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Determination of Genetic Diversity in *Apodemus mystacinus* (Mammalia: Rodentia) based on SSRs

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ABSTRACT: The aim of this study is to determine the genetic diversity of species in Rocky mouse, *Apodemus mystacinus*, using SSR (the simple sequence repeat) loci and to reveal the reasons of this variations, if any. In this study, 69 *A. mystacinus* samples were studied collected from 18 localities in Turkey. 7 SSR loci were used to determine the genetic diversity of *A. mystacinus*. As a result of this study, *A. mystacinus* includes 2 genetic groups that indicate the presence of two subspecies as *A. m. mystacinus* that is distributed in western Anatolia and *A. m. euxinus* in eastern Anatolia. This result also supports that one of the micro refugium areas is eastern Turkey and the other western and southern Turkey.

Keywords: *Apodemus mystacinus*, microsatellite, Turkey

*Apodemus mystacinus'*un (Mammalia: Rodentia) Genetik Çeşitliliğinin SSR ile Belirlenmesi

ÖZET: Bu çalışmanın amacı, SSR (basit dizi tekrarları) lokuslarını kullanarak kayalık faresinin, *Apodemus mystacinus*, genetik çeşitliliğini belirlemek ve varsa bu çeşitliliğin nedenleri ortaya koymaktır. Bu çalışmada, Türkiye'deki 18 lokaliteden toplanan 69 *A. mystacinus* örneği çalışılmıştır. *A. mystacinus*'un genetik çeşitliliğini belirlemek için 7 SSR lokusu kullanılmıştır. Bu çalışmanın sonucuna göre *A. mystacinus*, iki alttürün varlığını gösteren 2 genetik grup içermektedir; Anadolu'nun batısında *A. m. mystacinus* ve Anadolu'nun doğusunda *A. m. euxinus*. Bu sonuç aynı zamanda mikro sığınak alanlarından birinin Türkiye'nin doğusunda, diğerinin ise batısında ve güneyinde olduğunu desteklemektedir.

Anahtar kelimeler: *Apodemus mystacinus*, mikrosatellit, Türkiye

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INTRODUCTION

The rocky mouse, *Apodemus mystacinus*, is one of the rodent species widely distributed in Turkey. This species lives in rocky areas covered with forests reaching up to a height of 2700 m (Çolak et al., 2004). *A. mystacinus* can be easily separated from the other European *Apodemus* by different characters such as its larger body size and dark grey fur coloration (Michaux et al., 2005).

Although four subspecies were described as *A. m. mystacinus* Danford and Alston (1877) from Sebil, Turkey; *A. m. smyrnensis* Thomas (1903) from western Turkey; *A. m. rhodius* Festa (1914) from Rhodes and Crete; and *A. m. euxinus* Allen (1915) from Altındere, Turkey, the distribution areas and the validities of these subspecies are controversial.

According to some authors, *A. m. smyrnensis* is one of the subspecies that lives in western Anatolia (Çolak et al., 2007; Olgun et al., 2009; Olgun Karacan et al., 2015). Neuhauser (1936) reported that *A. m. smyrnensis* was distributed in western Turkey and in the Taurus mountains using morphological data. Çolak et al. (2007) suggested that İzmir, Aydın and Bursa specimens of *A. mystacinus* were different from the other specimens collected from Anatolia based on esterase variations and researchers claimed that these western specimens might be *A. m. smyrnensis*. The differentiation of the specimens that was distributed in western Turkey was supported using RAPD (Olgun et al., 2009) and mtDNA-RFLP (Olgun Karacan et al., 2015) variations. On the other hand, some researchers have claimed that *A. m. symrnensis* is synonymous with *A. m. mystacinus* based on morphological, biometrical, karyological, studies and bacular, phallic differentiations (Ellerman, 1948; Ellerman and Morrison-Scott, 1951; Çolak et al., 2004).

The other controversial subspecies is *A. m. euxinus* (Allen, 1915), and while some studies indicated that northern or northeastern Anatolia is the distribution area of this subspecies using morphological data (Neuhaser, 1936) and esterase variations (Çolak et al, 2007), others claimed the homogeneity of northeastern and eastern Anatolian populations depend on biometric measurements (Çolak et al., 2004) and genetic data Olgun et al., 2009; Olgun Karacan et al., 2015).

