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Orijinal araştırma (Original article)

PCR-RFLP variation of the oak gall wasp, *Andricus quercustozae* (Bosc, 1792) (Hymenoptera: Cynipidae) from Turkey[1]

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Summary

Oak gall wasp specimens collected in the summer of 2006-2008 from Turkey were studied using PCR-RFLP method for revealing intraspecific mitochondrial DNA variation of *Andricus quercustozae* (Bosc, 1792). A total of 28 haplotypes were detected among 95 individuals collected from 16 populations. The estimated average haplotype and nucleotide diversity were 0.45 and 0.05, respectively. The highest nucleotide divergence estimate among the analyzed oak gall wasp populations was 9.0% indicating an ancient split between *A. quercustozae* haplotypes.

Dendrogram obtained using PHYLIP program indicated that there was a significant relationship between geographical distribution of haplotypes and their clustering. AMOVA analysis for estimation of the partitioning of genetic differentiation was statistically significant. This study shows that *A. quercustozae* populations have high amount of genetic diversity and form geographically significant groupings. It seems that varied topography and evolutionary history of Turkey play an important role in shaping the current population genetic structure of the species.

Key words: *Andricus quercustozae*, Cynipidae, oak gall wasp, PCR-RFLP

Anahtar sözcükler: *Andricus quercustozae*, Cynipidae, meşe mazı arısı, PCR-RFLP

Introduction

Galls are abnormal growths composed of plant tissues caused by a variety of organisms including insects. Oak gall wasps (Hymenoptera: Cynipidae: Cynipini) is the second largest insect group with 1300 identified species preferring different oak species for gall formation (Ronquist & Liljeblad,

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2001). *Andricus* is a Holarctic genus of oak cynipid wasps and 80 species are known from western Palearctic, particularly from Europe and Turkey (Melika et. al., 2004). *Andricus quercustozae* (Bosc, 1792) is a widely distributed oak gall wasp species extending from Morocco through Turkey to Iran (Rokas et al., 2003). Brownish hard-shelled and unilocular asexual generation galls are formed by *A. quercustozae* on buds of oak trees in the late summer and early fall.

Mitochondrial DNA (mtDNA) has been extensively used as a molecular marker in various animal groups including gall wasps (Hayward & Stone 2006; Challis et al., 2007; Stone et al., 2007). Small size, relatively rapid rate of evolutionary change, maternal inheritance and the absence of recombination make mtDNA an ideal marker for diverse evolutionary studies including phylogenetic inference, identification of species origin, phylogeography, analysis of population history and structure (Avise, 2000, 2006). Associated with PCR amplification restriction fragment length polymorphism (RFLP) has been effectively used for phylogenetic and phylogeographic studies and revealed extensive intraspecific variation in a number of animal species (Martin & Simon, 1990; Fransisco et al., 2001; Bardakçı et al., 2006).

Turkey is one of the most important regions in the Mediterranean due to not only for its species richness but also its high genetic diversity. The reasons for this were explained by very complicated geologic history of Anatolia, heterogenous topography, varied climates, the presence of diverse phytogeographical regions, and its location between Europe and Asia (Çıplak et al., 1993; Avcı, 1993; Çıplak, 2003, 2004; Korkmaz et al., 2010). Turkey is also important for the recolonization processes of Europe after the last glacial cycles of Pleistocene (Hewitt, 1996, 1999, 2000). Furthermore, a large scale study on *Andricus quercustozae* showed that Anatolia is genetically distinct with refuge specific haplotypes and accepted as the center of genetic diversity for this species, with the Turkish lineages being sources to more western European populations. The greatest nucleotide diversity was observed in Turkey followed by the lower diversity and divergence estimates in the Balkans, Italy and Iberia. Moreover, as in this study a major genetic divide was observed between northeastern and southwestern lineages of *A. quercustozae* (Rokas et al., 2003).

In the present study, PCR-RFLP analysis of two mtDNA regions of *A. quercustozae* collected from most of its distribution range in Turkey was employed as a tool for assessing the levels of genetic diversity of the species across the sampled populations. Some implications for the current population structure and the effects of the varied topography of Anatolia shaping the current genetic structure of the studied species were discussed in detail.

