GENETIC ANALYSIS OF EGYPTIAN VULTURE (*NEOPHRON PERCNOPTERUS*) IN THE BALKANS AND TURKEY

LIFE+ PROJECT
"THE RETURN OF THE NEOPHRON"
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THE REPORT
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EXTENDED SUMMARY

Reduced populations are subject to stochastic environmental, demographic and genetic factors that can ultimately accelerate population extinction. Moreover, small population sizes and reduced gene flow lead to increased genetic drift, resulting in the loss of genetic diversity and an increase in inbreeding, which in turn reduces the adaptive potential of populations and the fitness of individuals. Within this scenario, genetic analyses are increasingly used to infer patterns of gene flow and population diversity and structure, and have become an essential tool in the management and conservation of threatened species. In this sense, it is essential to analyse contemporary and past genetic patterns to better calibrate the impact of genetic factors on population viability and to design efficient conservation measures.

The Egyptian vulture (*Neophron percnopterus*) provides a paradigmatic example of an endangered species that is going through a demographic decline. The species is considered as "Globally Endangered" with a total world population below 30,000 individuals. Formerly distributed throughout temperate and arid biomes of Palearctic, its populations dropped dramatically during the 20th century in India, the circum- Mediterranean, and the Middle East (>50% over the last three generations), and there are ongoing declines through much of the rest of its African range. In the early 20th century, the Egyptian vulture has been widespread and common in the Balkans, while in the last 30 years, the range of the species decreased and fragmented considerably with population declines estimated over 80% and an annual decline rate of 4-8%. Close to the Balkan population is Turkey's population, which is estimated at 1,500- 3,000 pairs and considered the second largest breeding pool of the species in Europe, after Spain. The recovery of the Egyptian vulture population in the Balkans necessarily involves the assessment of its genetics status in order to design appropriate conservation strategies, which could potentially involve the exchange of birds between neighbouring breeding areas. More specifically, knowing the Turkish population's genetic status and its level of differentiation from the Balkan population is essential to evaluate its potential use as a source for the genetic reinforcement of the Balkan population.

A genetic study of the Balkan and Turkish populations was conducted in the frame of LIFE+ project "The Return of the Neophron". Blood samples and growing feathers were collected from 32 contemporary specimens captured between 2010 and 2013 in Bulgaria and Greece, and 10 more samples were collected from birds in Turkey to explore the possible genetic differentiation with this population since it is geographically close and still holds large numbers of vultures. To explore changes in genetic diversity through time, 18 additional museum samples from Greece, Bulgaria and European Turkey dated as far back as 1853 and distributed throughout the 19th and 20th centuries.

Based on mitochondrial and nuclear data from historical and contemporary samples, obtained results suggest that recent fragmentation and decline has caused allele frequency fluctuations and an incipient loss of genetic diversity. Historical populations did not show any genetic structure indicating a panmictic scenario. In contrast, the current Balkan's population is showing signs of genetic loss and differentiation between geographic areas, with the northern being the least diverse and more differentiated compared to the historical one. The southern area does not show any sign of differentiation probably due to the small sample size. Comparing these populations with others reported in previous studies of Egyptian vultures, it becomes evident that the Balkan historical population was more diverse than any of the current Iberian populations. Even more, the northern and southern areas show less nuclear diversity than any of these populations. All of this evidence reflects a rapid process of genetic diversity loss as a consequence of the severe and rapid reduction of population size in recent decades.

The lack of relationship between genetic and geographic distances suggests the absence of current gene flow, and the relationship between genetic distances and time indicates allele frequency fluctuations in the area. Here, we provide yet another instance of the contraction and fragmentation of a formerly large historic population yielding similar results. Unfortunately, the process has likely not ended. Given the long generation time of this species (13 years), we would expect higher values of differentiation in a few more generations if the population decline and fragmentation persist. In this line, considering the small size of the Balkan popula-

tion, if the decline persists it will be almost extinct in <5 generations, therefore the lack of genetic variability will be less of an issue than the lack of birds. The Turkish population could be a possible source for reinforcement within ongoing conservation programs as it is less differentiated from the historical Balkan's than the contemporary Balkans population. However, we would expect an increase in this differentiation with time if there are no management actions that reverse this process. The low genetic diversity, the increase in structure and some evidence of bottlenecks in the different Balkans areas, especially in the northern one, indicate that translocations would be a short-term management solution.

In conclusion, this study supports that Balkan's population is differentiating from the historical one more quickly than Turkey's population. Given this, it would be feasible to reinforce the Balkan population with birds from Turkey. Of course, this type of active management cannot be approached without addressing the main causes of population decline, and taking into account potential negative effects associated with the movement of animals between isolated breeding nuclei, such as the risk of disease transmission, behavioural disruption, lower fitness in novel habitats in the presence of local adaptations, and negative demographic effects on the donor populations. Under this scenario, and not only due to genetic constraints but also demographic factors, the maintenance of the last Balkan populations of this scavenger seem to be extremely precarious and extinction may be unavoidable within a few decades, as has been predicted for other Mediterranean populations challenged with similar conservation problems. Therefore, urgent conservation measures should be taken to stop the main limiting factors associ¬ated with non-natural mortality as well as to revert the negative effects of genetic drift. Management programs should include the reinforcement of the populations and long-term genetic monitoring in order to avoid inbreeding depression, to maximize the genetic diversity and thereby, to increase the long-term population viability.

INTRODUCTION

The exponential growth of human populations over the last centuries and the increased consumption of resources have triggered an unprecedented global biodiversity crisis. Since 1500 AD, more than 300 vertebrate species have disappeared and a fifth of all vertebrate species are currently considered as "endangered" (Hoffman et al 2010, Dirzo et al 2014, Pimm et al 2014). This "anthropocene" defaunation is identified as the sixth extinction wave and it is leading to an irreversible loss in ecological functions and ecosystem services (Dirzo et al 2014). The process of decline of vertebrate populations often shares underlying factors inherent to low population size and increasing isolation. Reduced populations are subject to stochastic environmental, demographic and genetic factors that can ultimately accelerate population extinction. Moreover, small population sizes and reduced gene flow lead to increased genetic drift, resulting in the loss of genetic diversity and an increase in inbreeding (Frankham et al 2002, Méndez et al 2011), which in turn reduces the adaptive potential of populations and the fitness of individuals (Allendorf & Luikart, 2007).

Within this scenario, genetic analyses are increasingly used to infer patterns of gene flow and population diversity and structure, and have become an essential tool in the management and conservation of threatened species. These studies commonly analyse current genetic patterns only, but a comparison to pre-bottleneck conditions can help to distinguish alternative scenarios with contrasting consequences for population viability. If pre-bottleneck and current diversity are similar, population viability might be affected by the reduction in population size, but not by inbreeding depression. In this sense, it is essential to analyse contemporary and past genetic patterns to better calibrate the impact of genetic factors on population viability and to design efficient conservation measures. Nevertheless, studies that monitor the genetic status from the pre-bottleneck to the post-bottleneck stages are very scarce (Hellborg et al 2002; Spong & Hellborg 2002, Groombridge et al 2009, Packer et al 1991, Martínez-Cruz et al 2007).