Michaux et al. (2005) has revealed that there is a different lineage that is distributed in southwestern Turkey and Crete using molecular data, and researchers has called these specimens as *A. m. rhodius*. Similarly, Olgun et al. (2009) has showed two different lineages in western Turkey based on RAPD markers, and one of them has included Muğla specimens in the southwestern part. However, the validity of *A. m. rhodius* hasn't been supported by morphological (Ellerman, 1948; Vohralik et al., 2002; Çolak et al., 2004) and mtDNA-RFLP studies (Olgun Karacan et al., 2015).

Major fluctuations in the climate during late Pliocene and Pleistocene have affected populations (Hewitt, 1996). Also, the formation of biodiversity is directly related to climate change (Ficetola et al., 2017). Jandzik et al. (2018) have claimed that the climatic changes that occurred during the Pleistocene played important roles in shaping the diversity of Western Palaearctic biota and its present distribution. The refuges of the glacial periods during Pleistocene are the most important factors that clarify the biogeographic patterns in present day in Europe (Schmitt, 2007; Svenning and Skov, 2007). Turkey is one of the refugia including mountain ranges such as Anatolian Diagonal, and Taurus and Black Sea mountains and many seaways (Black, Aegean, and Mediterranean seas) that inhibited gene flow between populations (Bilgin, 2011). Especially, Anatolian Diagonal has acted as an important biogeographical barrier by dividing the lines or species into eastern and western parts (Hewitt,

1999, 2000; Veith et al., 2003; Çıplak, 2003, 2004; Mutun, 2010; Bilgin, 2011; Plötner et al., 2010; Kapli et al., 2013; Özdemir et al., 2014; Allegrucci et al., 2017; Riemsdijk et al., 2017; Yiğit et al., 2017).

DNA phylogenies may particularly be useful to present lineages, subspecies, and species (Hewitt, 1996). Animal mtDNA and nuclear genes are useful tools to understand the evolutionary history of these lineages, especially in Pleistocene (Avise et al., 1998; Michaux et al., 2002, 2003, 2004; Jaarola et al., 2004; Suzuki et al., 2003, 2004, 2008; Hewitt 2004; Deffointaine et al., 2005, 2009; Macholan et al., 2007; Hürner et al., 2010; Avise and Tatarenkov, 2012; Çolak et al., 2016). Microsatellite has high polymorphism, high and rapid mutation rate (10^{-3} – 10^{-4}) and small repetitive regions in genome and they are preferred to reveal differentiations between closer populations (Chiappero et al., 2011; Burgos-Pas et al., 2011; Gortat et al., 2013). Thus, in the present study we used microsatellites: (1) to determine the relationships among *A. msytacinus* specimens in Turkey, (2) to define the validity of the subspecies, (3) to explain the reasons of the speciation in *A. mystacinus*.

MATERIAL AND METHODS

A total of 69 specimens were collected from 18 different Turkish localities (Figure 1). Total DNA was extracted from kidneys by CTAB method (Doyle and Doyle 1991). The 7 microsatellites primers (GTTD8S, GTTF9A, GACAA12A, GACAD1A, GATAE10A, GTTD9A, MSAF-8) were chosen to amplify (Gockel et al., 1997; Makova et al., 1998). Primers were labelled by using 2 different fluorescent dyes, HEX and 6-FAM. Polymerase chain reaction (PCR) were carried out in final volumes of 25ul with 80 ng of the DNA samples (Çolak et al., 2016).

