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Population Genetic Structure of Testudo *hermanni boettgeri* (Hermann's Tortoise) in Türkiye

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Abstract: Multidisciplinary approaches for the conservation of endangered species have great importance in preparing management plans. In addition to ecological and population demographic parameters, genetic data provide vital information for conservation management plans for a species. The Hermann's tortoise (*Testudo hermanni*) suffers from illegal trade, fires, habitat fragmentation and destruction, and therefore, the genetic diversity of the regional populations has been affected. It was aimed herein to impart knowledge on the population genetic structure of *T. hermanni boettgeri*, listed as near threatened by the International Union for Conservation of Nature Red List as a subspecies and has limited distribution in European Türkiye. A study was conducted of 15 microsatellite loci of 221 tortoises from 15 different localities in the Thrace region in Türkiye. All of the examined loci were polymorphic, and the number of alleles varied from 2 to 13. The quantity of private alleles (*Pa*) at the localities ranged from 0 to 6. The average gene diversity was 0.31 (range: 0.25–0.38). The highest levels of allelic richness, *private alleles*, and genetic diversity (*Ar*, *Pa*, *He*) were observed at localities 3 and 7, close to each other. The total population ($p < 0.001$) and 12 of the 15 studied localities diverged from the Hardy–Weinberg equilibrium. Of the 15 localities studied, 6 had significantly different inbreeding coefficients. Furthermore, a 2-phased model of mutation (TPM) ($p < 0.001$) detected a recent bottleneck in the population. The population genetic results identified 8 groups with significant genetic structure ($F_{ST} = 0.166$, $p < 0.01$) in 2 large clusters ($K = 2$).

Key words: European Türkiye, microsatellite, genetic diversity, cluster, management units

1. Introduction

The leading anthropogenic causes for the worldwide decline in reptiles are habitat loss and degradation, the pet trade, introduced invasive species, disease and parasitism, environmental pollution, unsustainable exploitation and degradation, and global climate change (Todd et al., 2010). From these anthropogenic causes, the ecological perturbations and extensive habitat degradation caused by human activities are indisputably linked to the rapid loss of biological diversity (Đurakić and Milankow, 2019, 2020). According to the Turtle Taxonomy Working Group (2014), among reptiles, tortoises and turtles are the most endangered species, and over the past 20 years, over 2 million wild turtles and tortoises have been traded (Luiselli et al., 2016).

Two subspecies of Herman tortoises (*Testudo hermanni*, Gmelin, 1789), an endemic species to southern Europe, inhabit different geographical areas (Cheylan, 2001; Bour, 2004). *T. hermanni hermanni* (Gmelin, 1789) is located in the center and south of Italy, Spain, and France (west of the spread region). *T. h. boettgeri* (Mojsisovics, 1889) is located in the northeast of Italy, Balkan regions, Greece, and Türkiye (east of the spread region). *T. hermanni*, for which the distribution of the species is limited to Europe, the Balkans, and Turkish Thrace (Bertolero et al., 2011), was listed as Near Threatened on a global scale by the International Union for Conservation of Nature (IUCN)[1] in 2022.

The Hermann's tortoise that sprawls in fragmented populations across Mediterranean Europe is a species endangered and with an apparent genetic discontinuity between the eastern and the western subspecies (Pérez et al., 2014). *T. hermanni* populations showed two major clusters compatible with morphological classification according to the analysis of mitochondrial DNA (mtDNA) (van der Kuyl et al., 2002; Mirimin et al., 2004). Due to habitat

1 http://www.iucnredlist.org. [Accessed: Jun 21st, 2022].

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degradation, overfishing, and anthropogenic influences, the *T. h. hermanni* distribution is fragmented. (Zenboudji et al., 2016). *T. h. boettgeri* may have been affected by these historical climatic events and anthropogenic and ecological factors. According to the Cytochrome b (*Cytb*) gene from mtDNA, *T. h. boettgeri* was separated into three significant haplogroups in geographically isolated areas as the west coast of the Balkans, the Balkans, and southern Peloponnese (Fritz et al., 2006).

Conservation genetics is an essential component of species conservation and management plans since it provides information about the dynamics of genes in a population. Genetic studies contribute fundamental knowledge concerning the genetic structure and diversity of populations, inbreeding/outbreeding and introgression, and demographic trends, and ensure endangered (Zenboudji et al., 2016). Moreover, some researchers have used genetics to describe conservation units as evolutionarily significant units and management units (MUs) (Moritz, 1994), which have been suggested as crucial resources for determining which landscapes should receive the most protection (Smith and Grether, 2008). Microsatellite markers were often used to identify these genetic units (Salinas et al., 2011; Vargas-Ramírez et al., 2012; Zenboudji et al., 2016).

The fragmentation of a species' population causes a population reduction, often known as a bottleneck. This causes a decrease in genetic diversity with the loss of alleles through genetic drift (Frankhamn et al., 2009; Bouzat, 2010). Moreover, the reduced gene flow between isolated population fragments may increase the potential for inbreeding. Identifying population bottlenecks that lessen the chance of survival of populations is critical in conservation, and genetic bottleneck tests with sampling at a single point in time can be used to determine if a population decline has occurred (Peery et al., 2012). The effective population size (Ne) is another important statistic for managing populations and is required to predict the rate of inbreeding and loss of genetic variation in wildlife (Frankham, 2007).