Material and Methods

Sampling and laboratory protocols

A total of 95 specimens of *A. quercustozae* were collected in the summer of 2006-2008 from 16 localities covering most of the distribution range of the species in Turkey. The locations of collection sites are shown Table 1.

Total genomic DNA using the DNeasy Tissue Kit (QIAGEN) was isolated from single gall wasp individuals. The extracted DNA samples were checked by gel electrophoresis using % 1 agarose (Sigma) containing ethidium bromide with 1 × TBE running buffer (0,089 M Tris, 0,089 M Boric acid, 0,001 M disodium EDTA). Two mtDNA gene fragments were used for PCR-RFLP analysis; a 2540 bp mitochondrial DNA region covering ND4, ND6, Cyt B gene and a second 1800-bp fragment comprising the ATPases (6, 8) and COIII genes (Simon et al., 1994; Moretto & Arias 2005). All PCR reactions were carried out in 25µl volumes containing 0.5µl of the total DNA extraction, 2.5µl 10X PCR buffer (Promega), 2.0µl $MgCl_2$ (25mM), 1.0µl dNTPs (2mM each), 0.75µl of each primer (20µM) and 1.25 U of Taq DNA Polymerase (Promega) (Moretto & Arias 2005; Mutun, 2010). Thermocycling was performed following the steps given in Moretto & Arias (2005). Restriction digestion of the amplified mtDNA regions were performed with HindIII and HinfI, Hae III, HindIII and EcoRI (MBI Fermentas and TAKARA) restriction enzymes according to the manufacturer's instructions. Restriction fragments were separated by electrophoresis in 1% agarose gels containing ethidium bromide with 1 × TBE running buffer, visualized under UV light and photographed.

Data analysis

For both mtDNA fragments, variable restriction patterns for each enzyme were alphabetically designated as they were encountered. Haplotype and nucleotide diversity for each population were calculated based on restriction sites between all haplotypes (Nei & Li, 1979). Divergence among populations was estimated using the DA program contained in the software REAP (McElroy et al., 1991). The data were bootstrapped with 1000 replicates using the PHYLIP SEQBOOT program (Felsenstein, 1992). Unrooted DOLLO parsimony trees were constructed using the PHYLIP DOLLOP program. The majority rule consensus tree was generated using the PHYLIP CONSENSE program from these trees. The degree of geographic heterogeneity of mtDNA haplotype distributions was assessed using χ^2 statistics. Partitioning of genetic diversity among populations was estimated by analysis of molecular variance (AMOVA) using ARLEQUIN 3.1 (Excoffier et al., 2005). The partitioning of the variation was tested at three levels as i) among groups, ii) among populations within groups, and iii) within populations (Excoffier et al., 2005).

Table 1. Localities with their abbreviations and coordinates of the sampled populations

Population	Coordinates
Adıyaman (ADI)	N 37° 45.869' E 37° 43.136'
Aksaray (AKS)	N 38° 09.931' E 34° 11.463'
Balıkesir (BAL)	N 39° 35.623' E 27° 04.082'
Bitlis (BİT)	N 38° 21.019' E 42 ° 02.412'
Bolu (BOL)	N 40° 40.380' E 31° 25.991'
Çanakkale (ÇAN)	N 39° 53.213' E 26° 11.747'
Çankırı ÇKR)	N 40° 32.138' E 32° 38.418'
Elazığ (ELA)	N 38° 29.886' E 39° 22.599'
Kırklareli (KIR)	N 41° 56.657' E 27° 40.765'
Muş (MUŞ)	N 38° 36.793' E 41° 56.529'
Tekirdağ (TEK)	N 41° 25.829' E 28° 05.673'
Van (VAN)	N 37° 55.015' E 42° 57.828'
Kahramanmaraş (KAH)	N 37° 43.514' E 36° 40.038'
Bayburt (BAY)	N 40° 05.385' E 40° 25.327'
Sivas (SİV)	N 39° 56.082' E 37° 51.828'
Kayseri (KAY)	N 38° 40.512' E 36° 19.973'

Results and Discussion

A total of 28 composite haplotypes were detected from all specimens of *Andricus quercustozae* (Bosc, 1792) collected from 16 populations. The mtDNA fragments from 95 individuals had 67 recognition sites for the restriction enzymes used in this study representing 270 nucleotides. The present analysis of PCR-RFLP site variation of two mtDNA regions of *A. quercustozae* is in accordance with other Hymenopteran species (Fransisco et al., 2001; Moretto & Arias, 2005; Mutun, 2010). The composite haplotypes and their frequencies in each studied population are shown in Table 2. The most common haplotype was Type 6 detected in 21 individuals from 5 populations followed by Type 8 was found in 8 individuals from 2 populations.

