Greece: A Balkan Subrefuge for a Remnant Red Deer (Cervus Elaphus) Population


From the Department of Genetics, Developmental and Molecular Biology, School of Biology, Aristotle University of Thessaloniki, 54124 Thessaloniki, Greece (Karaiskou, Tsakogiannis, Gkagkavouzis, Pantis, Abatzopoulos, Triantaphyllidis, and Triantaphyllidis); Thrakomakedonon 136, Athens (Operator of Parnitha National Park); the WWF Greece, Philhelinon, Athens (Latsoudis); and the Department of Computer Science, Aristotle University of Thessaloniki, Thessaloniki, Greece (Kakaviotis).

*These authors contributed equally to the work.

Address correspondence to Nikoleta Karaiskou at the address above, or e-mail: nikolbio@bio.auth.gr

Data deposited at Dryad: http://dx.doi.org/doi:10.5061/dryad.222vq

Abstract

A number of phylogeographic studies have revealed the existence of multiple ice age refugia within the Balkan Peninsula, marking it as a biodiversity hotspot. Greece has been reported to harbor genetically differentiated lineages from the rest of Balkans for a number of mammal species. We therefore searched for distinct red deer lineages in Greece, by analyzing 78 samples originating from its last population in Parnitha Mountain (Central Greece). Additionally, we tested the impact of human-induced practices on this population. The presence of 2 discrete mtDNA lineages was inferred: 1) an abundant one not previously sampled in the Balkans and 2) a more restricted one shared with other Balkan populations, possibly the result of successful translocations of Eastern European individuals. Microsatellite-based analyses of 14 loci strongly support the existence of 2 subpopulations with relative frequencies similar to mitochondrial analyses. This study stresses the biogeographic importance of Central Greece as a separate Last Glacial Maximum period refugium within the Balkans. It also delineates the possible effects that recent translocations of red deer populations had on the genetic structuring within Parnitha. We suggest that the Greek red deer population of Parnitha is genetically distinct, and restocking programs should take this genetic evidence into consideration.

Subject area: Population structure and phylogeography

Key words: Balkan Peninsula, genetic lineages, genetic structure, microsatellites, mitochondrial DNA, translocations

The Balkans along with the Iberian Peninsula were 2 of the most important Pleistocene glacial refugia in the European continent for most taxa (Hewitt 2000). Although most studies have focused on the divergence among major refugial areas, recent phylogeographic analyses have revealed that a number of independent refugia must have indeed persisted within the Balkan Peninsula throughout the Quaternary (Poulakakis et al. 2005; Schmitt et al. 2006) reinforcing the “refugia-within-refugia” concept (Gómez and Lunt 2007). These areas maintain significantly differentiated genetic lineages for a number of species and have become hotspots of biodiversity, thus contributing to overall species diversity. However, further studies are required to determine whether this is a common pattern or only applicable to a few species.

Greece, as part of the Balkan Peninsula, has been reported to harbor genetically differentiated lineages from the rest of the Balkans for a number of animal species as a result of multiple separate refugia (Kasapidis et al. 2005; Stamatis et al. 2009; Alexandri et al. 2012). Limited information is available for an important game species, the red deer (Cervus elaphus) that has been reported in the area throughout the Last Glacial Maximum (LGM) period. During the LGM, the species managed to survive in the Iberian Peninsula, in adjacent regions...
of Southwestern France (Gascony, Dordogne, Languedoc), in the Italian Peninsula, in the Balkans including Greece and east of the Carpathians in Moldavia (Sommer et al. 2008) from where it has later expanded, following different recolonization routes.

Red deer have been abundant and part of Greek biodiversity throughout the centuries and found mostly in Central and Northern Greece. Fossils of red deer that date its presence to the LGM were reported in Greek Paleolithic sites of both Central and North Greece (Payne 1975; Bailey et al. 1983; Bailey 1997). Although there are almost no available historical demographic records, data from the last century have indicated drastic population decreases (Poirazidis and Paraschi 1992). Around 1950, the red deer were restricted mainly to Central Greece, with only one wild population left in Northern Greece (Sithonia Peninsula) that numbered around 100 individuals (Poirazidis and Paraschi 1992). This population has recently disappeared, and the only remnant wild population counting a few hundred animals is restricted nowadays to Parnitha Mountain (Central Greece, Figure 1). A small wild population of 20–40 individuals is also reported in North Greece, on Rodopi Mountain, but this could be considered as part of the neighboring Bulgarian population.