Hermann's tortoise is one of the target trade species and accounts for 13% of the global Testudo trade (Türkozan et al., 2008). The Turkish population was overharvested in the pet trade between 1974 and 2005 (Türkozan and Kiremit 2007). *T. hermanni* comprised 11% (of the total of 468,000 tortoises) of this trade. In addition to the illegal pet trade, wildfires, habitat degradation, agricultural activities, and other threats (Moreira and Russo, 2007; Couturier et al., 2011; Salinas et al., 2011; Maxwell et al., 2016) may risk the survival of natural populations of this species. The artificial selection of the illegal pet trade and other threats, such as habitat degradation and agricultural activities, may cause a reduction in the genetic diversity of the populations (Noël et al., 2007; Salinas et al., 2011). In such cases, genetic data help us to create successful captive breeding programs by managing genetic variation with reintroduction programs into the wild (Austin et al., 2011; Witzenberger and Hochkirch, 2011). A recent study in European Türkiye (T**ü**rkozan et al., 2019) reported a risk of population decline in *T. h. boettgeri* populations and patchy distribution due to settlements, and industrial and agricultural zones.

In this article, a follow-up study to that of T**ü**rkozan et al. (2019), it was aimed to identify the level of genetic variation and possible adverse effects of illegal trade in *T. h. boettgeri* using 15 nuclear markers. Furthermore, the conservation priorities of populations based on their genetic diversity levels were determined.

2. Materials and methods

2.1. Sampling and DNA isolation

Samples were collected from 15 different localities in the Turkish Thrace (Figure 1) from April to October 2014 and 2015. Toenail samples of 221 tortoises, taken with sterile pliers, were preserved by adding 96% ethyl alcohol in 2 mL-sterile tubes with a screw cap. The total genomic DNA was isolated using the standard phenol-chloroform DNA isolation method of Hillis and Moritz (1990). The isolated DNA was dissolved in 100 µL of TE (1 mM Tris-HCl, pH 7.5, 0.1 mM EDTA).

The 15 microsatellite loci used in the current study have been previously described for tortoises (Ciofi et al., 2002; Edwards et al., 2003; Schwartz et al., 2003; King and Julian, 2004; Roques et al., 2004; Forlani et al., 2005; Salinas et al., 2011). One of each primer pair (forward) was fluorescently labelled with Hex, 6-Fam, and Ned (Hex: *Test10*, *Test71*, *GmuB08*, *Gp55*, and *Goag5*; 6-Fam: *Test21*, *Test76*, *GmuD16*, *Gp61*, and *Gal50*; Ned: *Test56*, *Test88*, *GmuD51*, *Gp81*, and *Gp19*). Each locus was amplified in a total reaction volume of 25 µL [Reaction: 1X Taq buffer, 1.5 mM of MgCl2, 0.1 mM of dNTP, 0.5 U/µL of Taq polymerase (5 U/µL; Fermentas, MBI), and 0.24 pmol/µL of each of the oligonucleotide pairs]. A denaturation step was used to start the cycling for all of the microsatellite loci at 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 58 °C for 30 s, elongation for 45 s at 72 °C, and ending with a final elongation step at 72 °C for 5 min. An ABI 3730 automated DNA analyzer (Macrogen Inc., Seoul, South Korea) was used for the allele length determination, and Genemaker 1.8 (SoftGenetics, LLC., State College, PA, USA) was used to assign the allele sizes.

2.2. Data analysis

The Expectation Maximization (EM) technique of Dempster was used to assess the null allele frequencies estimated for each locus and population (Dempster et al., 1977). The null allele frequencies and the genetic