Haplotype and nucleotide diversity of each *A. quercustozae* population is given in Table 3. The average haplotype diversity for the studied populations was 0.45. The highest haplotype diversity (1.00) was estimated for the Kahramanmaraş and Bayburt populations followed by the Adıyaman population (0.90). The average nucleotide diversity for the analyzed populations was 0.054. The highest nucleotide diversity (0.152) was observed in the Kahramanmaraş population followed by Adıyaman (0.131) population. Amount of genetic diversity found for *A. quercustozae* species is concordant with the results of a study conducted on the same species (Rokas et al., 2003). Likewise, *A. caputmedusae* showed highest nucleotide and haplotype diversity

in the Kahramanmaraş and Adıyaman populations (Mutun, 2010). Thus, the presence of conspicuously high levels of genetic variation present both in Kahramanmaraş and Adıyaman localities may indicate that this area is not only important for species diversity but also for the genetic diversity because the area is known with its high species/lineage diversity (Çıplak, 2008). This may be a general pattern in Turkey for the oak gall wasp species and requires further studies. Populations with high haplotype and nucleotide diversity may imply the presence of a refuge area (Hewitt, 1996, 1999, 2000; Bennett & Provan, 2008).

Table 2. Composite haplotypes and their frequencies among *Andricus quercustozae* (Bosc, 1792) populations.

Haplotype	Composite Haplotype*	ADI	AKS	BAL	BIT	BOL	CAN	CKR	ELA	KIR	MUS	TEK	VAN	KAH	BAY	SIV	KAY	Total
Type 1	AABBA										1							1
Type 2	ABBBA										4							4
Type 3	ABCBA										1							1
Type 4	ACDBA										1							1
Type 5	ADABA		1															1
Type 6	AEABA		6		1	6				7		1						21
Type 7	AAAAA																2	2
Type 8	ALABA											4					4	8
Type 9	AGABA														1	5		6
Type 10	AFABA			4										1				5
Type 11	AKBBA						1											1
Type 12	AKEBA												2					2
Type 13	AEEBA				7													7
Type 14	AEFBA											2						2
Type 15	AEGBA					2												2
Type 16	AEHBA					3												3
Type 17	AFBBA						6											6
Type 18	AFDBA		1	1														2
Type 19	AFFBA			1														1
Type 20	AHABA							6										6
Type 21	AHACA								4									4
Type 22	AIAAA								2									2
Type 23	AJAAA	2																2
Type 24	BKABA	1																1
Type 25	BKDBA	1																1
Type 26	BKEBA	1																1
Type 27	BGEBA													1				1
Type 28	AGDBA														1			1
A.C.H	BBBBB																	2
A.L.H	BBBBB																	2
Sample Size		5	8	6	8	11	7	6	6	7	7	7	2	2	2	5	6	95

* Composite haplotypes detected in outgroups are also given as A. C. H and A. L. H. Composite haplotypes based on digestion pattern of restriction enzymes shown by capital letters are given in the following order for both mtDNA fragment: HindIII and HinfI, Hae III, HindIII, and EcoRI respectively

Table 3. Haplotype and nucleotide diversity calculated for the *Andricus quercustozae* (Bosc, 1792) populations with mean +/- S.E

Population	Haplotype Diversity	Nucleotide Diversity
Adıyaman	0.9000+/- 0.16100	0.131257
Aksaray	0.4643+/- 0.20001	0.043322
Balıkesir	0.6000+/- 0.21517	0.129451
Bitlis	0.2500+/- 0.18020	0.013416
Bolu	0.6545+/- 0.11148	0.059503
Çanakkale	0.0000+/- 0.00000	0.000000
Çankırı	0.0000+/- 0.00000	0.000000
Elazığ	0.5333+/- 0.17213	0.077950
Kırklareli	0.0000+/- 0.00000	0.000000
Muş	0.7143+/- 0.18090	0.095328
Tekirdağ	0.6667+/- 0.15983	0.072608
Van	0.0000+/- 0.00000	0.000000
K.Maraş	1.0000+/- 0.50000	0.152941
Bayburt	1.0000+/- 0.50000	0.070948
Sivas	0.0000+/- 0.00000	0.000000
Kayseri	0.5333+/- 0.17213	0.032437
Average	**0.4543+/- 0.00856**	**0.054948+/- 0.0001716**

