equilibrium and the signs of heterozygosity excess will be wiped out (LUIKART & CORNUET, 1998). N_e is the effective population size of the bottlenecked population (CORNUET & LUIKART, 1996) which is unknown for the captive Bozdağ population. However, parental population size is known to be about 40 to 50 (ARIHAN, 2000). It is possible to make a roughly estimate of N_e of bottlenecked population by using current estimations. N_e for current Bozdağ population was estimated and found to be 226.9 and 207.7 by linkage disequilibrium method (Table 3.7), whereas the actual population size for Bozdağ population is around 600 (Figure 1.1). Hence, there is nearly a 1/3 ratio between N_e and actual population size. If we use this information comparatively and assume that the actual size for parental population as 40 to 50, then N_e for bottlenecked population can around 10 to 15. To be more conservative, if it is even taken as 10, the detectability period should include at least the last 20 to 40 generations. However, maximum 10 generations have passed from the end of

bottleneck period since Bozdağ population was first established in 1988. Hence, bottleneck period is recent enough to fit this model assumption. On the other hand, if N_e for bottlenecked population was lower than 5, then model assumption is violated. But this is very unlikely situation with a parental population consisting of 40 to 50 individuals.

Also, PIRY *et al*. (1999) recommends at least 10 polymorphic loci to achieve high statistical power (> 0.80). We analyzed 11 loci and 10 of them were polymorphic, but 1 locus showed HW disequilibrium and was omitted. Thus, 9 polymorphic loci were evaluated by BOTTLENECK program. This also meets the model assumption. Also, according to PIRY *et al*. (1999), the method is useful even for 4 polymorphic loci, but totally unreliable for lower number of loci. This was also confirmed by TYPE I and TYPE II experiments. Beyond critical point (corresponds to ~4 loci), bottleneck detectability decreases, whereas detectability always persists for 9 loci under all models and types of experiments. Thus, detection failure can not also be due to the violation of this model assumption. Other methods for bottleneck analysis, the intralocus variance *k*-test and the interlocus *g*-test of REICH *et al*. (1999) and *M*-ratio method of GARZA & WILLIAMSON (2001), need much more loci (about 30 loci) than analyzed by this study to achieve high statistical power, and consequently these methods were not utilized. Briefly, the first and second alternate are unlikely to cause detection failure for Bozdağ population.

For the third alternate reason, gene flow may be the source of detection failure. If individuals from a different gene pool were migrated into Bozdağ PBS, this can significantly change allelic diversity and heterozygosity levels within Bozdağ population and heterozygosity excess can weaken. For instance, if somehow allelic diversity increases but heterozygosity remains nearly unchanged, then H_{eq} estimations will generally increase and fewer loci will show heterozygosity excess which in turn will decrease the probability to detect a bottleneck. However, migration into Bozdağ PBS is not possible due to the surrounding fences and consequently, Bozdağ population can be assumed as a closed population. But, from time to time, very small groups of individuals (2-3 individuals) were transferred into

Bozdağ PBS from outside by wardens (ARIHAN, 2000). Since these individuals were not transferred elsewhere but from Bozdağ WDA into Bozdağ PBS, they are part of a very similar gene pool. Briefly, detection failure due to the very low amount of gene flow from a very similar gene pool does not seem to be very possible, but this is not explicit and should be further supported with theoretical analyses.

On the other hand, simulations indicated type II error for distortion graphs and heterozygosity excess method extended to SMM especially as bottlenecks become severer. These experiments are expected to efficiently simulate bottleneck analysis based on microsatellite data, although the effect of selection, mutation, gene flow, and generation overlap were underestimated. Specifically, microsatellite loci are selectively neutral, and mutations can only cause minor effects even when simulation durations are very long. The absence of gene flow again should not cause unconformity, because the population is assumed to be closed. However, discrete generations may cause unconformity with Bozdağ population. Also, pre-bottleneck genetic diversity and population size (N_S) are unknown for Bozdağ population. All simulation experiments were started with identical pre-bottleneck genetic diversity and N_S. If different values for these parameters can cause variations in experimental results, this should be proportional variations especially for the extent of simulation generations. For instance, if pre-bottleneck genetic diversity was lower, the reversed bell-shaped structure of TYPE I and TYPE II results should still persist but would not spread over very long generations. Same conclusion is reasonable also for N_S and N_B. For this reason, simulation generations should not be thought as true indicators unless additional information for pre-bottleneck conditions is available.