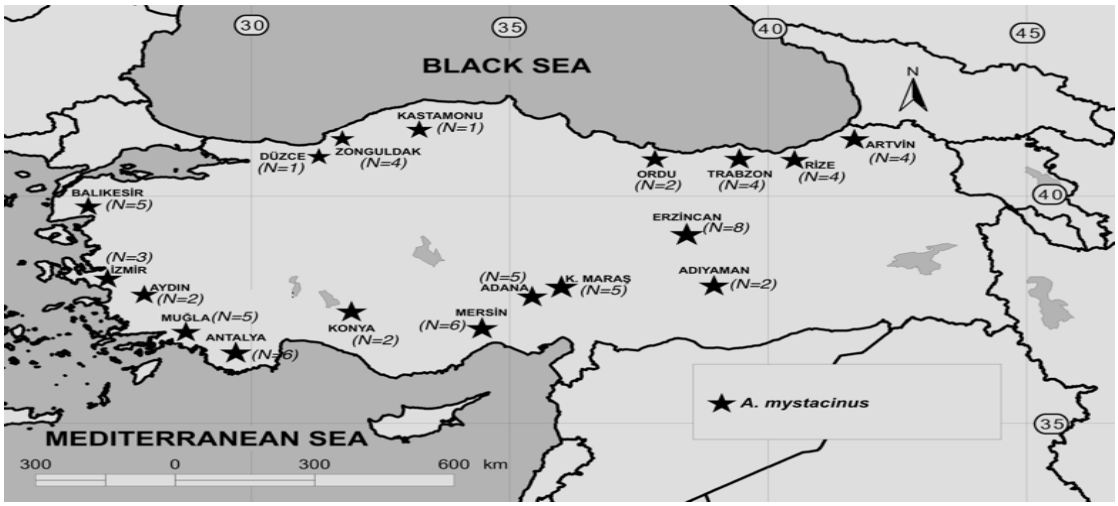

Figure 1. Collected areas and the number of specimens (N)

Amplification of microsatellite loci was performed as: an initial denaturation step at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 55-60 °C for 30-45 s, and extension at 72 °C for 45 s-1 min. After PCR, products were visualized on 2% agarose gel and analysed on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Electropherograms were evaluated using the Applied Biosystems Peak Scanner program (http://www. appliedbiosystems.com).

Firstly, microsatellite profiles were tested for the presence of null alleles to eliminate the scoring errors with FreeNA software (Chapuis and Estoup, 2007). Genetic polymorphism within specimens was determined as the mean

number of alleles per locus (A), polymorphic loci (P%), and observed (Ho) and expected heterozygosity (He) using GENETIX software version 4.05.2 (Belkhir et al., 2004). This program was also used to calculate allelic richness (AR). In addition, genetic structure was examined using the factorial correspondence analyses (FCA) of individual differentiation in GENETIX software. Private allelles and frequencies are calculated with GenAlex 6.5 (Peakal and Smouse, 2012). The degrees of genetic differentiation (FST) values and genetic distance (D_C) (Cavalli-Sforza and Edwards, 1967) among groups were analysed using FreeNA software. Neighbour-Joining (NJ) tree was constructed by MEGA 6.06 software using genetic distance data (D_C) (Tamura et al., 2013). The Analysis of Molecular Variance (AMOVA) test was executed for 2 different groupings on phylogenetic tree using ARLEQUIN v. 3.5 (Excoffier and Lischer, 2010). Bayesian structure analysis was run using STRUCTURE 2.1 (Pritchard et al.,2000; Falush et al., 2007). The result of STRUCTURE software was K that was indicated the number of populations calculated by allele frequencies.

RESULTS AND DISCUSSION

Initially, to determine the reliability of the microsatellite data set, the null (silent) allele frequencies for each population and locus were calculated. 0.2 and above are considered as invalid since the frequency values (r) will create problems for statistical analysis. Therefore, we didn't use the 4.7% of the performed tests (six out of 126) for the analysis.

The analysis of 7 microsatellite loci in 69 *A. mystacinus*, from 18 locations showed low levels of variability. The mean number of alleles (A) ranged from 1.00 (Kastamonu) to 2.8571 (Erzincan) and allelic richness (AR) from 7.00 (Kastamonu) to 9.78 (Erzincan) (Table 1).

Ho and He values were almost the same at different localities. The observed and expected heterozygosity values varied from 0.00 (Kastamonu) to 0.2857 (Ordu) and 0.00 (Kastamonu) to 0.3713 (Erzincan) (Table 1). The mean number of polymorphic loci (P%), and observed (Ho) and expected heterozygosity (He) were found to be significantly different between two groups. Besides, heterozygosity was higher than expected, that we might suspect an isolate-breaking effect (the mixing of two previously isolated populations) between the eastern and western groups of *A. mystacinus*.

Genetic relationships among populations were calculated by pairwise FST. The FST values ranged from -0.1185 (between Düzce and İzmir) to 0.7261 (between Konya and Kastamonu). FST values were indicated the possibility of 2 subpopulations among all samples. The number of clusters (K) varied from K= 1 to 7, the highest number of clusters supporting these two subpopulations was 2 (Figure 2).