The nucleotide divergence between the populations calculated using the DA program of REAP ranged from 0.001 to 9.0% (Table 4). Based on pair-wise comparisons of nucleotide divergence of *A. quercustozae* populations, Kırklareli and Bayburt populations are the most diverged populations (9.0%). Likewise, Muş and Çanakkale populations are diverged with 8.3% and Balıkesir is diverged from Kayseri population with 8.2%. The divergence estimates between pairs of populations in this study may well correlate with the geographic distances of the populations (Neigel & Avise, 1993).

A neighbor-joining tree resulting from the distance matrix among all mtDNA haplotypes including the divergence estimates between haplotypes is presented in Figure 1 with associated bootstrap values (>50 are shown on the branches). Dendrogram depicts three distinct groupings with respect to the geographic location of populations as A, B and C. The A clade includes Type 6 at the most basal position and found as the most common haplotype in five different locations. Furthermore, Type 10 and 12 are grouped together represented by the individuals from Kahramanmaraş-Balıkesir and Van populations, respectively. Type 9 shared between the Bayburt and Sivas populations are grouped with Type 5 from the Aksaray population. Type 22 from Elazığ and Type 18 shared between Aksaray and Balıkesir also make a small

group. Haplotype 8 from Kayseri and Tekirdağ is basal to this small group and further Type 7 from the Kayseri population is another basal haplotype in this cluster. The B group named as Western Clade comprises haplotypes only from westerly located populations including haplotypes from Tekirdağ, Balıkesir, Bolu, Çankırı and Çanakkale populations. On the other hand, C cluster named as Eastern clade due to the presence of those haplotypes represented only by easterly located populations from Muş, Elazığ Adıyaman, Bayburt, Bitlis and Kahramanmaraş localities.

A majority-rule consensus tree generated from *A. quercustozae* mtDNA haplotypes showed a similar branching pattern with the neighbor-joining dendrogram given in Figure 1; therefore it is not presented here. Haplotypes from different populations constituted three main groupings into eastern, western and eastern/western cluster. For many species it has been shown that the phylogeny of mtDNA haplotypes corresponds well to the geographical distribution of populations and geographical barriers have been found to shape current distribution of the lineages (Avise, 2000). Present study indicates the presence of an obvious correlation between the genetic relationships among mtDNA types to their geographical distribution into roughly east and west with an additional mixed groups of haplotypes. Complex geologic history of Turkey resulting in both local and larger scale isolation between faunal elements and the Anatolian Diagonal were accepted as the major geographic barriers dividing species/lineage distribution (Çıplak et al., 1993). An east-west separation (Figure 1) may support a significant geographic pattern considering the Anatolian Diagonal and fits well in most parts with the results of this study. The presence of a mixed group of haplotypes represented by the eastern/western populations may indicate that the species was widespread in Turkey for long time because under a commonly accepted phylogeographic approach more widely distributed haplotypes are ancestral when compared to the derived haplotypes that show more restriction in their distribution (Slatkin, 1991; Crandal & Templeton, 1993).

AMOVA analysis for estimating the extent of genetic differentiation at different hierarchical levels revealed highly significant amount of genetic variation (35.62%, $P < 0.001$) within populations and a significant partitioning of variance (25.50%) was found among groups (Table 5).