Beside parametric values, the structure of bottleneck period has critical importance, but unfortunately this is unknown for Bozdağ population. If the populations extirpated due to habitat destruction and competition with domestic livestock, then very slow bottlenecks (e.g. TYPE I in long generations) are more probable, whereas predation, disease, and poaching can accelerate bottleneck period. Also, as a rare case, populations can persist in small sizes after severe or not so severe bottlenecks. TYPE II bottlenecks gives simulations of this kind. During permanence period, due

to low $N_{e,}$ the rate of genetic drift is very high leading to faster reduction of genetic diversity. This was shown with the comparisons among different bottleneck types. Results represented a reversed bell-shaped distribution of significance in both TYPE I and TYPE II experiments, where type II error due to single step mutations can be observed prior to critical point. There is an increase in *p*-values after this critical point for all experiments under all models. These should not be taken as type II error, but as violation of model assumption since *p*-values increase due to the lack of bottleneck detectability caused by the insufficient number of polymorphic loci (about 4 loci). Hence, p_{SMM}, p_{TPM} (Wilcoxon test, Table 3.9) and distortion graph for Bozdağ population (Figure 3.5) may constitute a type II error, because SMM and TPM were based 100% and 70% single step mutations, respectively. In addition, the other alternate reasons were not supported by simulations and historical data.

Also, as mentioned, heterozygosity excess method extended to IAM should be underestimated for microsatellite data. However, both TYPE I and TYPE II experiments did not indicate type II error under IAM. But WILLIAMSON-NATESAN (2005) found out with simulations that as model departs from SMM to IAM, the degree of type I error (*i.e*. a non-bottlenecked population is assigned as bottlenecked) increases. According to the same study, under TPM with 70% single step mutations, the degree of type I error was higher and type II error was lower than under SMM in all types of tests. Also, as the proportion of multistep mutations for TPM was increased, the degree of type I error also increased. Hence, bottleneck analyses assuming multistep mutations determine higher heterozygosity excess (*i.e*. lower *p*-value) in analyzed loci also when non-bottlenecked populations are tested. Briefly, nonconformity of IAM to microsatellite loci (SHRIVER *et al*., 1993; ELLEGREN, 2004) and also the higher degree of type I error associated with multistep mutations indicate that bottleneck analyses with heterozygosity excess method extended IAM are not reliable.

Finally, heterozygosity excess and allele frequency distribution methods should be examined with more details during bottleneck analysis since there can be different sources of statistical error. First of all, the source of error should be determined by

researchers. If study population fits to model assumptions and any other factor (*e.g.* gene flow) do not constitute a source of error, then assumed mutation model should be correctly parameterized and the degree and type of error that is acceptable should be decided (WILLIAMSON-NATESAN, 2005). By this way, a more reliable inference on the results of bottleneck analysis is possible.

CHAPTER 5

CONCLUSION

According to the results, Bozdağ population and reintroduced populations suffer from a low genetic diversity. However, comparative analysis determined that differences in major genetic parameters among Bozdağ population and reintroduced populations are generally not significant and a very high percentage of genetic diversity within Bozdağ population was carried to both reintroduction sites. This is expected because Bozdağ population already has a low genetic diversity and major genetic parameters except allelic diversity are sensitive to changes in population size at a very low degree – even when source population shows a high genetic diversity.

Additionally, observed genetic diversity within reintroduced samples are in the ranges of expectation which indicates that these samples were randomly selected. And based on this finding, we can assume that both reintroduced populations were established entirely with randomly selected individuals since the same catching method was utilized for all translocations performed.

The lack of significant departure among traps in terms genetic diversity indicates homogeneity for the catching method. However, whether traps represent the actual genetic diversity within Bozdağ population is not explicit, but this is highly probable in the case of 172 samples. Thus, we can state that our data were not biased by the positions of traps and estimated genetic diversity for Bozdağ population is expected to be very close to the actual genetic diversity since sample size is very large.