Parnitha is a densely forested mountain range north of Athens, characterized as a National Park. It belongs to the Natura 2000 network (Dafis et al. 1996), harboring high biodiversity levels, with a lot of endemic plant and animal species. Red deer have recently faced various population changes in Parnitha mainly due to habitat fragmentation and disastrous fires (the most recent one in 2007). It has also been subjected to translocation practices, since the beginning of the 20th century, for hunting purposes, with very few official data regarding releasing events (Amorgianiotis 1997). Because Parnitha is a protected area and natural predators of deer (wolves, jackals, and brown bears) are extinct, an increase in its population size has been recorded over the last years. Thus, although population estimates differ between authors, there is an increase in the number of reported individuals from 150 in 1993–1994 (Amorgianiotis 1997) to 500–600 individuals in 2007 (Latsoudis and Kret 2008, 2009). The population today could be as high as 1000 individuals (Papika S, personal communication).

There are 2 main scenarios for the origin of the present day population: 1) the population consists mainly of individuals that always lived in Parnitha and 2) the old population has become extinct and was replaced by individuals either brought from Germany and Denmark by King George A’ in 1908–1913 or introduced from former Yugoslavia and Bulgaria during 1950–1960 (Amorgianiotis 1997; Latsoudis and Kret 2008). Human-induced practices can alter the genetic pool within initial refugial areas, eliminating in many cases historically indigenous populations (Harl et al. 2003). Thus, it is important to understand how natural factors (such as the LGM), along with anthropogenic factors, have shaped

the contemporary genetic structure of this species. Herein, using both microsatellite and mitochondrial DNA data (mtDNA), we aim: 1) to elucidate the phylogeographic history of the last remnant Greek red deer population, searching for signs of native lineages in agreement with the “refugia-within-refugia” hypothesis, 2) to investigate the effect of translocations on its present day population structure, and 3) to assess recent and historical patterns of demographic changes.

Materials and Methods

Sample Collection

Parnitha National Park is a protected area. Therefore, noninvasive methods were used for sample collection. In total, 78 samples from Parnitha were used in the analysis (63 dropped antlers and 15 blood and tissue samples of naturally dead animals stored at −20 °C). This is around one tenth of the population based on current estimates of 600–1000 individuals (Latsoudis and Kret 2008, 2009; Papika S, personal communication). Antlers were collected during the period 2007–2010, across a 280 km² area of Mountain Parnitha, whereas blood and tissue samples were collected in 2010. Because anonymous hikers were bringing in dropped antlers, exact sampling location has been recorded by Global Positioning System receiver for only a subset of 35 samples and mapped using ArcGIS 9.1 (Figure 1; Supplementary Table S1 online). For comparison reasons, 6 additional antlers from a Bulgarian red deer population, which reaches South (through the Rodopi Mountain) into North Greece, were included in the analysis.

DNA Extraction

We drilled out 30–50 mg of powder from antlers, and nuclear and mitochondrial DNA was extracted using the QIAGEN Investigator Kit following manufacturer’s protocol. The quality of DNA was sufficient, and in only few cases, repeats of DNA extraction were necessary. DNA from tissue and blood samples was extracted according to the cetyltrimethylammonium bromide method (Hillis et al. 1996).

Mitochondrial DNA Sequencing

PCR amplification was performed for a 992-bp fragment of the mtDNA control region using the primers and the amplification conditions described by Nussey et al. (2006). Subsequently, PCR products were purified using the Nucleospin® Extra kit (Macherey-Nagel), and Sanger sequencing was performed by Macrogen Company.