The Egyptian vulture (*Neophron percnopterus*) provides a paradigmatic example of an endangered species that is going through a demographic decline. The species has been closely linked to traditional agro-grazing economies for centuries (Gangoso et al 2013). Formerly distributed throughout temperate and arid biomes of Europe, Africa and Asia, its populations dropped dramatically during the 20th century in India, the circum-Mediterranean, and the Middle East (>50% over the last three generations), and there are ongoing declines through much of the rest of its African range. The species is currently considered as "Globally Endangered" with a total world population below 30000 individuals (BirdLife International 2014). The main drivers of this population decline (poisoning, accidents involving wind farms and power lines) all act to increase mortality rates of adult and immature birds in breeding and wintering areas, as well as during migration (Thiollay 2006, Hernández & Margalida 2009, Virani et al 2011, Angelov et al 2013, Wacher et al 2013, Ogada et al 2015, Sanz-Aguilar et al 2015). In addition, food shortages associated with changes in land use, pastoral systems, and veterinary and sanitary practices, mortality associated with suboptimal navigation during the first migration and direct persecution have also gained a prominent role in recent times (Kurtev et al 2008, Oppel et al 2015, Xirouchakis in prep.).

In the early 20th century, the Egyptian vulture was widespread and common in the Balkans (Patev 1950, Grubač 1989, Handrinos & Akriotis 1997). The first indications of decline in this region were reported in the 1960s (Nisbet & Smout 1957, Arabadzhiev 1962) and in the 1980s it became extinct in Croatia (Sušić 1993), Montenegro (Ljucović 1995), Bosnia and Herzegovina (Marinković et al 2007), and Serbia (Grubač 1999). In the last 30 years, the range of the species has decreased considerably and become fragmented in three core areas (with > 10 active territories in Bulgaria, Greece, FYR of Macedonia and Albania) and several relict clusters and single isolated pairs, with population declines estimated over 80% and an annual decline rate of 4-8% (Velevski et al 2015). Close to the Balkan population is Turkey's population, which is estimated at 1,500-3,000 pairs (BirdLife 2013) and considered the second largest breeding pool of the species in Europe, after Spain. Although the species is considered vulnerable and decreasing in this country (Şen 2013), the absence of monitoring programmes and population studies makes it impossible to estimate population trends.

The recovery of the Egyptian vulture population in the Balkans necessarily involves the assessment of its

genetics status in order to design appropriate conservation strategies, which could potentially involve the exchange of birds between neighbouring breeding areas. More specifically, knowing the Turkish population's genetic status and its level of differentiation from the Balkan population is essential to evaluate its potential use as a source for the genetic reinforcement of the Balkan population. These general objectives are thus specifically included in the LIFE Project "The Return of Neophron" (LIFE10 NAT/BG/000152) (http://lifeneophron.eu/en/news-view/hot-news/237.html). Here, we assess the genetic diversity and structure of the Balkan and Turkish populations in comparison with historical patterns to determine whether current patterns are the result of recent decline and fragmentation, and we evaluate genetic signals of past demographic changes.

MATERIAL AND METHODS

Study area and sample collection

The study area includes Bulgaria, Greece and Turkey (Fig. 1). Blood samples and growing feathers were obtained from 32 contemporary specimens captured between 2010 and 2013 in Bulgaria and Greece, distributed in 3 main areas: Northern (Varna, Ruse and their surroundings N=7), Central (Rhodope and Dadia N= 23) and Southern Balkans (Meteora N= 2). Additionally, we obtained 10 samples from birds captured in eastern and central Turkey to explore the possible genetic differentiation with this population since it is geographically close and still holds large numbers of vultures. To explore changes in genetic diversity through time, we also obtained 18 additional museum samples from Greece (n=8), Bulgaria (n=7) and European Turkey (n=3) dated as far back as 1853 and distributed throughout the 19th and 20th centuries (See Supplementary material: S-1). We collected the umbilical clot from feathers (Hovart et al 2005) and a piece of the foot pad when possible. Samples were preserved in 98% ethanol and stored at 4°C until analysis. DNA was extracted using standard phenol-chloroform methods (Sambrook et al 1989). Samples were collected with the official permissions of the Bulgarian Ministry of Environment and Waters, Greek Ministry of Environment, Energy and Climate Change and Turkish Ministry of Forestry and Water Affairs' General Directorate of Nature Conservation and National Park.

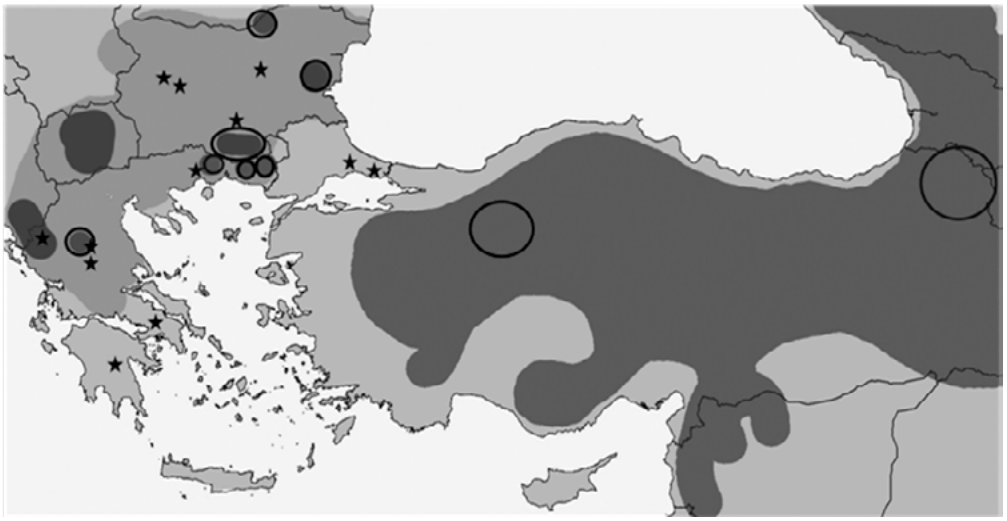


Figure 1. Sampling areas. Current (2010-2013) Egyptian vulture population is shown in dark grey and sampling areas in circles and ellipses. The distribution during the second half of the XXth century is shown in light grey (based on Velevski et al, 2015). Historically the species occupied the entire Balkans and Turkey. Stars represent localities of museum samples (1853-1979).