Although bottleneck analysis could not detect a recent genetic bottleneck for Bozdağ population, both heterozygosity excess method and allele frequency distribution method are based on correct assumptions. Thus, basic assumptions of methods can not constitute a source of error that can cause detection failure. Perceptibly, there is also no evidence for a source of error due to the violation of other model assumptions such as recentness of the bottleneck. However, it was shown that heterozygosity excess method under single step mutations (*i.e*. SMM) and allele frequency distribution method can lead to bottlenecked populations to be erroneously assigned as under equilibrium (*i.e*. type II error). Hence, a correct parameterization of mutation model and the degree of error that is acceptable should be determined to in order to make correct decisions.

Finally, the low genetic diversity estimated for Anatolian mouflon can greatly reduce resistance against pathogens. Bozdağ population significantly declined in number within the last 7-8 years and one of the most plausible reasons seem to be paratuberculosis. Consequently, paratuberculosis should be characterized with molecular markers and certain actions should be taken accordingly. Most importantly, the number of diseased individuals should be minimized during the selection for translocations.

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APPENDIX A

[17]Name - GenBank Accesion No. - *Origin* - Chromosome No. - # base pairs - *Repeat seq* [Repeat region]

1. OarCP20 - U15695 - *Ovis aries* - 21 - 75bp - *TG* [26-53]

```
1    ggcatttcat ggctttagca ggggc tgtgt gtgtgtgtgt gtgtgtgtgt gtgccgtttc
61   ctcctccagg ggatc
```

[Reverse Complemented Strand]

```
1    gatcccctgg aggaggaaac ggcacacaca cacacacaca cacacacaca gcccctgcta
61   aagccatgaa atgcc
```

2. OarJMP29 - U30893 - *Ovis aries* - 24 - 142bp - *AC* [35-72]

```
1    gtatacacgt ggacaccgct ttgtacacgg gtncacacac acacacacac acacacacac
61   acacacacac acatatacaa gcagctttgc taacaatngg aacaatcaac caaccctctt
121  cccctctgaa tcttgccact tc
```

[Reverse Complemented Strand]

```
1    gaagtggcaa gattcagagg ggaagagggt tggttgattg ttccnattgt tagcaaagct
61   gcttgtatat gtgtgtgtgt gtgtgtgtgt gtgtgtgtgt gtgtgtgtgn acccgtgtac
121  aaagcggtgt ccacgtgtat ac
```

3. OarFCB128 - L01532 - *Ovis aries* - 2 - 123bp - *GT* [51-94]

```
1    attaaagcat cttctcttta tttcctcgct ttgttcttat gactnactgc gtgtgtgtgt
61   gtgcgtgtgt gtgtgtgtgt gtgtgtgtgt gtgtgcagca tgtatgtctt agttgctcag
121  ctg
```

[Reverse Complemented Strand]

```
1    cagctgagca actaagacat acatgc(t)gca cacacacaca cacacacaca
cacacacacg
61   cacacacaca cacgcagtna gtcataagaa caaagcgagg aaataaagag aagatgcttt
121  aat
```

---

[17] Specification key.

Italic letters indicate repeat sequences.

Bold letters indicate primer sequences.

Oligos are sorted according to origins.

Source: http://www.ncbi.nlm.nih.gov/

4. OarFCB226 - L20006 - *Ovis aries* - 2 - 131bp - *AC* [64-91]

```
1    gtgagtccca tagagcataa gctcaaagag aaacgtgaaa agacacatat taatcaaatc
61   taaacacaca cacacacaca cacacacaca cagataaata ttaaaagcag gaagggaaag
121  gcaacatata g
```

[Reverse Complemented Strand]

```
1    ctatatgttg cctttccctt cctgcttta atatttatct gtgtgtgtgt gtgtgtgtgt
61   gtgtgtgttt agatttgatt aatatgtgtc ttttcacgtt tctctttgag cttatgctct
121  atgggactca c
```

5. MAF214 - M88160 - *Ovis aries* - 16 - 188bp - *GT* [26-138]

```
1    aatgcaggag atctgaggca gggacgtgtg tgtgtgtgtg tgtgtgcatg cagtgtgtgt
61   gtgtgtgcat gcagtgtgtg tgcgcgtgca tgcagtgtgt gtgcattttg tgtgtgtgca
121  ttttgtgtgt gtgtgtgtgc ttgcgtgcgt gcatttttt ctcctccaaa acctccctaa
181  gatcaccc
```