Microsatellite Genotyping

Fourteen microsatellite loci were analyzed by multiplexing in 5 independent reactions (2–3 loci per reaction; Supplementary Table S2 online, Georges and Massey 1992; Buchanan and Crawford 1993; Steffen et al. 1993; Bishop et al. 1994; Vaiman et al. 1994; Ede et al. 1995; Wilson et al. 1997; Roed and Midthjell 1998; Jones et al. 2002). Multiplex PCRs were performed in 10 µL reactions using 5 µL of Qiagen buffer from the Qiagen multiplex PCR kit and 30 ng of genomic DNA. Primer concentration for each amplified locus is given in Supplementary Table S2 online. Thermocycling was performed using an initial step of 95 °C for 15 min, followed by 35 cycles of 94 °C for 30 s, 56 °C for 1.5 min, 72 °C for 1 min with a final extension in 60 °C for 15 min. Microsatellite analysis was performed in a semiautomated Li-COR 4200 DNA® Analyzer, and genotypic data were obtained using the Saga Software® (Li-COR).

Mitochondrial DNA Data Analyses

Sequence alignment was performed using the CLUSTAL W algorithm (Thompson et al. 1994). The number of distinct haplotypes in the sequence data set was computed using the DNASP v5 software (Librado and Rozas 2009). Haplotype and nucleotide diversity were calculated with DNASP. The distribution of all pairwise haplotype differences (mismatch distributions) was calculated using the software Arlequin 3.11 (Excoffier et al. 2005) in order to investigate possible population expansion and decline. Additionally, to test for deviations from neutrality and possible population expansion, Tajima’s neutrality test was calculated using Arlequin 3.11. Significant negative values are attributed to recent population expansion.

A median-joining network (Bandelt et al. 1999) approach was used to investigate the relationships between haplotypes. Network was constructed using the software NETWORK 4.5.1.6 (Fluxus Technology Ltd 2010). A 331-bp subfragment of each sequence was used for direct comparison with 57 available haplotypes (accession numbers: DQ520200-DQ520256; Skog et al. 2009). Twenty-three additional haplotypes from Eastern Europe have also been previously published (Niedzialkowska et al. 2011). However, because a lot of informative sites would be lost due to their even shorter size (249 bp), they were not included. Only unique haplotypes were considered for network construction. To further investigate the relationships between haplotypes as detected in the median-joining network, phylogenetic analyses were carried out using the neighbor-joining (NJ; Saitou and Nei 1987) algorithm as implemented in MEGA 5 (Tamura et al. 2011).

Microsatellite Data Analyses

Given that antlers were used as the main DNA source, it was possible that some samples originated from the same individual. The DROPOUT software (McKelvey and Schwartz 2005) was used to calculate the number of unique individuals. Observed (H<sub>O</sub>) and expected (H<sub>E</sub>) heterozygosity values for each locus were calculated using GENEPOP 4.0 (Rousset 2008). Deviation from Hardy–Weinberg (HW) equilibrium was tested using Fisher’s exact tests (Rousset 2008) with the same software. The significant value for multiple tests was set using the sequential Bonferroni procedure (Rice 1989). FSTAT 2.9.3.2 (Goudet 2001) was used to compute inbreeding coefficient values (F<sub>IS</sub>) and allelic richness (A<sub>Rich</sub>). CERVUS 3.0.3 (Kalinowski et al. 2007) was used to evaluate polymorphic information content, null allele probability, and mean number of alleles for each locus.
The genetic structure of red deer was investigated using 2 different Bayesian clustering methods, STRUCTURE and GENELAND as well as a multivariate method, Discriminant Analysis of Principal Components (DAPC), so as to test consistency and reliability of the results. STRUCTURE 2.3 (Pritchard et al. 2000; Falush et al. 2007) was used to infer the number of genetic clusters ($K$). The log likelihoods of our data set [$\ln Pr(X|K)$] were estimated for different numbers of genetic clusters ($K = 1–5$) using an admixture ancestry model based on 100000 burn-in steps followed by 100000 Markov chain Monte Carlo (MCMC) replicates. We utilized a method developed by Evanno et al. (2005) to determine the number of populations present, based on the second order rate of change in the log probability of the data ($\Delta K$) among 20 runs of each assumed $K$ using the web-based utility “Harvest” (http://taylor0.biology.ucla.edu/struct_harvest/). We then evaluated the membership coefficient value ($q$) for each individual.