Nuclear data

Each individual was genotyped for 12 microsatellite loci selected due to their small product size, four of which were heterospecific (Bv6, 13, 14, 20; Gautschi et al 2000) and 8 species-specific (Np51, 163, 166, 229, 249, 257, 259, 296; Agudo et al 2008). In order to prevent genotyping errors associated with a low quantity and quality of DNA typical of museum samples, such as allelic dropout or false alleles, each sample was independently genotyped five times (Taberlet et al 1996). Consensus genotypes were then obtained; a heterozygote was noted when each allele was seen at least twice, and a homozygote when it was observed at least three times and no other allele was observed.

Fluorescently-labelled products were amplified in separate standard PCRs for each marker using end-labelled forward primers and analysed on an ABI 3130xl Genetic Analyzer (Applied Biosystems). Each PCR contained 1x PCR buffer, 2 mM MgCl2, 0.25 dNTPs, 0.4µl of each primer, 0.4U of Taq Polymerase, 0.01 (contemporary) and 0.08 (museum samples) mg/mL BSA and 50 ng of template (4 µl of the template from museum samples) in a total volume of 20µl. This PCR was run with a cycle of 2' at 94ºC, 40 cycles of 30" at 92º, 30" at the corresponding annealing temperature and 30" at 72ºC, and a final extension of 5' at 72ºC. Alleles were scored using GeneMapper v4 (Applied Biosystems).

Genetic nuclear diversity and structure

Observed (Ho) and expected (He) genetic diversity, mean number of alleles per locus (Na), allelic richness (Ar: the average number of alleles per locus rarefied to the smallest sample size), Hardy-Weinberg equilibrium, population inbreeding coefficient (Fis), and population differentiation (Fst) were estimated using Genetix (Belkir et al 1996-2004). Significance was determined at 95% confidence intervals based on 10000 permutations.

We performed an Isolation by Distance (IBD) analysis to investigate how the spatial distribution of samples can influence population differentiation patterns. We tested for IBD by performing a Mantel test between individual genetic distance and geographic distance with 9999 permutations to calculate significance using GeneAlEx (Peakall & Smouse 2001). We first performed the IBD with museum samples and later with contemporary samples.

We inferred the number of distinct genetic clusters (k) irrespective of the geographic origin of the samples using the Bayesian clustering analysis implemented in STRUCTURE 2.3.3 (Pritchard et al 2000). Simulations were run with a burn-in period of 50000 followed by an additional 500000 steps. We ran from 1 to 5 genetic clusters (k = 1-5) and 20 replicates for each k under an admixture model with correlated gene frequencies. We performed simulations with historical and contemporary samples, separately and all together. The probability of k populations was calculated based on Δk, as described in Evanno et al (2005) and implemented in STRUCTURE HARVESTER (Earl & vonHoldt 2011), and a visual inspection of the plot of the lnP(D) as a function of k. We performed these analyses with the pooled sample set, separately on museum and contemporary individuals, and finally with location information as a prior (Northern, Central and Southern).

Genetic nuclear diversity through time

To assess changes in genetic diversity through time we calculated individuals' observed homozygosity using cernicalin v.1.30 (Aparicio et al 2006). We used generalized linear models to assess the relationships between the individual's homozygosity and its date and location (Balkans or Turkey) in R 2.11.1 software (R-Development Core Team 2011). Models were built with a normal distribution of errors and the identity link function. There was no evidence of overdispersion, and residuals fitted to a normal distribution, indicating that distributions and error structures were appropriate. In order to assess not only the loss of genetic diversity but also the change in allelic frequencies and therefore the action of genetic drift within populations, we assessed the correlation of the linearized genotypic distance, as implemented in GeneAlEx (Peakall & Smouse 2012), and the temporal distances in years between each pair of individuals with the Mantel test. We used 10000 permutations to assess significance.

Bottleneck test

We tested whether the recent demographic decline of the Egyptian vulture populations left a detectable signal on contemporary genetic patterns using the software BOTTLENECK v.1.2.02 (Piry et al 1999), which tests whether there is a significant excess of heterozygosity in the sample with respect to that expected for the observed number of alleles under mutation-drift equilibrium (Cornuet & Luikart 1996). Since the power and reliability of the test is largely dependent on the mutation model and the mutation model of microsatellite markers is poorly known, we used three different mutation models to simulate data: the infinite allele model (IAM), the step-wise mutation model (SSM) and the two-phase mutation model (TPM). For the TPM, we used the parameterization recommended by Piry et al 1999 (5% multistep mutation events, distributed under a geometric function of variance =12), and a more relaxed model with 30% multistep mutations with variance 36. A one-tailed Wilcoxon signed-ranked test was performed to determine if the dataset exhibited a significant number of loci with heterozygosity excess, as expected in bottlenecked populations. This method has been shown to be capable of detecting recent population contractions (>4Ne generations ago).

Mitochondrial data

Genetic mitochondrial diversity and structure

To characterize the mitochondrial variation, we used partial sequences of the control region, which have already been shown to neatly discriminate between subspecies (Donázar et al 2002). Due to the low quality and small size of DNA obtained from museum samples, we redesigned a new pair of primers (Npe_CR2F: CTCTCTGCATGGYTCAGGAVTAG, Npe_CR2R: CAAGGAGTGCTAGGGGTGTA) for the amplification of a smaller (280 bp) but still informative fragment of Domain 1 spanning from R'4 to D-box, based on Roques et al (2004).

We calculated for each geographic partition and period and for the global sample the number of haplotypes (h), haplotypic diversity (Hd), number of variable sites (S), nucleotide diversity (Pi) and the mean number of pairwise nucleotide differences (k) using DNAsp v.4.10 (Rozas & Rozas 1999). To explore the historical demography, we used Fu's Fs and Tajima's D statistic, and also analysed the mismatch distributions and computed the raggedness index of this distribution to assess past demographic trends using Arlequin 3.0 (Excoffier et al 2005). We also calculated genetic differentiation as pairwise Fst for haplotype frequency values between populations with 10000 permutations for p-values. We constructed a distance-based phylogenetic tree using the K2P substitution model as it has the smallest AIC determined by jModelTest (Darriba et al 2012) and the neighbour-joining algorithm, as implemented in MEGA 6 (Tamura et al 2013). We evaluated the statistical support for the internal branches using 10000 bootstrap replicates.