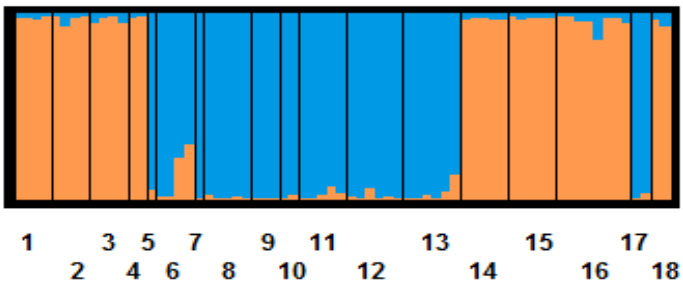

Figure 2. Bayesian structure analysis of individual genotypes in *A. mystacinus* samples* *1: Artvin. 2: Rize. 3: Trabzon. 4: Ordu. 5: Kastamonu. 6: Zonguldak. 7: Düzce. 8: Balıkesir. 9: İzmir. 10: Aydın. 11: Muğla. 12: Antalya. 13: Mersin. 14: Adana. 15: Maraş. 16: Erzincan. 17: Konya. 18: Adıyaman

Table 1. The overall level of variability in the 18 localities.

Group	Location	He	H	Ho	P	A	np	AR
	Ardanuç-Artvin	0.2659	0.3088	0.381	0.5714	1.8571	0	8.465
	Çamlıhemşin-Rize	0.2679	0.3061	0.4286	0.5714	1.7143	0	9.000
	Altındere-Trabzon	0.2589	0.2959	0.3214	0.5714	1.7143	0	8.467
	Gürgentepe-Ordu	0.2857	0.381	0.5	0.5714	1.7143	0	9.032
1 East	Feke-Adana	0.3571	0.3968	0.4286	0.5714	2.5714	0	9.500
	Püren Geçidi-Kahramanmaraş	0.3057	0.3397	0.4	0.5714	2.1429	0.1	9.756
	Kemaliye-Erzincan	0.3713	0.3971	0.3699	0.8571	2.8571	0	9.780
	Nemrut-Adıyaman	0.2857	0.381	0.4286	0.5714	1.7143	0	9.697
	Kastamonu	0	0	0	0	1	0	7
	Mean	**0.266**	**0.311**	**0.362**	**0.540**	**1.80**	**0.011**	**9.182**
	Zonguldak	0.183	0.2092	0.1786	0.4286	1.7143	0	9.000
	Akçakoca-Düzce	0.1429	0.2857	0.2857	0.2857	1.2857	0	8.467
	Kaz Dağı-Balıkesir	0.1886	0.2095	0.2571	0.2857	1.8571	0	9.032
	Kemalpaşa-İzmir	0.2361	0.2905	0.2143	0.7143	1.7143	0	9.500
2 West	Buharkent-Aydın	0.2679	0.3571	0.2857	0.5714	1.7143	0	9.756
	Köyceğiz-Muğla	0.3543	0.3937	0.3714	0.7143	2.7143	0.1	8.000
	Beyşehir-Konya	0.1071	0.1429	0.1429	0.2857	1.2857	0	9.697
	Çığılıkara Milli Parkı-Antalya	0.3532	0.3853	0.2619	0.7143	2.4286	0	9.531
	Sebil-Mersin	0.3313	0.3615	0.2143	0.7143	2.5714	0.083	9.049
	Mean	**0.270**	**0.327**	**0.285**	**0.583**	**2.232**	**0.021**	**9.115**

The phylogenetic analyses and the FCA indicate the existence of two lineages of rocky mouse in Turkey (Figure 3 and 4). The first lineage (the western Turkey) includes the specimens from Kastamonu, Zonguldak, Düzce, Balıkesir, İzmir, Aydın, Muğla, Konya, Antalya and Mersin. The second one (the eastern Turkey) comprises the specimens of Artvin, Rize, Trabzon, Ordu, Adana, Kahramanmaraş, Erzincan and Adıyaman. These lineages indicate two subspecies of *A. mystacinus* in the literature as: *A. m. mystacinus* (western Turkey) and *A. m. euxinus* (eastern Turkey). The SSR loci weren't useful to support the validity of *A. m. smyrnensis* that was claimed to live in western Turkey.