[Reverse Complemented Strand]

```
1    gggtgatctt agggaggttt tggaggagaa aaaaatgcac gcacgcaagc acacacacac
61   acacaaaatg cacacacaca aaatgcacac acactgcatg cacgcgcaca cacactgcat
121  gcacacacac acacactgca tgcacacaca cacacacaca cacgtccctg cctcagatct
181  cctgcatt
```

6. ADCYAP1 - NM 009625 - *Bos taurus* - 24 - 2641bp

```
1    gattctgtac ttaaaaggcc acaggcagac agatgttgac aagaaagtct cttttgaaac
61   cacgttcgga tagatttctg ctaactgccc agataaatag gagcagaggg ctggtcacct
121  ctgtaataac caccggcagc agtagaagaa accgcagctt cagacgcagc cagagagact
181  tctgagcagc gaaggcgctt gcctgctcga gctgcctggc cgggcggctg accagacgc
241  cgacttcgct gaggccctct ctctttctct ctctctctct ctttgcttct ttccttatca
301  cttctttctt ctcggtggac ttcaggccac tttgtctccc acccacactc agctcgtcgc
361  ctcctccgtc ttccttctcc atctctcccc tcgcccccct tctctcggtg tcacgctccg
421  tcctagttcc gagcgtcgtc aaacttttga acagaataac aggactcagc aaacaagtcc
481  tccagctcct cccgcggctc cggctcgttc ctgcggctcc tgctcagacg ctaacgccaa
541  acggcgatga ctcttgggtt gtgactgcag cgcacaaact tggagaagcc ctttgcccgc
601  cgtcctactt ggcagcaaac cctctcctgg cagcgaatga ccatgtgtag cggagcgagg
661  ctggccctgc tcgtttacgg gatactgatg cacagcagcg tctacggctc acctgccgcc
721  tccggactcc ggttcccggg gatcaggccg gagaacgagg tgtacgacga ggacggaaac
781  ccgcagcagg acttctacga ttcggagtct ccgggcgtgg ggagccccgc ctccgcgctg
841  cgcgatgcct acgcgctcta ctaccccgcg gaggaaagag atgtcgccca cgggatcctt
901  aataaggcct accgcaaagt gctggaccag ccgtccgcca ggagatacct gcagacgctc
961  atggccaagg gcttgggtgg gaccccgggc ggcagcgcgg acgacgactc ggagccgctc
1021 tccaagcgcc actcggacgg catcttcact gacagctaca gccgctaccg gaaacaaatg
1081 gctgttaaga aatacttggc tgctgttcta gggaaaaggt ataaacaaag ggttaaaaac
1141 aaaggacggc gaataccgta tttgtagcga cgagttgcca gctatcctgt gtatacggcc
1201 ctgacacaat gagaagtcgt ttttcccaac tgactgaact gtcattgctc ctgtgttctg
1261 tcccacatgt atttatgtat gaagtcaagc cattaaatga atattttgat aataatattg
1321 tttttctttt tacgaagcac tagagaatgc acagatatac tttgtggacc aattattgat
1381 attgacatat atattacgaa tatataaaga gtatatatat atatatatat aaagtataat
1441 agagagccgt tcatacagtg tgcacaagga ttgaagattc gcctgagctg tttgttttta
1501 tataaaataa atagaaaaat agacaatcat tgttttgaat attactccta tttttgtaaa
1561 ctggaattaa aaggatagta tttttatcca cgataggcct gaagatatta atcctgacca
1621 tttgctactg tacataaaca gtgatgccct gctccaggga ctttgaggta atgatttggg
1681 aggattgctg aaggtctgtc tttcccaggg agtctctagg gcaggctgct tcaatcccag
1741 ctgaactcaa ctgaggctct gtctacccct tgctgggtgg caatgccaat acttccgctt
1801 tctttgattc tatttttatg tgtatttgtc tctcttcaga ctctcagccc acaaggaaat
```