GENELAND 3.1.4 (Guillot et al. 2005) in R 2.8.1 incorporates geographical coordinate information for genotyped individuals to estimate the most likely number of genetic groups ($K$) and their spatial boundaries. All the parameters (including $K$) are processed simultaneously. As suggested in Guillot et al. (2005), our data set was first analyzed over 10 independent runs, assuming a $K_{\text{max}} = 1$ and a $K_{\text{max}} = 5$ using the Dirichlet model and the following parameters: 100000 MCMC iterations, collecting data at every 100th iteration. Then, we performed a second series of 100 independent MCMC runs of 100000 iterations, each with $K$ fixed to the value identified in the first step. The average logarithm of the posterior probability for each of the 100 runs was computed, and the 10 runs with the highest mean posterior probability values were kept for inference. Spatial distribution of genetic clusters was created with the help of Grass software (Neteler and Mitanova 2008) using Parnitha raster map, GENELAND posterior probability maps, and an R script.

The multivariate method DAPC uses both Principal component analysis (PCA) and Discriminant Analysis (DA) to identify and describe clusters of genetically related individuals. PCA is used as a prior step to DA because it can convert a set of possibly correlated variables into a set of linearly uncorrelated variables. When groups are lacking, DAPC uses the popular machine learning algorithm (K-means) in collaboration with the BIC criterion (Bayesian information criterion) in order to decide the number of the clusters in the data. It was implemented with the Adegenet package (Jombart 2008) within the statistical package R v2.12.1.

To test for recent genetic bottlenecks in Parnitha, deviations from expected heterozygosity were inferred under the assumption of mutation drift equilibrium by either stepwise mutation model (SMM), infinite alleles (IAM), or the 2-phase model (TPM) with 80% and 90% SMM (Di Rienzo et al. 1994) using the program BOTTLENECK 1.2.02 (Cornuet and Luikart 1996). Data were analyzed with the recommended settings (Piry et al. 1999). The sign test and the Wilcoxon signed-ranks test implemented in the software were used to test for significant heterozygosity excess. The standardized differences test was not used, because it requires at least 20 polymorphic loci. The allele frequency distribution (“mode shift”) test included in BOTTLENECK 1.2.02 was also performed (Luikart et al. 1998).

A Bayesian coalescent-based technique designed to assess historical demographic changes implemented in MSVAR 1.3 (Storz and Beaumont 2002) was applied on the Parnitha data-set using the later refined method of Beaumont (1999). Both the exponential and the linear model were used for comparison purposes. For each model, 4 independent runs were conducted, using different starting points and seeds for the chains to test for stability of the estimates. The generation time was set to 8 years (following Kruuk et al. 2002). Each chain had 20000 thinned updates and a thinning interval of 10000 steps, leading to a total number of $2 \times 10^8$ updates. An initial 10% of the data points were discarded as burn-in, whereas the rest of the data points of the 4 different runs were combined. All other parameters were set to software defaults.

Finally, in order to investigate the probability of inbreeding in Parnitha population, the $N_e$ (effective population size) of red deer Parnitha population was estimated using one-point estimate methodologies implemented in NeESTIMATOR 1.3 software (Peel et al. 2004). $N_e$ was calculated using the linkage disequilibrium method option of NeESTIMATOR. Results were also compared with the one-point $N_e$ estimate given by ONeSAMP 1.2 (Tallmon et al. 2008).

### Results

#### Genetic Variation of Parnitha Population

A total of 69 individuals from all studied regions were successfully screened for variation in the mitochondrial control region. Sequencing of 859 bp revealed 28 variable sites and resulted in the detection of 12 haplotypes (Supplementary Table S3a online). Haplotypes CP1 and CP12 were the most common, with overall frequencies of 67.2% and 18.8%, respectively. The 6 samples analyzed from Rodopi Mountain possessed only haplotype CP12. Eight polymorphic sites divide the obtained haplotypes into 2 main groups, hereafter called Haplogroups I and II. Haplogroup I contains 7 haplotypes (CP1–CP7), whereas Haplogroup II contains 5 haplotypes (CP8–CP12) with overall frequencies of 75.4% and 24.6%, respectively. When focusing on the common 331-bp fragment reported by Skog et al. (2009), only haplotypes of Haplogroup II were identical with other European haplotypes (Supplementary Table S3b online). Haplogroup I has never been detected before, despite being the most frequent. The most frequent CP1 haplotype was close only to CD5 (separated by 2 parsimony informative sites fixed in all European haplotypes, Skog et al. 2009). The clustering of haplotypes was also supported by NJ analysis, where bootstrap values for Haplogroups I and II were 67% and 82%, respectively (Supplementary Figure S1 online). Low haplotypic diversity values (0.435 ± 0.005) and nucleotide diversity (0.004 ± 0.001) values were detected for the Parnitha sample (Table 1).