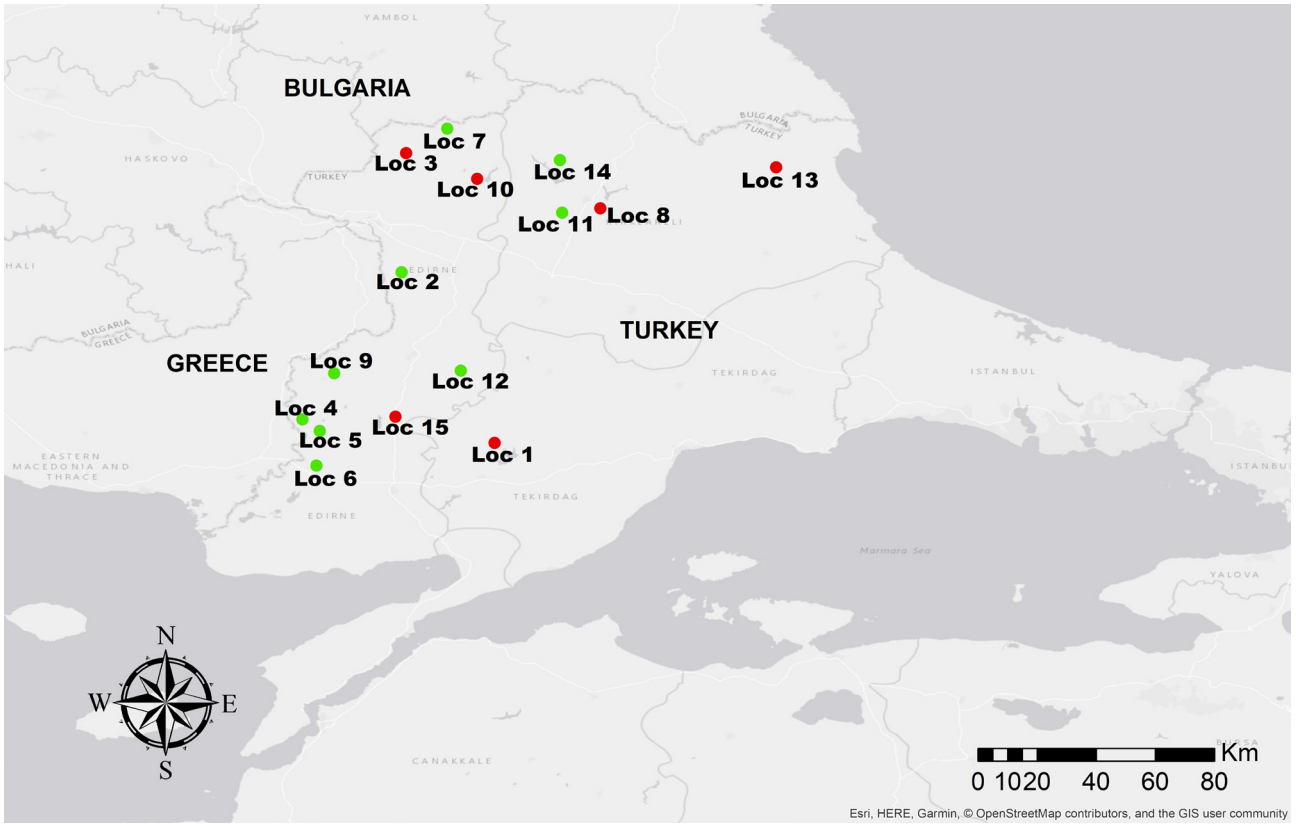

Figure 1. Sampling localities of *T. h. boettgeri* (Loc 1: Malkara, Loc 2: Orhaniye, Loc 3: Hanlıyenice, Loc 4: Adasarhan, Loc 5: Balabanlı, Loc 6: İpsala, Loc 7: Hacılar, Loc 8: Şeytanderesi, Loc 9: Meriç, Loc10: Taşlısekban, Loc 11: Kırklareli, Loc 12: Çöpköy, Loc 13: Demirköy, Loc 14: Erikler, and Loc 15: Keşan; the colorations symbolize the clusters).

differentiation (F_{ST}) estimate, which used the excluding null alleles (ENA) correction method, to correct the positive bias induced by the presence of null alleles on the F_{ST} estimation were calculated using FreeNA [number of replicates: 1000, computation of the bootstrap 95% confidence interval (95% CI)] (Chapuis and Estoup, 2007). Since the null allele frequency of the 15 loci was low at the localities, all of the loci were used in the analysis. The number of alleles (*Na*), number of private alleles (*Pa*), allelic richness (*Ar*), expected heterozygosity/gene diversity (*He*; Nei's genetic diversity, Nei 1973), and observed heterozygosity (*Ho*) in each locus and each population, were calculated using Genepop 4.0 (Rousset, 2008) and Fstat 2.9.3.2 (Goudet, 1995). For estimating the effective population (95% CI, the minimum acceptable frequency of the analysis for the alleles was P_{Crit} = 0.02), the linkage disequilibrium (LD) approach was applied (Hill, 1981; Waples, 2006; Waples and Do, 2010), as implemented in NeEstimator 2.0 (Do et al., 2014). The inbreeding coefficient (F_{IS}) deviation from the Hardy–Weinberg equilibrium (*HWE*) in each locus and sampling location and detection of the LD between the loci were determined using the probability test approach (Guo and Thompson, 1992) implemented in the program Genepop 4.0 (Rousset, 2008). The Fisher procedure was used for determining probability values across all loci and all populations. A pairwise test for population differentiation (F_{ST}) was carried out with Fstat 2.9.3.2 (Goudet, 1995). The significance levels of these analyses were estimated using a Markov chain with 1000 batches and 1000 iterations per batch. Bonferroni correction was used for tests involving multiple comparisons at all significance levels (Rice, 1989). The correlation between the genetic differentiation (F_{ST} value) and geographic distance was tested using the Mantel test (10,000 iterations at a 95% CI) with Arlequin 3.1 (Excoffier et al., 2005). Moreover, Bottleneck 1.2 was used to detect bottlenecks at the localities (Cornuet and Luikart, 1996). Fluctuations in the locality size were assessed using the Wilcoxon signed-rank test with 5000 permutations under the 2-phase mutation model (TPM; single-step mutation: 95%, multiple step mutation: 5%, variance among multiple steps: 12), which is more suitable for microsatellite data than the strict 1-step stepwise mutation model (SMM) (Piry et al., 1999).

The most significant objective standard for determining early conservation decisions for livestock breeds is phylogenetic diversity based on microsatellite loci (Barker, 1999). The unweighted pair group method with arithmetic mean (UPGMA) distance tree based on the F_{ST} data was carried out to reveal the genetic relationship between the *T. h. boettgeri* localities using Populations 1.2.32 (Langella, 1999) with the Reynolds et al. (1983) weighted model, which calculated the genetic distance for short-term evolution. Structure 2.3, which uses a Bayesian clustering method to estimate the most likely number of populations (K) without utilizing previous knowledge of the geographic location of the individuals collected, was used to analyze the population structure (Pritchard et al., 2000). This program defines clusters based on allele frequencies. Twenty runs were carried out by setting the number of clusters (K) from 1 to 15 and from 1 to 8 (the groups formed according to the UPGMA distance tree) with 10,000 burn-in periods followed by 100,000 Monte Carlo Markov Chain (MCMC) replicates, assuming an admixture model that allows individuals to have mixed ancestry and correlated allele frequencies. The best estimation of the K value was inferred by looking at the variation of the likelihood of the data and following the approach based on likelihood ratios (Evanno et al., 2005). Also assessed was the genetic structure using Geneland 3.1.5, which incorporates geographic information to estimate the spatial extent of each population (Guillot et al., 2008). The most probable K value for the 15 localities was inferred using spatial, null allele, and correlated allele frequency models with the MCMC (iterations: 100,000 replications: 10, thinning: 100, burn-in: 200, maximum rate of Poisson process: 100, spatial uncertainty: 0.1 km).