1861 tctcctgata aaacaacagc tggatctaaa ttgtgcttct ccccagaatt catactactc
1921 cctgggggag gagttggggg actgtacaga gaagagagac ttgaatagga agctctcttt
1981 tctgtacttc cagggacccc agtatcccaa ggttagggca attggaacaa agtgggacta
2041 ttggaaaagg cagagcataa ggcagtaggg ggaggaccct ggagagggac tggctgcagg
2101 cagccccagc ctgggggctg gcggtaagcc cagtcccacc ataggtcccc tgcctgcctg
2161 actttgggcg ctgggtattg gaaatggatg caaagtacaa tgtgtttttc tccagtgcta
2221 tccatgctgc tcatgttgtg aaatggccag gatcctcccc tttgaaccct gctgtgtagg
2281 agcctccctg ttcttccgtg gttttcctga agactcctct ttcccacctt ctcgcactgt
2341 ttaagtactg tttgccgttt tttattcact tctcttaaac ttgtgaatgc ttctttttcc
2401 tgttgattga tgctagcact tattgtaaag tgtaggaacc cctgtgtggt taccactaag
2461 taattatgca ctatatatga atcttttgtt tcttgttgat tgagtttgta ggtaaaatgt
2521 atttttctac atttatggct tattgcttag taaaatttat ttcataaaac caacctttgt
2581 gatattagaa tgtgtagtgt tcacatgttg ctcagttgta ctaactgata aatcatttaa
2641 accccaaaaa aaaaaaaaaa a

[Reverse Complemented Strand]
1   ttttttttttt ttttttgggg tttaaatgat ttatcagtta gtacaactga gcaacatgtg
61   aacactacac attctaatat cacaaaggtt ggttttatga aataaatttt actaagcaat
121 aagccataaa tgtagaaaaa tacattttac ctacaaactc aatcaacaag aaacaaaaga
181 ttcatatata gtgcataatt acttagtggt aaccacacag gggttcctac actttacaat
241 aagtgctagc atcaatcaac aggaaaaaga agcattcaca agtttaagag aagtgaataa
301 aaaacggcaa acagtactta aacagtgcga gaaggtggga aagaggagtc ttcaggaaaa
361 ccacggaaga acagggaggc tcctacacag cagggttcaa aggggaggat cctggccatt
421 tcacaacatg agcagcatgg atagcactgg agaaaaacac attgtacttt gcatccattt
481 ccaataccca gcgcccaaag tcaggcaggc aggggaccta tggtgggact gggcttaccg
541 ccagccccca ggctggggct gcctgcagcc agtccctctc cagggtcctc cccctactgc
601 cttatgctct gccttttcca atagtcccac tttgttccaa ttgccctaac cttgggatac
661 tggggtccct ggaagtacag aaaagagagc ttcctattca agtctctctt ctctgtacag
721 tcccccaact cctcccccag ggagtagtat gaattctggg gagaagcaca atttagatcc
781 agctgttgtt ttatcaggag aatttccttg tgggctgaga gtctgaagag agacaaatac
841 acataaaaat agaatcaaag aaagcggaag tattggcatt gccacccagc aaggggtaga
901 cagagcctca gttgagttca gctgggattg aagcagcctg ccctagagac tccctgggaa
961 agacagacct tcagcaatcc tcccaaatca ttacctcaaa gtccctggag cagggcatca
1021 ctgtttatgt acagtagcaa atggtcagga ttaatatctt caggcctatc gtggataaaa
1081 atactatcct tttaattcca gtttacaaaa ataggagtaa tattcaaaac aatgattgtc
1141 tatttttcta tttattttat ataaaaacaa acagctcagg cgaatcttca atccttgtgc
1201 acactgtatg aacggctctc tattatactt tatatatata tatatatata ctctttatat
1261 attcgtaata tatatgtcaa tatcaataat tggtccacaa agtatatctg tgcattctct
1321 agtgcttcgt aaaaagaaaa acaatattat tatcaaaata ttcatttaat ggcttgactt
1381 catacataaa tacatgtggg acagaacaca ggagcaatga cagttcagtc agttgggaaa
1441 aacgacttct cattgtgtca gggccgtata cacaggatag ctggcaactc gtcgctacaa
1501 atacggtatt cgccgtcctt tgtttttaac cctttgttta tacctttcc ctagaacagc
1561 agccaagtat ttcttaacag ccatttgttt ccggtagcgg ctgtagctgt cagtgaagat
1621 gccgtccgag tggcgcttgg agagcggctc cgagtcgtcg tccgcgctgc cgcccggggt
1681 cccacccaag cccttggcca tgagcgtctg caggtatctc ctggcggacg gctggtccag
1741 cactttgcgg taggccttat taaggatccc gtgggcgaca tctctttcct ccgcggggta
1801 gtagagcgcg taggcatcgc gcagcgcgga ggcggggctc cccacgcccg gagactccga
1861 atcgtagaag tcctgctgcg ggtttccgtc ctcgtcgtac acctcgttct ccggcctgat
1921 ccccgggaac cggagtccgg aggcggcagg tgagccgtag acgctgctgt gcatcagtat
1981 cccgtaaacg agcagggcca gcctcgctcc gctacacatg gtcattcgct gccaggagag
2041 ggtttgctgc caagtaggac ggcgggcaaa gggcttctcc aagtttgtgc gctgcagtca
2101 caacccaaga gtcatcgccg tttggcgtta gcgtctgagc aggagccgca ggaacgagcc
2161 ggagccgcgg gaggagctgg aggacttgtt tgctgagtcc tgttattctg ttcaaaagtt
2221 tgacgacgct cggaactagg acggagcgtg acaccgagag aaggggggcg aggggagaga
2281 tggagaagga agacggagga ggcgacgagc tgagtgtggg tgggagacaa agtg**gcctga**
2341 **agtccaccga gaagaaagaa** gtgataagga aagaagcaaa gagagagaga gagagaaaga
2401 gagagggcct cagcgaagtc ggcgtctggg tcagccgccc ggccaggcag ctcgagcagg
2461 caagcgcctt cgctgctcag aagtctctct ggctgcgtct gaagctgcgg tttcttctac
2521 tgctgccggt ggttattaca gaggtgacca gccctctgct cctatttatc tgggcagtta
2581 gcagaaatct atccgaacgt ggtttcaaaa gagactttct tgtcaacatc tgtctgcctg
2641 tggcctttta agtacagaat c