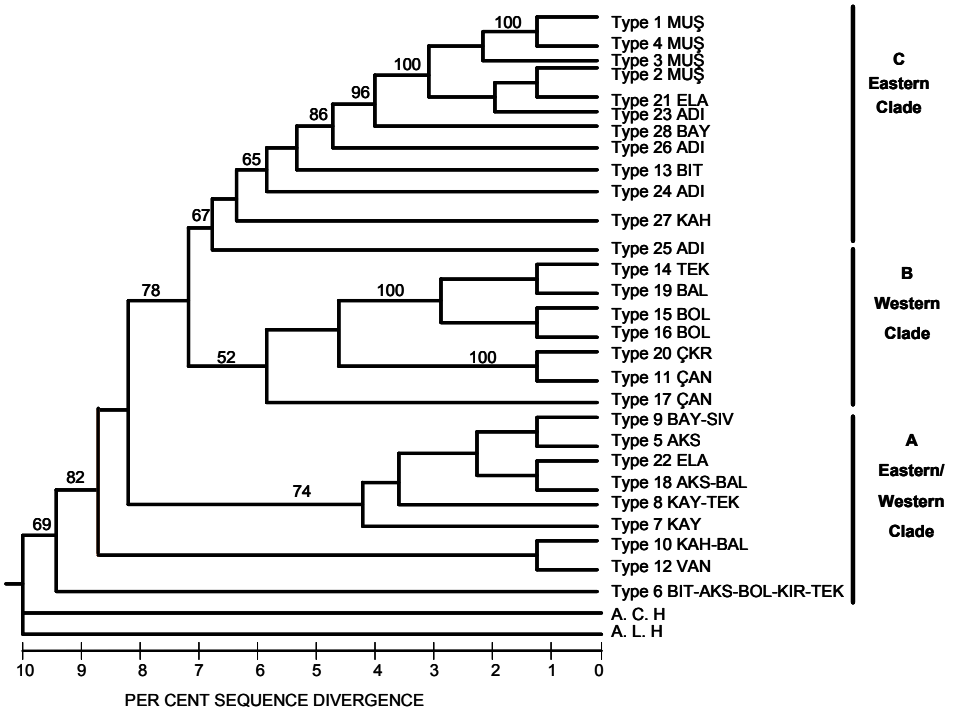

Figure 1. Neighbor-joining dendrogram of the 28 haplotypes of *Andricus quercustozae* (Bosc, 1792). Numbers above the branches represent the bootstrap values 1000 replicates of the restriction fragment data between the haplotypes. Support values <50% are not represented.

On the other hand, 38.88% of genetic variance was present within groups indicating that each population developed its own genetic variation through time in addition to having an obvious differentiation among groups. Current data may also indicate that each population developed its own genetic variation through time (Avise, 2006) in addition to having differentiation among groups which may be due to the effects of the environmental fluctuations occurred in the area.

Analysis of mtDNA PCR-RFLP was useful in inferring phylogeographic relationships of *A. quercustozae* populations of Turkey. The oak gall wasp lineages were well defined according to their geographical origin, and data provided evidence that their contemporary distribution was greatly influenced by major past geological events. More studies on different taxa will facilitate to identify a general pattern in the area and the proposed conclusion drawn in this study can be proven by future and more detailed work.

Table 4. Pair-wise nucleotide diversity (above diagonal) and net nucleotide divergence (below diagonal) among the populations of *Andricus quercustozae* (Bosc, 1792)