All 78 Parnitha samples were successfully genotyped for microsatellite analysis. Error-checking procedures aided in
identifying and removing genotyping errors before they were incorporated into multilocus genotypes. Thus, the Dropout software revealed 2 pairs of identical samples (sampled in different years), leading to the identification of 76 unique individuals. All microsatellite loci amplified successfully and were highly polymorphic (Supplementary Table S4 online). Three loci (RT1, ILST06, and T193) deviated from HW equilibrium. When these loci were excluded from further analysis, the HW test for the Parnitha population (initially statistically significant) was nonsignificant (P = 0.09). The inbreeding coefficient (F<sub>IS</sub>) for Parnitha was close to 0 for each locus, indicating lack of heterozygosity deficiency. The heterozygosity value (0.625) was moderate compared with observed values for other European populations (Zachos and Hartl 2011), though quite low based on expected values (Table 1).

The above parameters were not calculated for the Rodopi Mountain population due to its small sample size.

**Phylogeography and Fine-Scale Population Structure**

According to the network (Figure 2) built based on the present data incorporating genetic information from 39 red deer populations (Skog et al. 2009), Greek haplotypes were grouped into Haplogroup C, which is restricted to Eastern Europe. No Greek haplotypes belong to the western lineage (Haplogroup A) or the Sardinian–North African lineage (Haplogroup B) of Skog et al. (2009). Haplogroup C is further substructured into CA haplotypes spread throughout the range of Haplogroup C, CB haplotypes more or less confined to the western part of the range (mainly Italy) and CD haplotypes occurring in the eastern part of the range (Skog et al. 2009). Greek Haplogroup I forms a separate clade within Haplogroup C, whereas Haplogroup II is grouped with CA haplotypes.

**Table 1.** Number of samples analyzed (N) for Parnitha population, number of haplotypes/alleles (A), haplotypic (h) and nucleotide diversity (π) of mtDNA data, and observed and expected heterozygosity values (Hₜ, and Hₑ, respectively) of microsatellite data

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>A</th>
<th>h</th>
<th>π</th>
<th>Hₜ</th>
<th>Hₑ</th>
</tr>
</thead>
<tbody>
<tr>
<td>mtDNA</td>
<td>69</td>
<td>12</td>
<td>0.435</td>
<td>0.004±0.001</td>
<td>0.625</td>
<td>0.604</td>
</tr>
<tr>
<td>Microsatellite</td>
<td>76</td>
<td>97</td>
<td>0.625</td>
<td>0.604</td>
<td>0.625</td>
<td>0.604</td>
</tr>
</tbody>
</table>

**Figure 2.** Median-joining network of mtDNA control region haplotypes. Three haplogroups were obtained (A, B, and C) when comparing Greek haplotypes with the haplotypes of Skog et al. (2009) based on a common 331-bp fragment. Greek haplotypes (CP haplotypes) fall within Haplogroup C and are split in 2 lineages (I and II). Because this is a shorter fragment than the original analysis, haplotype CP1 is identical to CP4 and CP7 (CP1 is denoted), whereas CP12 and CP8 is identical to CA1 (CA1 is denoted).
groups is 90% and 75%, respectively. Only 3 individuals were assigned to the third cluster when $K = 3$. Finally, DAPC analysis of Parnitha sample gave similar support for $K = 1–3$ (Supplementary Figure S3 online). For $K = 2$, results are >85% similar to STRUCTURE and GENELAND analyses with the 2 main groups including 52 and 24 individuals, respectively (Supplementary Figure S4 online).

**Bottleneck Tests and Inference of Past Demographic History in the Parnitha Sample**

Recent demographic history was inferred based on mismatch distribution analysis of mtDNA data. The distribution was not unimodal (Supplementary Figure S5 online), and, moreover, Tajima's $D$ values were positive and nonsignificant ($D = 1.05$, $P = 0.152$), a pattern probably consistent with a recent genetic bottleneck or stable population at equilibrium. Similar results were obtained when only individuals belonging to Haplogroup I (the most frequent and possibly native one) were analyzed. Based on microsatellite data, evidence for a recent bottleneck was found in Parnitha using the SMM test ($0.002 < P < 0.0046$) and the TPM model with 80% SMM (0.04), whereas the IAM did not support such an event. Mode shift analysis did not detect any distortion of allele frequency and showed normal L-shaped distributions, which is a typical property of a population at equilibrium.