7. BM415 - G18413- *Bos taurus* – 6 – 138bp - *GT?*-(?-?)*

```
1    tg gctacagc ccttctggtt tg catgtgtc cgtgtgtgtg tgtgtgtgtg tgtgtgtgtg
61   tgtgtgtgtt tgtttggctt gaagatgtca gcaagtactg atgattagag ttcttgctgt
121  tggtgattag ctctctaa
```

[Reverse Complemented Strand]

```
1    tta gag(ag) ct aatcaccaac agcaag aact ctaatcatca gtacttgctg
acatcttcaa
61   gccaaacaaa cacacacaca cacacacaca cacacacaca cacacacgga cacatgcaaa
121  ccagaagggc tgtagcca
```

8. BM1443 - G18438 - *Bos taurus* - 254bp

```
1    atcaagggaa tggggcagga gtgcctccca ggaggcagtc acacgtggct gtcag aataa
61   agagacatgg tcaccgg ctg gcaaagagca accaaaatcc attgcagttc actcctctgg
121  gctgcccacc acacacacac acacacatac acacacatgc acacacacac acacacacac
181  acacacacac acccttcctc ccacacctcg aaaagttgcc ccgtcaactt aaaatgcatt
241  caccacttct ttat
```

[Reverse Complemented Strand]

```
1    ataaagaagt ggtgaatgca ttttaagttg acggggcaac ttt tcgaggt gtgggaggaa
61   g ggtgtgtgt gtgtgtgtgt gtgtgtgtgt gtgtgcatgt gtgtgtatgt gtgtgtgtgt
121  gtgtggtggg cagcccagag gagtgaactg caatggattt tggttgctct ttgccagccg
181  gtgaccatgt ctctttattc tgacagccac gtgtgactgc ctcctgggag gcactcctgc
241  cccattccct tgat
```

9. ILSTS011 - L23485 - *Bos Taurus* - 14 - 234bp – TC/CA [121-160]

```
1    agt gcttgct acatggaaag tgc tcagtga aaaggggatt gaggttgaaa taattgattg
61   attataggtt nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn
121  tctctctctc tctc ttat ca cacacacaca cacacacaca aanntgatga attttatgac
181  ntcatgttgc tgnnnacttt ttggtagggc tctgcatttt agagcactaa atca
```