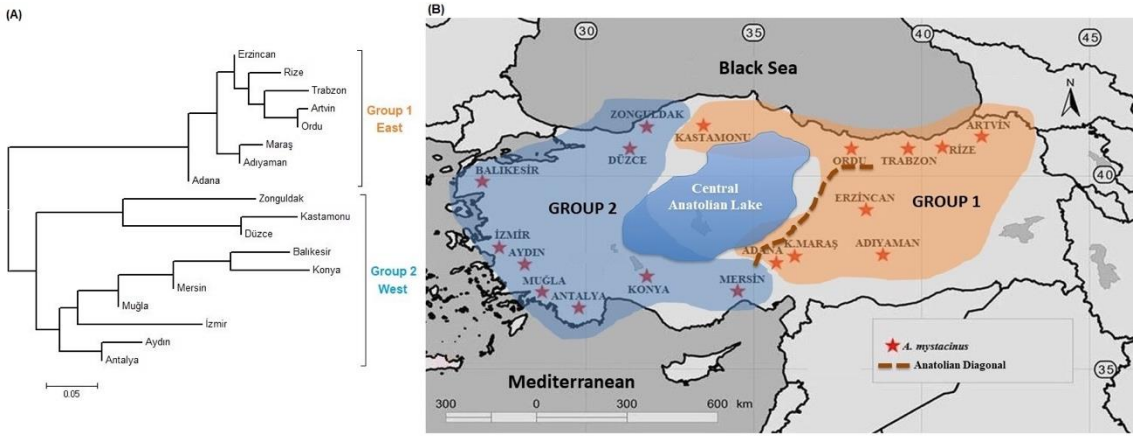

Figure 3. The unrooted Neighbour-Joining tree (A), and the distribution patterns and the isolation barriers of the groups in Anatolia (B)

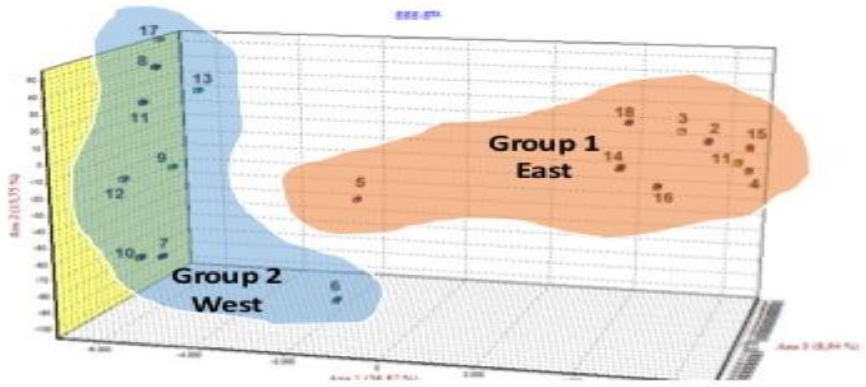

Figure 4. The Factorial Correspondence Analysis of *A. mystacinus* indicated two Turkish groups

Two of 4 new private alleles were determined in the Group 2 (western turkey) (Table 2). The less frequency of private alleles in *A. mystacinus* groups (5%<) indicate the high level of gene flow. Besides, AMOVA displayed these two major groups of *A. mystacinus* namely the most percentage of variations were shared among groups (29.63%) while it was low within groups (10.16%) (Table 3). This result also indicate that the ongoing gene flow between two *A. mystacinus* subpopulations.

Table 2. Distribution and the frequencies of the private alleles

Population	Loci	Allele	Frequency
Muğla	GTTD9A	201	0.100
Mersin	GTTD9A	205	0.083
Kahramanmaraş	GTTF9A	102	0.100
Kahramanmaraş	GACAD1A	150	0.100

Table 3. Analysis of molecular variance between *A. mystacinus* subpopulations

Source of variation	DF*	Sum of squares	Variance components	Percentage of variation (%)
Among groups	1	41.178	0.55442 Va	29.63
Among populations within groups	16	40.649	0.19011 Vb	10.16
Within groups	120	135.158	1.12632 Vc	60.2
Total	137	216.986	1.87085	
Fixation index				
F_{ST} 0.39796 Vc (P = 0.00000+-0.00000)				
F_{SC} 0.14441 Vb (P = 0.00000+-0.00000)				
F_{CT} 0.29635 Va (P = 0.00000+-0.00000)				