3. Results

3.1. Genetic diversity within the localities

In total, 74 alleles were identified for the 15 loci in 221 samples. All of the loci examined were polymorphic, with a mean of 4.93 alleles (range: 2–13). From a total of 74 alleles, 18 *Pa* were found, and the highest number of *Pa* was found in the 2 locations in the northwest (Localities 7 and 3) (Table 1). The null allele frequencies at the localities ranged from 0.01 to 0.07 (Table 1). At all of the investigated localities, the *Test56* (0.226) and *Gp19* (0.103) loci had the highest null allele frequencies. The total population and 12 of the study localities diverged from the *HWE* (Table 1). Furthermore, 8 of the studied loci diverged from the *HWE* (*Test21* chi-squared, $p < 0.01$; *Test56* chi-squared, $p < 0.01$; *Test71* chi-squared, $p < 0.01$; *GmuB08* chi-squared, $p < 0.01$; *GmuD51* chi-squared, $p < 0.01$; *Gp81* chi-squared, $p < 0.01$; *Gp19* chi-squared, $p < 0.01$; and *Goag5* chi-squared, $p < 0.01$). All of the loci were assumed to be independent

Table 1. Descriptive statistics of the analyzed loci for the 221 samples.

Localities	N	*Na*	*Pa*	*Ar*	*Nu*	*Ne*	*Ho*	*He*	F_{IS}	*HWE*
Locality 1	44	46	1	1.82	0.05	13.0	0.26	0.29	0.13***	***
Locality 2	43	43	2	1.92	0.04	131.1	0.29	0.31	0.04	***
Locality 3	18	50	4	2.20	0.05	20.9	0.30	0.37	0.19***	***
Locality 4	13	34	1	1.80	0.03	-	0.34	0.28	–0.22	***
Locality 5	14	32	1	1.84	0.04	11.2	0.29	0.30	0.03	***
Locality 6	13	36	1	1.88	0.05	31.3	0.28	0.30	0.07	***
Locality 7	8	41	6	2.22	0.07	1.9	0.29	0.38	0.24***	**
Locality 8	12	38	2	2.03	0.04	1.8	0.28	0.33	0.15*	***
Locality 9	8	31	0	1.79	0.03	-	0.23	0.25	0.09	*
Locality 10	10	34	0	1.92	0.03	2.2	0.29	0.32	0.09	
Locality 11	6	30	0	1.80	0.02	2.1	0.27	0.26	–0.03	
Locality 12	11	39	0	2.10	0.06	2.6	0.29	0.36	0.18**	***
Locality 13	9	37	0	2.08	0.05	4.0	0.27	0.34	0.19*	*
Locality 14	4	27	0	1.80	0.01	-	0.32	0.26	–0.23	
Locality 15	8	35	0	2.03	0.04	2.6	0.31	0.34	0.10	*
Overall	**221**	**74**	**18**	**1.95**	**0.04**	**11.5**	**0.29**	**0.31**		***

N: sample size, *Na*: number of alleles, *Pa*: number of private alleles, *Ar*: allelic richness, *Nu*: null allele frequency, *Ne*: effective population size ($P_{Crit} = 0.02$), *Ho*: observed heterozygosity, *He*: expected heterozygosity/gene diversity, F_{IS}: inbreeding coefficient (statistically significant F_{IS} and *HWE* values: *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

because no LD was found between the loci pairs (chi-squared, $p > 0.05$). The mean *Ar* per locality varied from 1.79 (Locality 9) to 2.22 (Locality 7), with an average of 1.95 (Table 1). While the *Ho* per locality varied from 0.26 (Locality 1) to 0.34 (Locality 4), with an average of 0.29, the expected heterozygosity (*He*) per locality ranged from 0.25 (Locality 9) to 0.38 (Locality 7), with an average of 0.31 (Table 1). Localities 3 and 7 had higher *Ar*, *Pa*, and *He* (Table 1). The effective population sizes (*Ne*) at 8 localities varied from 1.8 (Locality 8) to 131.1 (Locality 2), with an average of 11.5 (Table 1) for the localities overall.

A deficit in heterozygotes with the F_{IS} value showed a significant difference at Locality 1 ($F_{IS} = 0.13$), Locality 3 ($F_{IS} = 0.19$), Locality 7 ($F_{IS} = 0.24$), Locality 8 ($F_{IS} = 0.15$), Locality 12 ($F_{IS} = 0.18$), and Locality 13 ($F_{IS} = 0.19$) (Table 1). The whole population showed evidence of a recent bottleneck under the 2-step mutation model (Wilcoxon test, $p < 0.001$), while only 4 localities (127 samples) showed evidence of a current bottleneck under the 2-step mutation model (Locality 1 Wilcoxon test, $p < 0.01$; Locality 2 Wilcoxon test, $p < 0.01$; Locality 3 Wilcoxon test, $p < 0.05$, and Locality 8 Wilcoxon test, $p < 0.01$).