	Adıyaman	Aksaray	Balıkesir	Bitlis	Bolu	Çanakkale	Çankırı	Elazığ	Kırklareli	Muş	Tekirdağ	Van	K.Maraş	Bayburt	Sivas	Kayseri
Adıyaman		0.112817	0.13754	0.12325	0.11607	0.15191	0.17746	0.15183	0.10031	0.16077	0.12656	0.16667	0.15866	0.14813	0.11913	0.12956
Aksaray	0.02552		0.10188	0.08147	0.06667	0.10576	0.19734	0.16672	0.02166	0.11314	0.11964	0.10062	0.14883	0.12398	0.06037	0.14557
Balıkesir	0.00719	0.01549		0.11068	0.10977	0.15679	0.20929	0.18314	0.08293	0.16661	0.14577	0.18219	0.13798	0.16308	0.11209	0.16391
Bitlis	0.05092	0.05311	0.03925		0.08036	0.19541	0.22047	0.18097	0.04695	0.15132	0.07106	0.20901	0.15381	0.16926	0.11806	0.11444
Bolu	0.02069	0.01526	0.01529	0.04391		0.13503	0.13917	0.13137	0.04695	0.12361	0.10727	0.12878	0.14239	0.12287	0.06692	0.12103
Çanakkale	0.05477	0.05261	0.06056	0.05719	0.07374		0.19641	0.17423	0.10576	0.16268	0.16869	0.12688	0.21957	0.14451	0.08088	0.15297
Çankırı	0.06818	0.06946	0.06456	0.06376	0.06994	0.03091		0.06295	0.19906	0.22471	0.24588	0.22353	0.22892	0.22353	0.22353	0.25368
Elazığ	0.04722	0.06068	0.07944	0.01359	0.06264	0.01037	0.02394		0.16665	0.20811	0.19717	0.18302	0.19557	0.18302	0.18302	0.20225
Kırklareli	0.03466	0.00025	0.01821	0.04024	0.01701	0.07426	0.06996	0.06276		0.10262	0.09899	0.10732	0.13698	0.12636	0.05366	0.13338
Muş	0.04747	0.04382	0.05421	0.06915	0.04619	0.08351	0.06773	0.06014	0.05995		0.16087	0.15859	0.14515	0.15511	0.10901	0.16361
Tekirdağ	0.02463	0.06116	0.04474	0.02805	0.04121	0.06088	0.06295	0.06218	0.06269	0.07691		0.13861	0.147	0.11524	0.07878	0.06256
Van	0.00103	0.07896	0.06177	0.02029	0.09003	0.00953	0.05235	0.06144	0.00507	0.05193	0.01023		0.13928	0.03099	0.03099	0.09296
K.Maraş	0.01656	0.05071	0.00321	0.07063	0.03616	0.06115	0.06524	0.06022	0.06051	0.02101	0.03422	0.06282		0.12565	0.11201	0.13393
Bayburt	0.04702	0.06685	0.06288	0.06208	0.05764	0.07757	0.06885	0.05085	0.09089	0.07196	0.04347	0.00482	0.01372		0.03547	0.06526
Sivas	0.05351	0.03871	0.04736	0.06135	0.03717	0.04938	0.06635	0.05144	0.05366	0.06134	0.04277	0.03199	0.03555	0.00001		0.05087
Kayseri	0.04771	0.05076	0.08296	0.00951	0.07506	0.05052	0.06274	0.05176	0.05171	0.09073	0.01004	0.07675	0.04125	0.01357	0.03466	

Table 5. Analysis of molecular variance (AMOVA) among the studied oak gall wasp populations. * $P < 0.001$ after 1000 permutations. Va, Vb, and Vc are the associate covariance components

Source of Variation	d.f.	Sum of Squares	Variance Components	Percentage of Variation	Fixation indices
Among groups	4	89.093	0.82780Va	25.50*	F_{CT}=0.25500
Within groups	11	92.877	1.26201Vb	38.88*	F_{ST}=0.64377
Within populations	78	90.201	1.15642Vc	35.62*	F_{SC}=0.52183

Özet

Türkiye' den *Andricus quercustozae* (Bosc, 1792) (Hymenoptera: Cynipidae) mazı arılarının PCR-RFLP varyasyonu

Bu çalışmada ülkemizde bulunan *Andricus quercustozae* (Bosc, 1792)'nin tür içi mitokondriyal DNA çeşitliliği 2006-2008 yılları arasında yapılan arazi çalışmalarında toplanan örneklerle PCR-RFLP yöntemi kullanılarak çalışılmıştır. Onaltı populasyondan toplanan 95 bireyde toplam 28 haplotip belirlenmiştir. Haplotip ve nükleotid çeşitliliği sırasıyla 0.45 ve 0.05 olarak hesaplanmıştır. Analiz edilen populasyonlar arasında en fazla nükleotid farklılaşması % 9.0 olarak hesaplanmıştır bu da *A. quercustozae* haplotipleri arasında oldukça eski bir ayrılmayı ifade etmektedir.

PHYLIP programı kullanılarak elde edilen dendrogram haplotiplerin coğrafik dağılımları ile oluşturdukları gruplar arasında bir bağıntının olduğunu göstermektedir. Genetik farklılaşmanın dağılımını belirlemek için yapılan AMOVA analizi istatistiksel olarak anlamlı çıkmıştır. Bu çalışma *A. quercustozae* populasyonlarının oldukça fazla genetik çeşitliliğe sahip olduğunu ve coğrafik olarak belirli gruplaşmalar yaptığını ortaya koymaktadır. Elde edilen veriler ışığında çalışılan türün populasyon genetik yapısının şekillenmesinde ülkemiz topografyasının ve evrimsel tarihinin etkili olduğu sonucu çıkarılabilir.

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