Looking to past events, the MSVAR analysis revealed a strong population decline in Parnitha population; posterior distributions of current effective population size log($N_0$) have no overlap with the posterior distributions of ancestral population size log($N_1$), when assuming either a linear or exponential model of population decline (Supplementary Figure 3).

**Figure 3.** Individual-based clustering results: (a) STRUCTURE results for $K = 2$ when considering Parnitha and Rodopi Mountain individuals, (b) STRUCTURE results for $K = 2$ when considering only Parnitha individuals, and (c) spatially derived genetic clusters by GENELAND 3.0 when considering Parnitha individuals. Light color indicates the sampling sites within the same cluster.
Figure S6 online). Our analysis suggested that the contemporary red deer population size of Parnitha is roughly 0.3–0.6% of its historical size, indicating severe population collapse and that the ancestral population size was around 280,000 individuals prior to its drastic decline. The time since the onset of the population decline (T) was approximately 15,000–25,000 years ago.

Two different approaches were used to estimate the current effective population size (Nₑ) in order to evaluate the population status and the risk of inbreeding depression. Single point estimates of Nₑ using NeEstimator gave a mean value 69 for the Parnitha sample (95% confidence interval [CI] = 49.1–79). When applying the OneSAMP software, values of Nₑ were similar (63, 95% CI = 46.31–116.1). In fulfillment of data archiving guidelines (Baker 2013), we have deposited the primary data underlying these analyses with Dryad.

Discussion
Our study sheds light on the origin of the last remnant red deer population in Greece. Most scenarios until now have supported a drastic decline of this population, highly affected by human activities like habitat alterations, hunting, and translocation of animals. We indicate that a native population is still present in Parnitha Mountain along with a translocated one.

Discrete mtDNA Lineages and Substructuring in a Small Geographic Area

All available studies analyzing red deer phylogeography in Europe (Ludt et al. 2004; Skog et al. 2009; Niedzialkowski et al. 2011; Carden et al. 2012) have supported 3 deeply divergent mitochondrial DNA lineages that correspond to 3 possible refugia: a Eastern European or Balkan lineage (C), a Western European or Iberian lineage (A), and an enigmatic lineage restricted to Sardinia and Africa (B). Greek haplotypes fall within the eastern lineage (C), reinforcing the theory of a Balkan refugium (that included the Dinaric Mountains and extended to the Southern Alpine at their northeastern limit, Sommer et al. 2008). Red deer naturally expanded northwards from this refugium, after the last ice age and recolonized countries mainly east and southeast of Europe (Skog et al. 2009; Niedzialkowski et al. 2011).

The present study reveals a very interesting pattern: 2 mitochondrial lineages were detected in Parnitha, with the most frequent one (Haplogroup I) being unique to Central Greece (never reported in the Balkans before). Because red deer are known to have occurred in Greece throughout the LGM (Sommer et al. 2008), our data support that the Parnitha population is a possible remnant of a Greek refugial population. The strong phylogeographic differentiation of this population reinforces the concept of multiple centers within the main Balkan refugium that lead to strong substructuring (Schmitt 2007). This pattern has been reported in Greek territory for some other mammal populations such as the brown hare (Kasapidis et al. 2005; Stamatis et al. 2009), wild boar (Alexandri et al. 2012), and stone marten (Papakosta et al. 2012) due to the existence of 2 differentiation centers (a northern and a central), with the Pindos mountain range playing a decisive role in the creation of distinct refugial areas where locally adapted populations have survived (Tzedakis et al. 2002). The restricted presence of this Greek lineage in Southern Balkans could be the result of prolonged geographic isolation and no or limited dispersal to the north. However, the extinction of all wild red deer populations from Northern Greece does not allow us to localize the northern boundary of this refugium.