RESULTS

Genetic nuclear diversity and structure

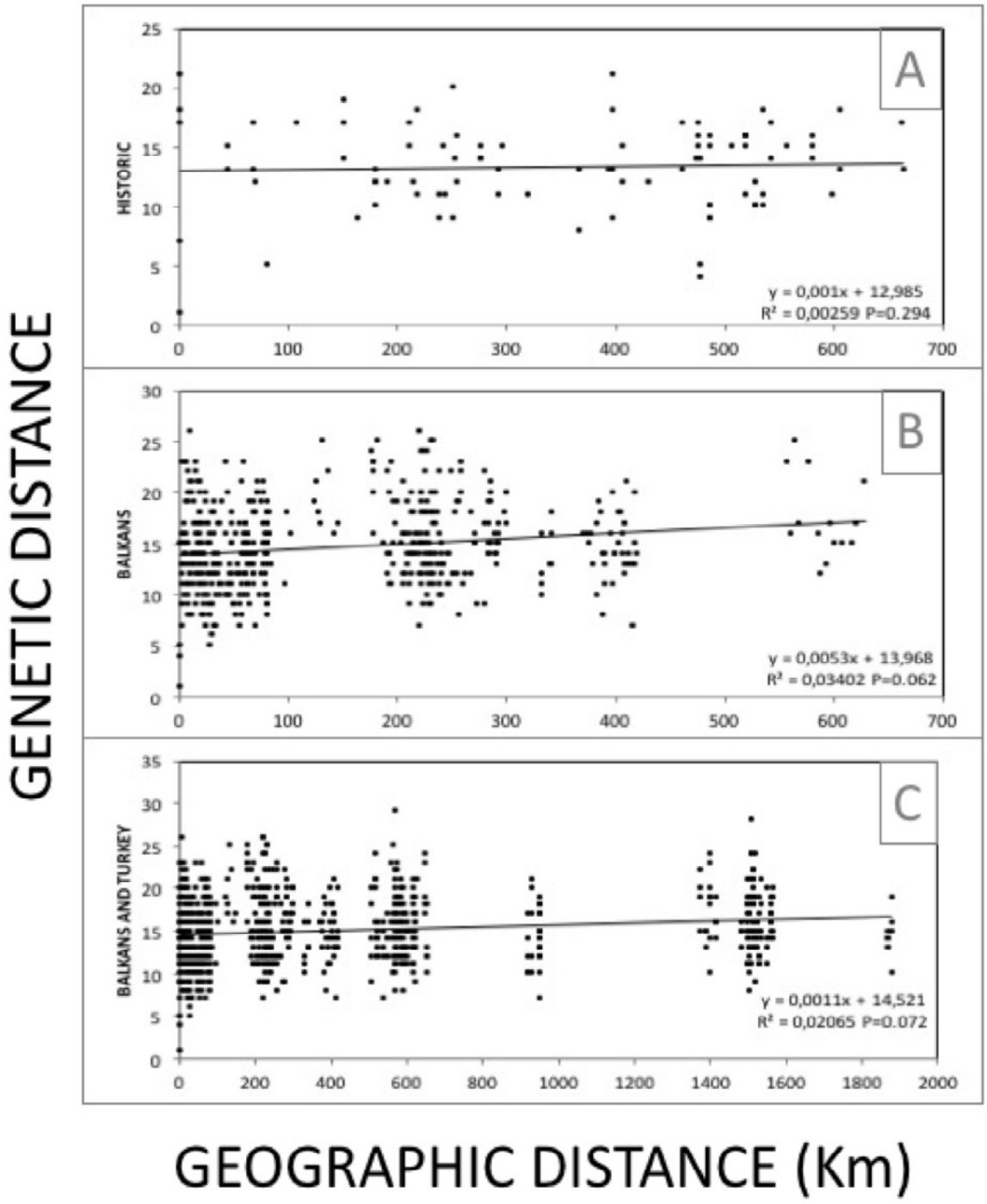
All populations were in Hardy-Weinberg equilibrium and had genetic diversity values ranging from He = 0.586 in the historical population to 0.479 in the southern one (probably due to the small sample size), with the overall diversity for the contemporary Balkan's population being 0.557. Similarly, the highest allelic richness adjusted for the minimum sample size was found in the historical population and the smallest one in the northern (AR= 2.365 and 2.208, respectively), with the overall allelic richness of the contemporary Balkan's population being 2.278 (Table 1). Only the historical population showed values of Fis significantly different from zero, probably due to the historical structure in this area.

We did not find a significant relationship between genetic and geographic distance in the contemporary Balkan's population, regardless of whether the nearest population (Turkey) was included. We did not detect this relationship in historical samples either (Fig 2).

		Nuclear data						Mitochondrial data					
		N	He	Ho	NA	AR	Fis	N	h	S	Hd	Pi	k
HISTORICAL		18	0.586	0.477	4.750	2.365	0.232	20	13	13	0.942	0.013	3.611
CONTEMPORARY	Northern	7	0.506	0.527	3.083	2.208	0.043	7	5	9	0.905	0.014	3.905
	Central	23	0.541	0.583	4.417	2.248	-0.054	22	17	20	0.974	0.017	4.636
	Southern	2	0.479	0.708	2.333	2.333	-0.172	2	2	5	1.000	0.018	5.00
	All Balkan's	32	0.557	0.579	4.583	2.278	-0.024	31	21	20	0.968	0.016	4.443
Turkey		10	0.550	0.567	3.750	2.296	0.024	9	9	10	1.000	0.013	3.500

Table 1. Attributes of historic and contemporary Greece, Bulgaria and Turkey populations. N= sample size, He= Expected heterozygosity, Ho= Observed heterozygosity, NA= Number of alleles, AR= Allelic richness weighted for the smallest sample size, h= number of haplotypes, S= number of polymorphic sites, Hd= haplotype diversity, Pi= nucleotide diversity, k= average number of nucleotide differences.

Population differentiation measures (Fst) showed significant differentiation between historic and contemporary populations (Table 2; Above diagonal), except in the case of the southern Balkan population. Indeed, the historical Balkan population was more differentiated from the contemporary Balkan population (Fst = 0.050) than from the contemporary Turkish population (Fst = 0.030), but was still differentiated from the latter. This may indicate that genetic drift could have been affecting the Balkans in historical times or that there was a geographical pattern in this area. We also found significant differentiation values between the Northern and the Southern partition of the Balkan's population.



Figire 2. Isolation by distance patterns in historic (A), contemporary Balkan's population (B) and contemporary with Turkish population (C). All plots show geographic distance (km) on the x-axis and the genetic distance on the y-axis.

		HISTORICAL	CONTEMPORARY				
			Northern	Central	Southern	Total	Turkey
HISTORICAL		-	0.055*	0.049*	0.071	0.050*	0.030*
CONTEMPORARY	Northern	0.074*	-	0.026	0.092*	0.001	0.036
	Central	0.031*	0.012	-	0.054	-0.015	0.039*
	Southern	-0.007	0.068	0.019	-	0.033	0.020
	All	0.034*	-0.024	-0.033	-0.008	-	0.030*
TURKEY		-0.003	0.015	0.004	0.000	0.003	-

Table 2. Genetic differentiation between pairs of populations, below the diagonal: mitochondrial Fst for haplotype frequencies; above the diagonal: pairwise differentiation Fst values for microsatellite markers. Asterisk indicates significant values after 10000 permutations.