[Reverse Complemented Strand]

```
1    tgatttagtg ct ctaaaatg cagagcccta cc aaaaagtn nncagcaaca tgangtcata
61   aaattcatca nntt tgtgtg tgtgtgtgtg tgtgtg ataa gagagagaga gaga nnnnnn
121  nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnaaccta taatcaatca
181  attatttcaa cctcaatccc cttttcactg agcactttcc atgtagcaag cact
```

10. SRCRSP8 - L22200 - *Capra hircus* - 2 - 341bp

```
1    gtctcttcgg ctgcagaaga gacagg tgcg gtctggttct gatttcac tg gtcttaattt
61   cttatctgac ctggtagtca atcaagggca tgggagaaag agaacaagag agagagagag
121  aggtgggtgg gaaaagggag ctgggcactc tgagctgaag ggggaggggt ctccgtggga
181  cc tgtgtgtg tgtgtgtgtg tgtgtgtgtg tgtgtgtgtg tg agctaagc atcgactttc
241  tcatgcagga ctggcagcct gcctgtcatt ccgcacttct gtgtgtacac gctgcctgtg
301  caaatgattc gagtgttaca attctcttca gctctgtaga t
```

[Reverse Complemented Strand]

```
1    atctacagag ctgaagagaa ttgtaacact cgaatcattt gcacaggcag cgtgtacaca
61   cagaagtgcg gaatgacagg caggctgcca gt cctgcatg agaaagtcga tgcttag ctc
121  acacacacac acacacacac acacacacac acacacacag gtcccacgga gacccctccc
181  ccttcagctc agagtgccca gctccctttt cccacccacc tctctctctc tctcttgttc
241  tctttctccc atgcccttga ttgactacca ggtcagataa gaaattaaga ccagtgaaat
301  cagaaccaga ccgcacctgt ctcttctgca gccgaagaga c
```

11. SRCRSP3 - L22195 - *Capra hircus* - 10 – 187bp

```
1    gatcggtacc cggggatctg ttctatgaac tgatgtgtgt gtgtgtatat gtgtgtgtgt
61   gtgtgtgtgt gtttgtgtgt gagtgtgcat gcacaaaggt ggttctttgg acattcagcc
121  agctaatcag gtaactggta tttccaacat ggaacataat gtctggcatg cagatacagt
181  taacatg
```

[Reverse Complemented Strand]

```
1    catgttaact gtatctgcat gccagacatt atgttccatg ttggaaatac cagttacctg
61   attagctggc tgaatgtcca aagaaccacc tttgtgcatg cacactcaca cacaaacaca
121  cacacacaca cacacacata tacacacaca cacatcagtt catagaacag atccccgggt
181  accgatc
```

APPENDIX B

Table B.1. Molar values of oligonucleotides.

LOCUS	Sequence	OD-260 nm	Micrograms	Picomoles	Backbone mol. weight [Da]*
OarCP20	*F*	7.4	240	35387	6781
	R	19.8	614	89837	6835
OarFCB226	*F*	7.0	217	29510	7362
	R	30.5	1087	112717	9640
BM1443	*F*	8.1	238	34924	6802
	R	20.0	610	107325	5685
OarFCB128	*F*	10.2	340	38852	8744
	R	33.5	1032	124481	8286
BM415	*F*	10.5	362	59623	6075
	R	26.0	781	121664	6417
SRCRSP3	*F*	9.2	295	47969	6148
	R	27.6	900	139486	6452
ADCYAP1	*F*	7.0	232	34603	6706
	R	19.1	573	71029	8062
OarJMP29	*F*	7.4	236	29708	7946
	R	27.3	808	102620	7869
MAF214	*F*	7.0	213	26194	8134
	R	32.6	1029	125636	8193
SRCRSP8	*F*	6.7	228	33914	6723
	R	21.2	667	86572	7706
ILSTS011	*F*	6.1	197	31992	6157
	R	25.4	800	132160	6055

<Abbr/> *F*, forward; *R*, reverse.

*****daltons.

Table B.2. Composition of oligonucleotides.