3.2. Identification of the locality units

The Mantel test results of the 15 localities revealed no correlation between the genetic divergence (pairwise F_{ST}) and geographic distances ($r = 0.38$, $p > 0.05$). The UPGMA distance tree created using the Reynolds (1983) weighted model grouped the studied localities into 8 groups (Group 1: Locality 1; Group 2: Localities 3 and 10; Group 3: Localities 8 and 13; Group 4: Locality 15; Group 5: Localities 2, 7, and 12; Group 6: Localities 4, 5, and 6; Group 7: Locality 9; and Group 8: Localities 11 and 14) (Figure 2). The pairwise comparison (F_{ST} values) of the groups varied from $F_{ST} = 0.015$ (between the Groups 1 and 3) to $F_{ST} = 0.316$ (between Groups 4 and 8) (Table 2). The F_{ST} values of the groups indicated genetic structuring with significant differences in all of the pairwise comparisons (overall $F_{ST} = 0.166$, $p < 0.01$) (Table 2). The F_{ST} values estimated using the ENA correction method showed little difference from the F_{ST} values calculated not using the method (Table 2).

The populations of *T. h. boettgeri* were divided into 2 main clusters as in the UPGMA distance tree created using the Reynolds et al. (1983) weighted model (Figures 2, 3a, and 3b). The Bayesian clustering method revealed a strong structure for K = 2, corresponding to the highest value of ΔK (Figure 3c). While the first cluster consisted of Groups 1–4, the second cluster included Groups 5–8) (Figures 2 and 3b). The results obtained with Geneland which used the geographic locations of the localities, were consistent

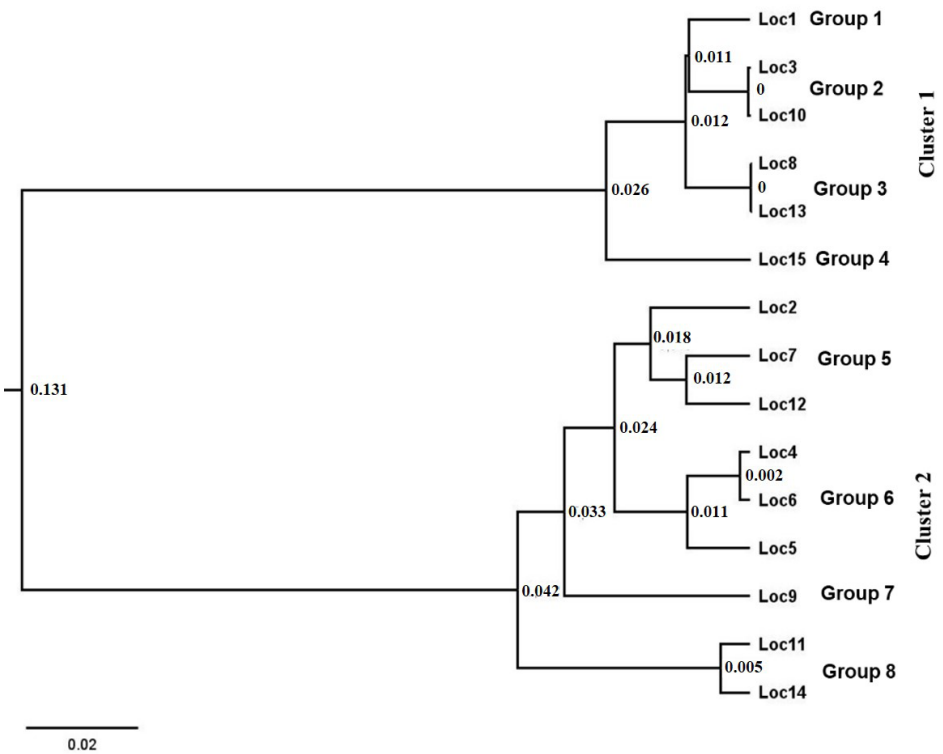

Figure 2. UPGMA distance tree created using the Reynolds (1983) weighted model (the node values are bootstrap values estimated with 1000 permutations).

Table 2. Pairwise comparison of the 8 determined groups.

	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7	Group 8
Group 1		0.021	0.013	0.056	0.143	0.202	0.241	0.228
Group 2	0.022[b]		0.024	0.042	0.118	0.200	0.172	0.223
Group 3	0.015[b]	0.020[b]		0.122	0.098	0.159	0.204	0.182
Group 4	0.048[c]	0.031[a]	0.108[c]		0.211	0.291	0.282	0.310
Group 5	0.153[c]	0.120[c]	0.101[c]	0.207[c]		0.047	0.061	0.069
Group 6	0.210[c]	0.204[c]	0.160[c]	0.290[c]	0.049[c]		0.162	0.090
Group 7	0.246[c]	0.165[c]	0.201[c]	0.263[c]	0.051[a]	0.168[c]		0.195
Group 8	0.258[c]	0.239[c]	0.207[c]	0.316[c]	0.078[c]	0.102[c]	0.203[c]	

Bottom section: genetic distance values (F_{ST}) between groups, upper section: genetic distance values (F_{ST}) between the groups using the ENA correction, significance p-values of the chi-squared test, [a]$p < 0.05$; [b]$p < 0.01$; [c]$p < 0.001$.

with the 2 major clusters indicated by Structure and the UPGMA distance tree (Figures 4a and 4b).