STRUCTURE analyses showed that all samples clustered in only one group, either when analysing all samples together or when independently analysing historical or contemporary samples. In addition, the most likely number of clusters was 1 even when setting locality or period as a prior.

We could not assess changes in individual homozygosity among localities or periods with generalized linear models, probably due to the small sample size degree of freedom in each model. Nevertheless, we found evidence for changes in allelic frequencies when relating genetic and temporal distance (Fig. 3). This analysis showed that those individuals that were more distant in time, were also more distant genetically. This analyses and Fst values indicated changes in allelic frequencies through time between and within populations, an expected consequence of drift in declining populations.

Bottleneck tests did not detect evidence of heterozygote excess, either when TPM or SMM mutation models were assumed. Heterozygote excess, indicative of recent declines, was only detected under an infinitive allele model in the northern of the Balkans partition ($p<0.05$).

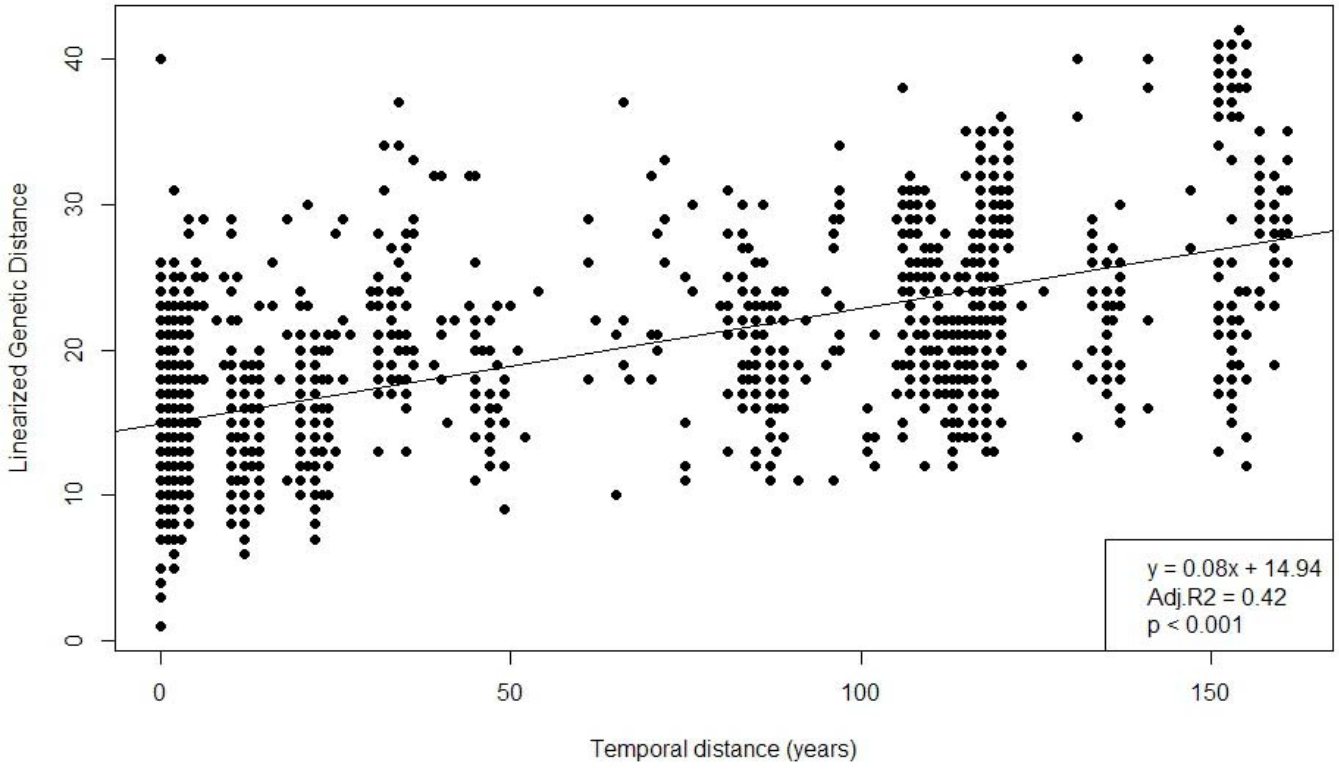


Figure 3. Relationship between individual observed heterozygosity and time in Balkans and Anatolia.

Genetic mitochondrial diversity and structure.

We found a total of 36 haplotypes (21 for the current population and 13 for the historic one) defined by 22 variable sites (Table 1, Supplementary material: S-2), all of them being nucleotide substitutions (G+C = 0.536). Haplotype and nucleotide diversity were similar in historical and contemporary populations and both were lower than the one found in the Turkish population (Table 1). Most of the haplotypes were present at a low frequency and were unique for each area or population (Fig. 4, Supplementary material: S-3). We found that historical populations shared only 3 out of 13 haplotypes with the contemporary population. Although the same proportion was shared with the Turkish population, these haplotypes were not the same. Moreover, the contemporary population shared only two out of nine possible haplotypes with Turkey.