LOCUS	Sequence		A (%)	C (%)	G (%)	T (%)	Td*	Tm†	Tm‡
OarCP20	*F*	22 -MER	18.18	18.18	31.82	31.82	69.45	71.32	66.00
	R	22 -MER	27.27	22.73	40.91	9.09	74.13	76.91	72.00
OarFCB226	*F*	24 -MER	29.17	25.00	25.00	20.83	64.78	73.88	72.00
	R	32 -MER	6.25	31.25	12.50	50.00	77.12	78.34	92.00
BM1443	*F*	22 -MER	40.91	18.18	27.27	13.64	64.65	69.45	64.00
	R	18 -MER	22.22	5.56	55.56	16.67	62.08	69.06	58.00
OarFCB128	*F*	29 -MER	20.69	27.59	6.90	44.53	71.09	72.36	78.00
	R	27 -MER	37.04	25.93	22.22	14.81	71.83	76.24	80.00
BM415	*F*	20 -MER	10.00	30.00	25.00	35.00	63.33	70.30	62.00
	R	21 -MER	42.86	28.57	19.05	9.52	60.80	68.88	62.00
SRCRSP3	*F*	20 -MER	20.00	20.00	30.00	30.00	60.68	68.25	60.00
	R	21 -MER	19.05	19.05	28.57	33.33	63.32	68.88	62.00
ADCYAP1	*F*	22 -MER	18.18	40.91	31.82	9.09	81.16	80.64	76.00
	R	26 -MER	38.46	19.23	30.77	11.54	71.89	76.04	78.00
OarJMP29	*F*	26 -MER	23.08	26.92	23.08	26.92	69.95	76.04	78.00
	R	25 -MER	36.00	8.00	44.00	12.00	72.30	75.82	76.00
MAF214	*F*	26 -MER	30.77	15.38	42.31	11.54	78.04	79.19	82.00
	R	26 -MER	15.38	3.85	50.00	30.77	74.50	77.62	80.00
SRCRSP8	*F*	22 -MER	9.09	22.73	27.27	40.91	68.09	71.32	66.00
	R	25 -MER	28.00	20.00	28.00	24.00	70.17	74.18	74.00
ILSTS011	*F*	20 -MER	25.00	20.00	30.00	25.00	61.41	68.25	60.00
	R	20 -MER	35.00	35.00	15.00	15.00	60.65	68.25	60.00

<Abbr/> *F*, forward; *R*, reverse.

***** modified nearest neighbor method.

† %GC method.

‡ 2*(A+T) + 4*(C+G).

APPENDIX C

Composition of Solutions

(*i*) 10x TBE (Tris-Borate-EDTA) Electrophoresis Buffer

	per liter	Final 1x Concentration
Tris	108g	89mM
Boric Acid	55g	89mM
0.5M EDTA (pH 8.0)	40ml	2mM
H_2O	to 1 liter	

(*ii*) 6x Loading Dye Solution

10mM Tris-HCl (pH 7.6), 0.03% bromophenol blue,
0.03% xylene cyanol FF, 60% glycerol, and
60mM EDTA.

(*iii*) Ethidium Bromide (EtBr) Solution for Gel Staining

400ml dH_2O
40µl EtBr

APPENDIX D

Table D.1. Chemicals.

Chemical	Brand	Catalogue No.
Albumin, Bovine (BSA)	Sigma	9048-46-8
dNTP, mix	Fermentas	#R0192
MgCl2	Fermentas	#R0971
Primers	Alpha DNA	-
Taq DNA polymerase	Fermentas	#EP0402
H_2O, nuclease free	Fermentas	#R0582
H_2O, molecular biology grade	AppliChem	A7398
EDTA	AppliChem	6381-92-6
Tris base	Sigma	77-86-1
Boric acid	Sigma	10043-35-3
Ethidium bromide	AppliChem	A1151
Ladder	Fermentas	#SM0361
Bromophenol Blue	AppliChem	A2331
Taq buffer	Fermentas	#B33
Agarose, low EEO	AppliChem	A2117
Dneasy® Tissue Kit	Qiagen	69506

APPENDIX E

Table E.1. Equipments.

Equipment	Brand	Model
Centrifuge	Eppendorf	5415R
Power supply	APELEX	PS90009TX
Electrophoretic gel system	MAXICELL	EC 360M
Palm-Cycler	Corbett	C91-96
Gel documentation system	INFINITY	INFINITY-3000
Microwave oven	Arçelik	MD552
Incubator/Shaker	Zhicheng	ZHWY-200B
Precision balance	Sartorius	ED2245
DNA sequencer	Applied Biosystems Instr.	ABI310