In this study, we exhibit that the rocky mouse has two lineages and also support the existence of regional variations or refuge areas as western and eastern in Anatolia. Two possible reason could cause this genetic differentiation: the central Anatolian Lake and the Anatolian Diagonal. One of the reasons of these regional variations could be the existence of the central Anatolian lake during the Pliocene (Figure 3). This lake system partitioned Anatolia and this might have interrupted the gene flow among populations around. Additionally, the effect of the Kızılırmak Delta, that was connected with central Anatolian lake system in Pliocene (5.3-2.6 Mya), on gene flow among *A. mystacinus* specimens was presented using *Cytb* gene and

RFLP markers (Olgun Karacan PhD thesis, 2013; Olgun Karacan et al., 2015).

The other potential reason is the Anatolian Diagonal extends from northeastern Turkey (Bayburt, Gumushane close) to southwestward and is divided into two branches toward the Mediterranean: Central Taurus Mountains and the Nur Mountains (Gür, 2017). This mountain range is a physical and an ecological barrier between populations in East-West side of it (Gündüz et al., 2007; Mutun, 2010, 2016; Mutun and Atay, 2015; Gür, 2016). Furthermore, Gündüz et al., (2007), found that the two phlogroups of the Anatolian squirrel were located on the Anatolian Diagonal, and that these two phylogroups were likely to have occupied the LGM refuges around the Anatolian Diagonal. Similarly, Mutun (2016) revealed that there were glacial refuges on both sides of the Anatolian Diagonal. In this study, it has been shown that the Anatolian Diagonal serves as a barrier in separating the two lineages of *A. mystacinus* (Figure 3). Regions where genetic diversity is high could be considered as a refuge area that the populations could survive during glacial periods in Pleistocene (Hewitt, 1996; Michaux et al., 2005). Climate and vegetation changes during Plio-Pleistocene caused cooling down and drought in the Mediterranean. Different habitats in low places functioned as glacial refugees and populations in these refugees were differentiated into new lineages during interglacial period. (Hewitt, 1996). Kosswigg (1955), suggested two Anatolian glacial refugia, one in western Anatolia and one in eastern Anatolia, based on non-genetic data. These regional variations was also supported by the genetic and morphometric studies on some animals distributed in Turkey like small mammals and amphibians (Çıplak, 2004; Fritz et al., 2009; Gvozdik et al., 2010; Çolak et al., 2016; İbiş et al., 2017; Gür et al., 2017; Şeker et al., 2018). Gvozdik et al. (2010) revealed that the Anatolian and Caucasus–Caspian were an important Pleistocene refuge for *H. orientalis*. Also, phylogeographic and demographic data suggest Anatolia as an ancient glacial refuge for turtles (Fritz et al. 2009). Furthermore, the existence of different micro refugium areas especially in the Black Sea region were also demonstrated (Fritz et al., 2009). Çıplak (2004), indicated that southern Anatolia was a refuge for Anterastes populations. Çolak et al. (2016) presented the regional refuges in northern Anatolia. Gür et al. (2017) showed that the Taurus Mountains served as a refuge where the grand squirrels of the inhabitants moved to the higher latitudes in warm periods since the Last Glacial Maxima. Moreover, Şeker et al. (2018) has considered Anatolia as a potential glacial refuge in Pleistocene because of the high genetic variability in Turkish water vole populations. In this context, Adana and Erzincan specimens from the eastern side of diagonal and Antalya and Muğla specimens from the western side of the diagonal show higher genetic variability than from the other specimens indicate that these regions might be the potential refuge areas (Table 1).

CONCLUSION

Thus, we achieved two important results in this study: One is the subspeciation of *A. mystacinus*: the SSR loci show the validity of only two subspecies distribute in Turkey: *A. m. mystacinus* and *A. m. euxinus*. The other important result is the supporting the presence of glacial refugium areas in Turkey using SSR loci. More information about the central Anatolian specimens will also help us to clarify rocky mouse distribution and subspeciation process or the hybrid zones where the refugium specimens make contact refugium in Anatolia in future.

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