4. Discussion

4.1. Gene diversity

The current study is the first comprehensive study of the population genetic structure of *T. h. boettgeri* after the illegal trade of this species from European Türkiye. The total *Na* in the recent study (74 alleles, 15 loci, 221 samples) was lower than that of Zenboudji et al. (2016) (148 alleles, 17 loci, 372 samples: *T. h. hermanni*: 357/*T. h. boettgeri* 15). However, the *Na* in studies of *T. hermanni* have varied with the number of samples studied, the number of loci, and the number of polymorphic loci (Salinas et al., 2011; Cutuli et al., 2013; Zenboudji et al., 2016). Among all of the studied localities, 3 (*Pa* = 4, *Ar* = 2.20, *He* = 0.37), and 7 (*Pa* = 6, *Ar* = 2.22, *He* = 0.38) showed the highest *Pa*, *Ar*, and *He* values. *Pa* found in only a single genetic cluster or sampling location have proven informative for population genetic studies in such areas as molecular ecology and conservation genetics (Szpiech and Rosenberg, 2011). Therefore, populations with the most significant number of privative alleles may be prioritized for protection. The high genetic diversity in these localities could be explained by the high genetic diversity of the founder individuals having different alleles. The highest values in the number of *Pa*, *Ar*, and *He* found in these localities suggest they may have harbored a population of *T. h. boettgeri* isolated in the past because of patchy distribution due to settlements, and industrial and agricultural zones. The average total genetic diversity in the present study was higher than that in France (t-value: 2.15, $p < 0.05$), smaller than that in Spain (t-value: –1.65, $p > 0.05$), and in Italy (t-value: –10.19, $p < 0.001$) from *T. h. hermanni* populations and smaller than that in Gonfaron/France (t-value: –16.83, $p < 0.001$) from *T. h. boettgeri* populations (Table 3). Furthermore, this value observed in other endangered species in Testudines was similar to that of *Podocnemis lewyana* (*He* = 0.32, t-value: –0.69, $p > 0.05$) (Vargas-Ramírez et al., 2012) and relatively smaller than that of *Chelonoidis petersi* (*He* = 0.74, t-value: –40.58, $p < 0.001$), *Chelonoidis chilensis* (*He* = 0.59, t-value: –26.33, $p > 0.001$), *Chelonoidis donosobarrosi* (*He* = 0.51, t-value: –18.74, $p > 0.001$) (Fritz et al., 2012), *Emys orbicularis* (*He* = 0.71, t-value: –37.73, $p > 0.001$), and *Emys trinacris* (*He* = 0.68, t-value: –34.88, $p > 0.001$) (Pedall et al., 2011).

The deviation from the *HWE* in the total population and 12 of the studied localities was similar to a study conducted with the *T. h. hermanni* population in France, which was performed with 15 microsatellite loci (Salinas et al., 2011) and differed by only one locus according to their research. T**ü**rkozan et al. (2019) reported patchy distribution in *T. h. boettgeri* populations in the Thrace region of Türkiye due to settlements, and industrial and agricultural zones. The isolation of populations caused by this patchy distribution and trading as a pet animal may have led to deviation from the *HWE*, with an increase in the probability of inbreeding in populations that lead to a decrease in genetic diversity. The distribution of both *T. h. hermanni* (Western Europe) and *T. h. boettgeri* (the Balkans) was affected by two critical factors: the first, climate fluctuation; and the second, agricultural activities, urbanization, forest fires, and poaching (Cheylan, 2004; Bertolero et al., 2011; Couturier et al., 2011; Santos and Cheylan, 2013). Adding to these factors, collection for the pet trade can be included in poaching and result in the loss of large numbers of wild animals of *T. h. boettgeri* subspecies (Zenboudji et al., 2016). Before Türkiye was included in the Convention on International Trade in Endangered Species agreement, *T. h. boettgeri* was excessively traded as a pet animal (Türkozan and Kiremit, 2007). The deviation from the *HWE* and the low *He* (*He* = 0.31) of *T. h. boetgeri*