Apart from the native lineage that managed to survive despite past population oscillations, a second one was also found in the area. This haplogroup is shared with other Balkan populations (Figure 2) and is possibly the result of successful translocations of Eastern European red deer individuals (Amorgianiotis 1997; Latsoudis and Kret 2008). Although there are anecdotal records of translocations of red deer (15 breeding individuals) from Germany and Denmark at the beginning of the 20th century (which would belong to lineage A of Skog et al. 2009), genetic traces of these introduced individuals were not detected. Maybe, these animals did not survive in Parnitha habitat. However, we cannot rule out the possibility that they were not sampled, though we have sampled approximately one tenth of the current population.

Although having a different pattern of inheritance and rate of evolution from mtDNA, microsatellite-based clustering approaches strongly support the existence of 2 subgroups in Parnitha. Despite the fact that the 3 different statistical methods differ in the capacity to perform assignment tests (Chen et al. 2007), they all agree that the main cluster is almost 50% more frequent. Most individuals of this frequent cluster seem more confined in the area of the core of the National Park (Figure 3c) according to Geneland analysis, whereas individuals of the small cluster are spread across a larger geographical area probably due to local competition for resources or mates. However, this result needs deeper examination, because only half of the individuals were included in the Geneland analysis due to the lack of geographic coordinates for many samples. It should be stressed that all individuals of the most frequent cluster carry haplotypes that belong to the native Haplogroup I, whereas both native and introgressed lineages coexist in the other smaller cluster (Supplementary Table S1 online). We should also bear in mind that the much higher frequency of cluster I does not directly preclude higher abundance. Frequencies may reflect abundance under certain sampling conditions. Because sampling was mostly dependent on availability of antlers from independent collectors, we cannot rule out the possibility that, for example, these samples do not belong to one herd or originate from places within the habitat, where cluster I was more frequent. The above limits our ability to make such a solid inference at this point.

Although the introduced individuals from neighboring country/countries have survived and hybridized to some degree with the native ones, they do not seem to have yet fully introgressed after the roughly 8 generations that have passed since their arrival (probably after 1950). This lack of
complete admixture could not be the result of physical barriers between subpopulations, such as geographic distance or other landscape features like type and density of vegetation, slope, and elevation (data not shown). A possible explanation could be the existence of premating reproductive barriers between the 2 subpopulations based on differences in acoustic cues or odor profiles (Charlton et al. 2007) that can act as isolation factors and thus prevent complete breeding between subpopulations. More data are needed to support such a hypothesis, because Cervus species are in general not known to show such particular mating preferences, allowing even cross-species hybridization (red and sika deer, for example, Senn and Pemberton 2009).

Short- and Long-term Demographic Changes in the Greek Red Deer Population

Detecting historical demographic bottlenecks, especially of small populations, is of major concern, because they can lead to loss of genetic variability, inbreeding depression, and thus increased risk of extinction. Our study does not reveal a unimodal mtDNA haplotype distribution and thus a sudden expansion of the population. However, mismatch distribution as well as the low haplotypic and nucleotide diversity values (see review in Zachos and Hartl 2011) are indicative of a bottleneck signal. Moreover, a population bottleneck is supported based on microsatellite results by models depending on heterozygosity excess methods such as SMM and TPM (with 80% SMM). Though the IAM and mode shift models failed to detect any reduction in the Parnitha population, such discrepancies between the different models have been documented before, even for species that have experienced severe reductions in population size (Rossa de Oliveira et al. 2009; Hoffman et al. 2011). These discrepancies may be attributed to a number of factors, such as migration or hybridization that can mask the detection of bottleneck signals (Cornuet and Luikart 1996): No data exist to support recent migration of Northern red deer individuals into Parnitha. On the other hand, as previously stated, individuals from Eastern European populations were used to enrich the existing population in the 20th century. According to Cornuet and Luikart (1996), nonnative individuals bringing with them rare alleles could erase a bottleneck signal within a few generations, without substantially affecting overall heterozygosity.

Severe historical population decline was also detected by the MSVAR simulations that quantify and date the deer population decline circa 15–25 kyr (Senn and Pemberton 2009). From 26 500 to 19 000 cal. yr BP (Clark et al. 2009), the ice sheets were at their maximum extension. Nevertheless the Greek population managed to survive these severe climatic changes, although its population sizes were naturally affected.