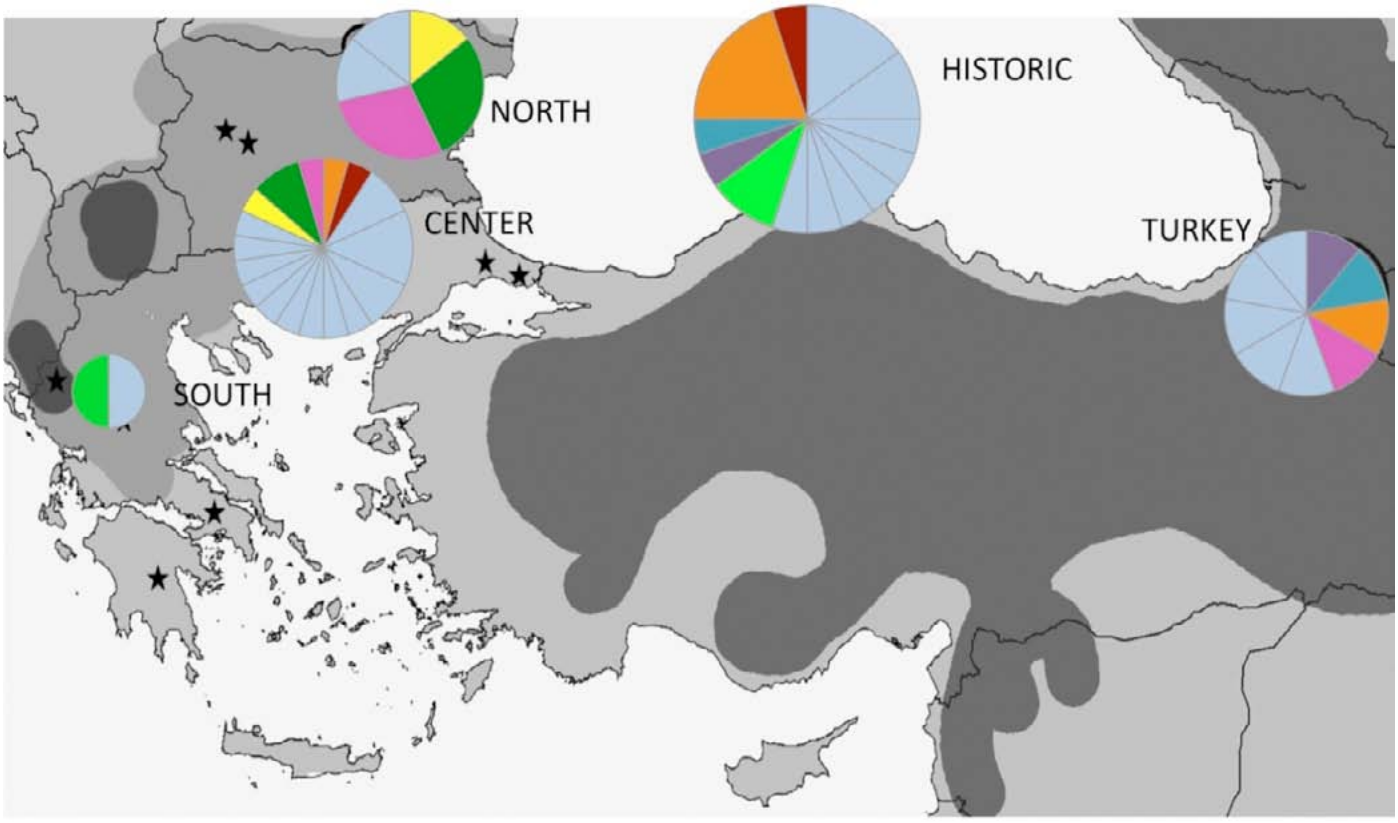


Figure 4. Frequencies of haplotypes in each area. Blue portions indicates exclusive haplotype for each area, other colours represent shared haplotypes. Size of partitions indicates the frequencies of the haplotype.

We found a significant differentiation between the historic and the contemporary Balkan's population (Fst=0.034; p = 0.027). This differentiation was lost when including the Turkish population (Table 2; Below diagonal). We did not find any significant mitochondrial differentiation between geographic sites in the Balkans.

We present the Neighbour-Joining tree with bootstrap supports over 50%, and the *Neophron percnopterus ginginianus* haplotypes as outgroups (Fig. 5). The mitochondrial tree highlights a reciprocal and well-supported monophyletic grouping of N.p.p and N.p.g. We found evidence for population expansion due to an excess number of alleles as indicated by a negative and significant Fu's in the Historic, Contemporary and Turkey populations (Supplementary material: S-4). We did not find any evidence with Tajima's D.

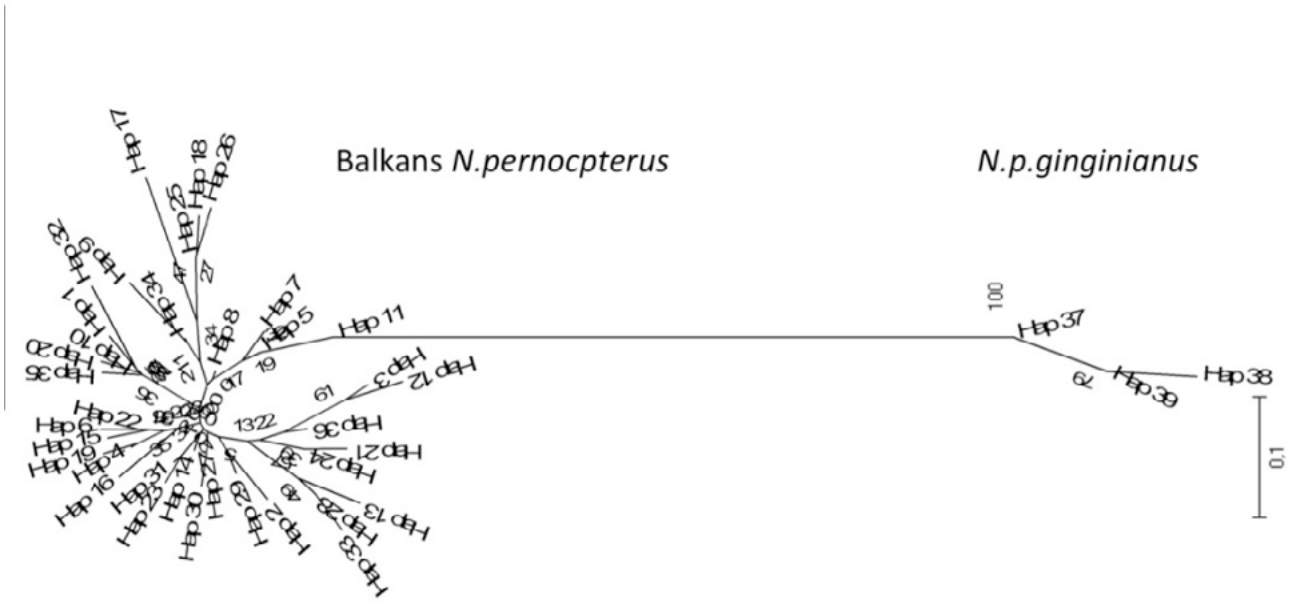


Figure 5. Unrooted neighbour-joining tree of *Neophron pernocpterus pernocpterus* mitochondrial control region haplotypes found in Balkans and Anatolia and *Neophron pernocpterus ginginianus* as outgroups. Values correspond to bootstrap support.

DISCUSSION

Based on mitochondrial and nuclear data from historical and contemporary samples, our results suggest that recent fragmentation and decline has caused allele frequency fluctuations and an incipient loss of genetic diversity. Historical populations did not show any genetic structure indicating a panmictic scenario. In contrast, the current Balkan's population is showing signs of genetic loss and differentiation between geographic areas, with the northern being the least diverse and more differentiated compared to the historical one. The southern area does not show any sign of differentiation probably due to the small sample size. Comparing these populations with others reported in previous studies of Egyptian vultures, we see that the Balkan historical population (He = 0.59) was more diverse than any of the current Iberian populations, in which expected heterozygosity using the same markers ranged from 0.53 to 0.57 (Agudo et al, 2011). Even more, the northern and southern areas show less nuclear diversity than any of these populations. All of this evidence reflects a rapid process of genetic diversity loss as a consequence of the severe and rapid reduction of population size in recent decades.