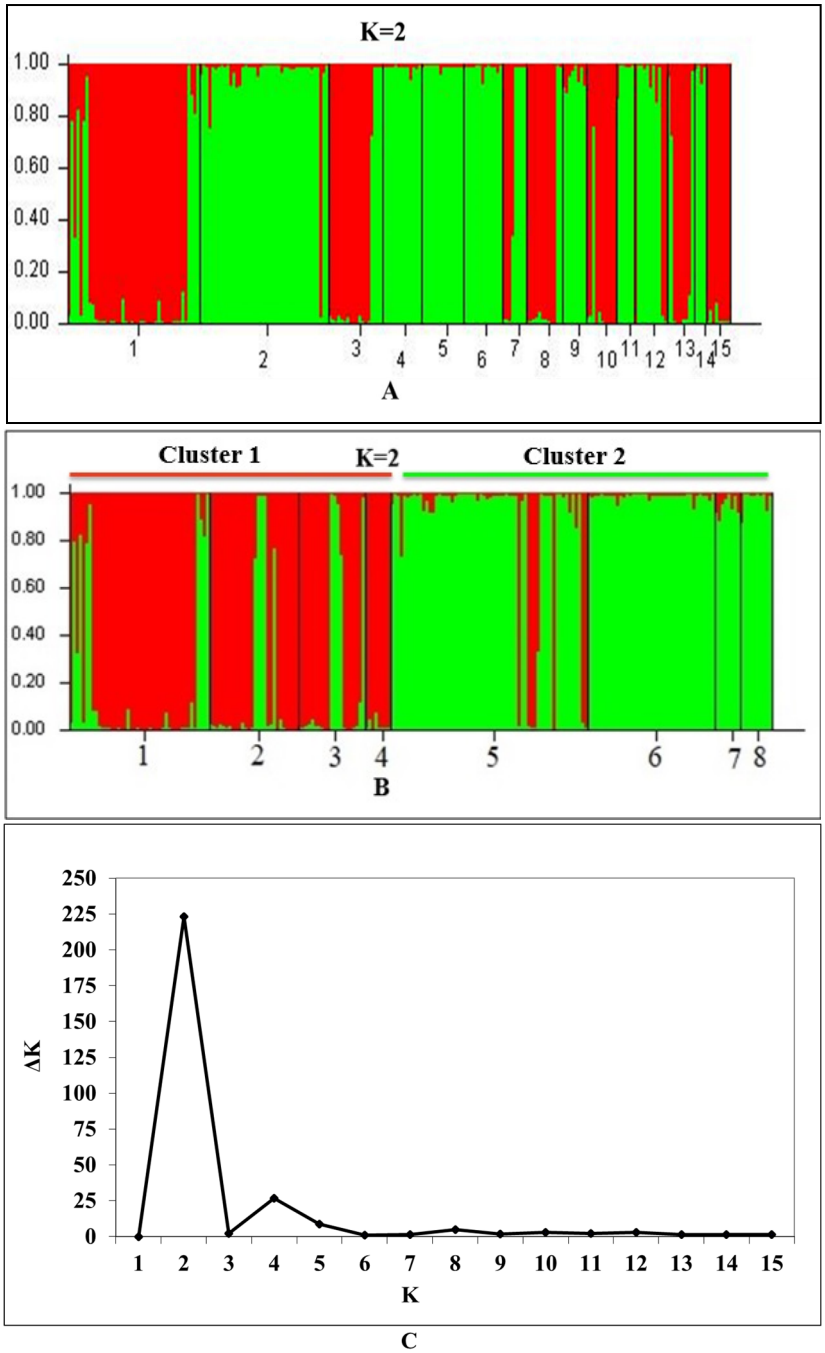

Figure 3. Population assignment test performed with Structure. (A) Barplots that estimated membership coefficients of the analyzed individuals in each locality. (B) Barplot, K = 2, clusters for 8 groups in the UPGMA distance tree. (C) Graph of ΔK as a function of the number of groups K, (Evanno's method) (the numbers on the barplots symbolize the sampling localities).

could occur with a bottleneck due to climatic fluctuations or activities caused by human factors. The detection of a bottleneck (TPM, $p < 0.001$) (likely because of patchy distribution due to settlements, industrial and agricultural zones, gene flow, and the intensive trade as pet animals) in the *T. h. boettgeri* population in Türkiye explains the deviation from the *HWE* and low genetic diversity.

The heterozygous deficiency with significant differences in the mean F_{IS} value was detected in Clusters 1 and 2, created with Structure analysis and the UPGMA distance