Changes in population size continued after the ice sheet retreat in the Holocene period up until today, due to both natural (predation) or human-induced factors such as deforestation of the mountain, intensive agriculture practices, habitat fragmentation, and illegal hunting in the area. These population changes followed by reintroductions in the last century have formed the present day population with an estimated effective population size of \( N_e >60 \) individuals. Considering that 1) in polygynous mammals, \( N_e \) is a priori relatively small, 2) \( N_e \) of the Greek population is higher than the values proposed as indicative of possible inbreeding depression (Franklin 1980), and 3) its population size has grown considerably during the last decades, from 150 in 1993–1994 (Amorgianiotis 1997) to a current estimate of 500–1000 (Latsoudis and Kret 2008, 2009; Papika S, personal communication), red deer should not be considered as under immediate threat for extinction in the near future. However, demographic estimations may not always reflect actual genetic size of the breeding population (Hmwe et al. 2006). Management of red deer should focus on maintaining its effective size as large as possible, by reducing risks like illegal hunting, habitat fragmentation, as well as intentional disastrous fires, as in 2007, which resulted in the death of numerous individuals.

Translocations and Their Impact on the Native Population

Until recently, reductions in the population size of game species in Europe were simply counterbalanced by reintroductions. Several mammal species were restocked using either farmed animals or wild individuals collected from geographically distant regions, for example, the cases of European hare (Mamuris et al. 2001; Kasapidis et al. 2005; Andersen et al. 2009), wild rabbit (Delibes-Mateos et al. 2008), roe deer (Randi 2005), and wild boar (Randi 2005). However, little attention was paid to the potential consequences for the native populations, such as loss of genetic variation and changes to their genetic composition (Lairkar et al. 2010). These are due to resource and breeding competition of translocated individuals, as well dispersal, migration, antipredator behavior, and morphological differences (Champagnon et al. 2012). Studies on the genetic impact of these translocations on wild populations have revealed that restocking practices have affected the genetic structure of these wild populations even to a small degree (Vernesi et al. 2003; Nikolov et al. 2009). As far as red deer is concerned, the effects of translocation have been studied in 2 threatened populations, the Tunisian and the Corsican (Hajji et al. 2007; Hajji et al. 2008) as well as in the largest population in Europe, the Scottish red deer (Pérez-Espona et al. 2009). Introductions led to significant genetic differentiation only in the case of the Corsican population due to founder effects.

Translocations in the present study have resulted in subtle genetic substructuring. The fact that the native subpopulation is still strong could probably be due to small numbers of nonnative breeding individuals initially used. Reintroductions that took place in the past do not seem to have blurred the history of the native population, because they have not managed to replace the indigenous gene pool. However, the demographic expansion of both autochthonous and
translocated individuals in recent years might pose a serious threat for the integrity of the population. Admixture that has been limited in the past could now be quickened from the expansion process. Thus, future monitoring programs should take genetic evidence into consideration.

**Conclusion**

The Greek red deer population is genetically distinct from other Balkan populations and is probably the remnant of an original Greek gene pool. This finding stresses the importance of Central Greece as a separate refugium within the Balkans during the LGM, supporting discreet genetic lineages for a number of species. The existence of different glacial refugia within a classical refuge area was also reported for the Spanish roe deer (Royo et al. 2007). In this case, the Northwestern and the Central–Southern part have acted as separate refugia areas within the Iberian Peninsula.

Future analysis of ancient DNA could confirm the originality of Greek red deer lineage and provide a deeper investigation of past events. Another autochthonous red deer population was reported in Mesola, Italy with very low diversity levels due to repeated bottleneck events (Lorenzini et al. 2005; Zachos et al. 2009). These 2 cases underline the need for further conservation attention to species inhabiting refugial areas. Considering its unique genetic status, the conservation of the only Greek remnant population of red deer, located in Parnitha National Park, should be a priority.

**Supplementary Material**


**Funding**

Operator of Parnitha National Park under contract 38269/26-5-2008 with Research Committee of Aristotle University Of Thessaloniki.

**Acknowledgments**

We thank the staff of the Operator of Parnitha National Park and all the anonymous hikers for their help with sampling. We also thank Dimitris Bourmpoundalis for his help with geographical statistical analyses.

**References**


Received June 19, 2013; First decision July 31, 2013; Accepted January 8, 2014
Corresponding Editor: Jennifer Jackson