The lack of relationship between genetic and geographic distances suggests the absence of current gene flow (Hutchison & Templeton 1999) and the relationship between genetic distances and time indicate allele frequency fluctuations in the area (e.g. Demandt 2010). Evolution into different demographic units due to reductions in gene flow and increases in genetic drift is often observed in wild populations following natural or human-induced fragmentation limiting connectivity and ultimately gene flow (Godoy et al 2004, Martínez-Cruz & Godoy 2007, Méndez et al 2014). Here, we provide yet another instance of the contraction and fragmentation of a formerly large historic population yielding similar results. Unfortunately, the process has likely not ended. Given the long generation time of this species (13 years, see Grande et al 2009), we would expect higher values of differentiation in a few more generations if the population decline and fragmentation persists. However, considering the small size of the Balkan population, if the decline persist it will be almost extinct in <5 generations, so the lack of genetic variability will be less of an issue than the lack of birds. A relatively similar pattern was observed for Egyptian vultures inhabiting the southernmost region of

the Iberian Peninsula, which also suffered isolation of neighbouring breeding areas and a strong population decline (Agudo et al 2011). It seems unlikely that this situation will be reversed by natural dispersal: Balkan and Turkish populations mix during migration and in the Sahelian wintering areas (see Meyburg et al 2004; Oppel et al 2015, VCF 2014), but the probability of individuals moving between distant breeding nuclei could be very low, based on evidence from other populations. In fact, Egyptian vultures are highly philopatric with natal dispersal distances less than 50 km (Grande et al 2009), although some individuals may disperse up to 550 km (Elorriaga et al 2009, Lieury et al. 2015).

The Turkish population could be a possible source for reinforcement within ongoing conservation programs as it is less differentiated from the historical Balkan's than the contemporary Balkans population. However, we would expect an increase in this differentiation with time if there are no management actions that reverse this process. The low genetic diversity, the increase in structure and some evidence of bottlenecks in the different Balkans areas, especially in the northern one, indicates that translocations would be a short-term management solution. The question that arises here is whether the Balkans population is the one differentiating from the historic and the rest of the Egyptian vulture populations or, on the contrary, it is the Turkish population that is differentiating. To answer this question, it is essential to include more samples of other closer populations, such as those in the Middle East or other populations in Europe. Nevertheless, the limited available information on Anatolia suggests that this area is well-connected and that the population that remains there is still dense. Moreover, there is no sign of a disconnection from other areas. On the contrary, it has been shown that the Balkans population is largely reduced and disconnected from other areas, in most cases due to the extinction of the closest local populations in Italy, Montenegro and Croatia. This study supports that Balkan's population is differentiating from the historical one more quickly than Turkey's population. Given this, it would be feasible to reinforce the Balkan population with birds from Turkey. Of course, this type of active management cannot be approached without addressing the main causes of population decline, and taking into account potential negative effects associated with the movement of animals between isolated breeding nuclei, such as the risk of disease transmission, behavioural disruption, lower fitness in novel habitats in the presence of local adaptations, and negative demographic effects on the donor populations (Pérez et al 2012).

The characterization of the vanishing Balkan population of Egyptian vultures demonstrates the importance of understanding structure at a fine spatial scale following rapid fragmentation and general decline in the entire surviving breeding nucleus. Historically, there was a unique panmictic population in the Balkans and Anatolia, and today the Balkans populations are isolated and extremely small. Bottlenecks increase rates of inbreeding, loss of genetic variation and fixation of deleterious mutations, thereby reducing adaptive potential and increasing the probability of extinction (Frankham et al 2002). In addition, as we explain above, the natural connection between the Balkan and the Turkish populations is becoming increasingly unlikely with only few pairs left in between (Velevski et al 2015). Under this scenario, and not only due to genetic constraints but also demographic factors, the maintenance of the last Balkan populations of this scavenger seem to be extremely precarious and extinction may be unavoidable within a few decades, as has been predicted for other Mediterranean populations challenged with similar conservation problems (Sanz-Aguilar et al 2015). Therefore, urgent conservation measures should be taken to stop the main limiting factors associated with non-natural mortality (Velevski et al 2015) as well as to revert the negative effects of genetic drift. Management programs should include the reinforcement of the populations and long-term genetic monitoring in order to avoid inbreeding depression, to maximize the genetic diversity and thereby, to increase the long-term population viability (Gautschi et al 2003).

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SUPPLEMENTARY MATERIAL

Table S-1. Relationships of the samples used in this study.

Id_Laboratory	Sample Type	Field_Code	Year	Site	Supplier
87	Feather in alcohol	BG1 20.7.2010	2010	Bulgaria	BSPB / BirdLife Bulgaria
89	Feather in alcohol	BG2 29.7.2012	2012	Bulgaria	BSPB / BirdLife Bulgaria
90	Feather in alcohol	BG3 29.7.2012	2012	Bulgaria	BSPB / BirdLife Bulgaria
91	Feather in alcohol	BG4 27.7.2012	2012	Bulgaria	BSPB / BirdLife Bulgaria
92	Feather in alcohol	BG5 29.7.2012	2012	Bulgaria	BSPB / BirdLife Bulgaria
94	Feather in alcohol	BG6 27.7.2012	2012	Bulgaria	BSPB / BirdLife Bulgaria
95	Feather in alcohol	BG7 28.7.2012	2012	Bulgaria	BSPB / BirdLife Bulgaria
96	Feather in alcohol	BG8 26.7.2012	2012	Bulgaria	BSPB / BirdLife Bulgaria
97	Feather in alcohol	BG9 2.8.2010	2010	Bulgaria	BSPB / BirdLife Bulgaria
99	Feather in alcohol	BG10 12.7.2010	2010	Bulgaria	BSPB / BirdLife Bulgaria
103	Feather in alcohol	BG11 2.8.2010	2010	Bulgaria	BSPB / BirdLife Bulgaria
106	Feather in alcohol	BG12 26.7.2012	2012	Bulgaria	BSPB / BirdLife Bulgaria
107	Feather in alcohol	BG13 27.7.2012	2012	Bulgaria	BSPB / BirdLife Bulgaria
108	Feather in alcohol	BG14 27.7.2010	2010	Bulgaria	BSPB / BirdLife Bulgaria
110	Feather in alcohol	BG15 2.8.2012	2012	Bulgaria	BSPB / BirdLife Bulgaria
114	Feather in alcohol	BG16 26.7.2012	2012	Bulgaria	BSPB / BirdLife Bulgaria
117	Feather in alcohol	BG17 29.7.2012	2012	Bulgaria	BSPB / BirdLife Bulgaria
118	Feather in alcohol	BG18 14.7.2010	2010	Bulgaria	BSPB / BirdLife Bulgaria
119	Feather in alcohol	BG19 27.7.2012	2012	Bulgaria	BSPB / BirdLife Bulgaria
120	Feather in alcohol	BG20 6.8.2010	2010	Bulgaria	BSPB / BirdLife Bulgaria
121	Feather in alcohol	BG21 1.8.2012	2012	Bulgaria	BSPB / BirdLife Bulgaria
122	Feather in alcohol	BG22 15.7.2010	2010	Bulgaria	BSPB / BirdLife Bulgaria
123	Feather in alcohol	BG23 5.8.2010	2010	Bulgaria	BSPB / BirdLife Bulgaria
126	Feather in alcohol	BG24 16.7.2010	2010	Bulgaria	BSPB / BirdLife Bulgaria
146	Umbilical clot	NMNH3	1903	Bulgaria	National museum of Natural History Sofia
149	Umbilical clot	NMNH1	1895	Bulgaria	National museum of Natural History Sofia
152	Umbilical clot	NMNH5	1898	Bulgaria	National museum of Natural History Sofia
155	Umbilical clot	NHM-Kotel2	1925	Bulgaria	Natural History Museum of Kotel
156	Umbilical clot	NMNH6	<1910	Bulgaria	National museum of Natural History Sofia
167	Umbilical clot	NMNH4	1904	Bulgaria	National museum of Natural History Sofia
1	Blood in ethanol	GR1	2012	Greece	WWF Greece
5	Blood in ethanol	GR2	2012	Greece	WWF Greece
7	Feather in alcohol	GR3	2012	Greece	WWF Greece
8	Feather in alcohol	GR4	2012	Greece	WWF Greece
9	Feather in alcohol	GR5	2012	Greece	WWF Greece
12	Feather in alcohol	GR6	2013	Greece	HOS / BirdLife Greece
128	Feather in alcohol	GR7	2013	Greece	HOS / BirdLife Greece
129	Blood in ethanol	GR8	2013	Greece	WWF Greece
138	Umbilical clot	528	1877	Greece	Athens Zoological Museum
140	Umbilical clot	344	1853	Greece	Athens Zoological Museum
144	Umbilical clot	332	1859	Greece	Athens Zoological Museum
166	Umbilical clot	NMNH8	1899	Greece	National museum of Natural History Sofia