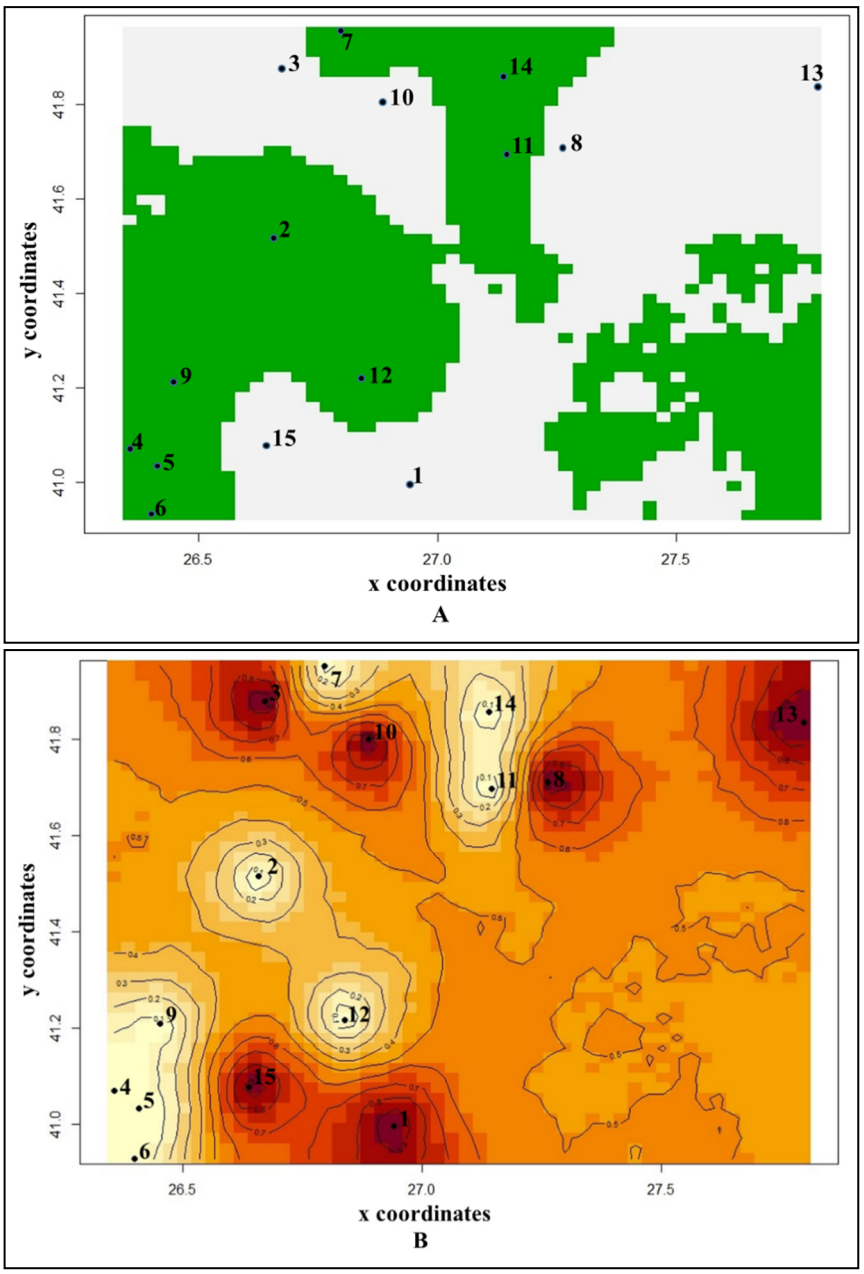

Figure 4. Maps of the population clusters (K) identified by GENELAND. (A) Map spatial distribution of each group defined, K = 2. (B) Map of the posterior probability defined, K = 2 (the numbers symbolize the sampling localities, the colors in A and B symbolize the clusters inferred in STRUCTURE).

tree, as in Corsica, Var1, and Var2 (*T. h. hermanni*, France), Ganfaron (*T. h. boettgeri*, France), and Alberta and Minorca (*T. h. hermanni*, Spanish) (Table 3). A possible recent founder effect may explain the substantial significance in the F_{IS} value (Zenboudji et al., 2016). This would suggest that these populations are of a recent origin. These populations could have formed due to habitat degradation and historical climatic conditions interrupting the gene flow. The founder effect causes genetic drift, leading to the loss of genetic diversity in a new population created by a minimal number of individuals with low heterozygosity from a larger population. Detecting a bottleneck in *T. h. boettgeri* supported this recent founder effect.

Table 3. Genetic diversity indices of the present study and other *T. hermanni* populations (Zenboudji et al., 2016).

	Populations		N	*Ar*	*Ho*	*He*	F_{IS}
T. h. hermanni	Italy	Puglia	14	3.40	0.41	0.39	0.02
		Abruzzo	6	3.20	0.56	0.47	−0.04
		Tuscany	12	3.60	0.45	0.46	−0.06
		Sicily	11	3.40	0.38	0.38	0.05
		Sardinia	24	4.30	0.39	0.40	0.03
		Overall	67	3.58	0.44	0.42	
	France	Corsica	73	4.40	0.34	0.39	0.1***
		Var 1	60	2.60	0.24	0.25	0.11***
		Var 2	16	2.00	0.22	0.24	0.11*
		Overall	149	3.50	0.27	0.29	
	Spain	Albera	30	2.00	0.17	0.22	0.2***
		Ebro Delta	31	3.90	0.41	0.40	−0.01
		Minorca	75	3.30	0.33	0.38	0.14***
		Overall	136	3.60	0.30	0.33	
	France	Gonfaron	15	4.50	0.43	0.49	0.15*
T. h. boettgeri	Present study	Cluster 1	99	2.53	0.28	0.33	0.15***
		Cluster 2	122	2.34	0.29	0.32	0.10***
		Overall	221	2.39	0.29	0.31	

*p < 0.05; **p< 0.01; ***p < 0.001.

4.2. Structure of the localities

The UPGMA distance tree created using the pairwise F_{ST} values of 15 the localities was divided into 8 groups with 2 significant clusters (Figure 2). The localities of *T. h. boettgeri* showed patchy distribution due to dense regions of agricultural fields, industrial zones, and settlements. Therefore, the distances between some localities were pretty large (for example, the distance between Localities 6 and 13 was 153.9 km, Figure 1). The genetic distance matrix (F_{ST}) and the geographic distance matrix were not compatible (r = 0.38, p > 0.05), which may have been due to the recent separation of the localities from each other and, hence, their complete differentiation.

All of the pairwise comparisons of the identified groups exhibited significant differences (Table 2). According to the Structure and Geneland analysis results, the most appropriate K value was 2 (K = 2) (Figures 3b and 4). This result supports the 2 large clusters in the UPGMA distance tree (Figure 2). The observed genetic structure in the groups reflects a weak genetic structure with discernable population differentiation. While groups 1–4 were located in the first cluster, groups 5–8 were in the second cluster (Figures 2 and 3b). Gene flow was interrupted among these clusters because of dense agricultural fields, industrial zones, and settlements, especially between clusters 1 and 2. As a result, the population of *T. h. boettgeri* in Türkiye constitutes 2 independent genetic units, and the 8 groups are weakly differentiated.

4.3. Identification of the MUs

The complex evolutionary history of mtDNA and microsatellite markers was used to identify conservation units (Salinas et al., 2011). The loss of the genetic diversity that resulted in the extinction of genetically differentiated populations brings about the destruction of evolutionary diversity in a conservation context.

This study revealed for *T. h. boettgeri* a clear population structure with crucial implications for conservation with low levels of genetic diversity and *Ar*. To preserve genetic diversity, 2 large clusters (cluster 1: groups 1–4; cluster 2: groups 5–8) should be treated as independent MUs (Moritz, 1994).

In conclusion, many polymorphic microsatellite markers with more samples (221) were used. It was found that 12 of the studied localities and 8 of the studied loci have diverged from the *HWE*. Genetic diversity and *Ar* were low in the whole population and at the studied localities. The whole population showed evidence of a recent bottleneck. The studied localities were divided into 8 groups, and the pairwise comparison indicated genetic structuring with significant differences in all of the pairwise comparisons.

The populations of *T. h. boettgeri* were split into 2 main clusters, and these clusters can be proposed as 2 MUs, as the most relevant for conservation purposes.

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