Id_Laboratory	Sample Type	Field_Code	Year	Site	Supplier
927	Umbilical clot	GR9	1990	Greece	Natural History Museum of Vitsa
928	Footpad tissue	GR10	1965	Greece	Natural History Museum of the University of Thessaloniki
929	Feather	NHMC 80.4.45.8	1929	Greece	Natural History Museum of Crete
150	Umbilical clot	NMNH7	1893	Turkey	National museum of Natural History Sofia
164	Umbilical clot	NMNH2	1893	Turkey	National museum of Natural History Sofia
165	Umbilical clot	NMNH9	1894	Turkey	National museum of Natural History Sofia
1532	DNA	Np1532	2014	Turkey	Local collaborators
1533	DNA	Np1533	2014	Turkey	Local collaborators
1534	DNA	Np1534	2014	Turkey	Local collaborators
1535	DNA	Np1535	2014	Turkey	Local collaborators
1536	DNA	Np1536	2014	Turkey	Local collaborators
1553	DNA	Aroj	2014	Turkey	Koç University
1554	DNA	Egypt	2014	Turkey	Koç University
1555	DNA	ná	2014	Turkey	Koç University
1556	DNA	Rgdv	2014	Turkey	Koç University

Table S-2. Differences in the 36 haplotypes sequences (406bp) found in Greece, Bulgaria and Turkey. H = haplotype, N = Haplotype frequence. Column headers indicate nucleotide positions.

H	N	Variable Sites																					
		23	24	26	34	40	46	47	48	50	51	55	59	60	75	80	84	99	119	138	144	160	261
1	3	A	G	T	C	T	G	A	T	G	T	G	C	G	G	A	A	C	T	A	A	C	C
2	2	.	A	.	T	.	A	.	.	.	C	.	.	.	.	.	.	T	.	.	.	T	T
3	6	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	T
4	1	.	A	.	T	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	T	T
5	1	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	T	.	.	.	T	T
6	1	.	.	.	T	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	T	T
7	1	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	T
8	1	.	.	.	T	C	A	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.
9	1	.	.	.	.	.	A	.	.	.	C	.	.	.	.	.	.	.	.	.	.	T	T
10	2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	T
11	2	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.
12	6	.	.	.	.	C	A	.	.	.	C	.	.	.	.	.	.	.	.	.	.	T	T
13	2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	T
14	2	.	.	C	.	.	A	.	C	.	C	.	.	.	.	.	.	T	.	G	.	T	T
15	3	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	T	T
16	2	.	.	.	T	.	A	.	.	.	.	.	.	.	.	.	G	.	C	.	.	T	T
17	1	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	T	.	.	.	.	.
18	1	.	.	.	.	C	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
19	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	T
20	1	.	.	.	.	C	A	.	.	.	C	.	.	.	.	.	.	.	.	.	.	T	T
21	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.
22	1	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	T	.
23	1	.	.	.	T	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	T	T
24	1	T	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	T	T
25	1	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	T	.	.	.	T	T
26	2	.	.	.	T	.	.	.	.	.	.	.	.	.	A	.	G	.	C	.	.	T	T
27	4	.	A	.	T	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	T	T
28	4	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	T
29	1	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	T	T
30	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	T
31	1	T	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.
32	1	.	.	.	T	.	.	.	.	.	C	A	.	A	.	.	G	.	.	.	.	T	T
33	1	.	.	.	.	C	A	.	.	.	C	.	.	.	.	.	.	.	.	.	.	T	.
34	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	T
35	1	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.
36	1	.	A	.	T	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.

Table S-3. Frequencies of the 36 haplotypes found in historic and contemporary populations. N = number of sequences, h = number of haplotypes.

		N	h	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
Historic		20	13	3	2	2	1	1	1	1	1	1	1	1	4	1																							
Contemporary	North	7	5																										1	2	2	1	1						
	Centre	22	17												1	1	2	3	2	1	1	1	1	1	1	1	1	1	1	2	1			1	1				
	South	2	2			1																												1					
Turkey		9	9										1	1	1																1			1	1	1	1	1

Table S-4. Genetic variability in current and Historic populations. N = number of sequences, h = number of haplotypes, S = number of segregating sites, Hd = haplotipic diversity, Pi = nucleotidic diversity, k = mean nucleotidic diversity, * indicates significant values.

		Tajima's D	Fu and Li's D	Fu's	Tau	Raggedness
HISTORIC		-0.053	-0.439	-5.571*	3.611	0.029
CONTEMPORARY	North	0.035	0.460	-0.185	3.905	0.218
	Centre	-0.576	0.484	-9.463*	4.63	0.055
	South	0	-	1.609	5	2
	All	-0.389	0.370	-12.206*	4.443	0.039
TURKEY		-0.230	0.049	-6.256*	3.50	0.074