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Using ISSR markers in determination of genetic relationship between 2*n* = 54 and 2*n* = 60 cytotypes of *Nannospalax xanthodon* (Nordmann, 1840) (Mammalia, Rodentia) from Central Anatolia

Tuba Yağcı*, Eda Şen & Rafig R. Gurbanov

ABSTRACT. A total of 36 samples of 2*n* = 54 and 2*n* = 60 cytotypes of *Nannospalax xanthodon*, distributed in the Central Anatolia region of Turkey, were analyzed for the first time by using inter-simple sequence repeat-polymerase chain reaction (ISSR-PCR) technique. The analysis revealed 112 ISSR bands, 101 of which were polymorphic. Seven ISSR primers ($(AG)_8$ T, $(GGAGA)_5$, $(GACA)_4$, $(TG)_8$ A, $(CAG)_5$ GC, $(CAG)_4$ AC and $(GA)_8$ AC)) were optimized from total 20 primers. $(AG)_8$ T and $(GA)_8$ AC primers were most informative to distinguish cytotypes by producing specific bands for 2*n* = 54 and 2*n* = 60. The cytotypes in genetically close relationships were separated into three different groups by UPGMA cluster analysis, in which, the highest genetic diversity was measured for 2*n* = 60. Our results showed that ISSR markers can be used as a simple and reliable molecular tool, for the estimation of genetic diversity in cytotypes of *Nannospalax* at low range genetic distances.
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KEY WORDS: cytotype, ISSR, *Nannospalax xanthodon*, Turkey.

Tuba Yağcı [tuba.yagci@bilecik.edu.tr], Department of Molecular Biology and Genetics, Faculty of Science and Arts and Biotechnology Application and Research Center of Bilecik Şeyh Edebali University, Bilecik 11230, Turkey; Eda Şen [edasenmbg@bilecik.edu.tr], Department of Molecular Biology and Genetics, Faculty of Science and Arts, Bilecik Şeyh Edebali University, Bilecik 11230, Turkey; Rafig R. Gurbanov [rafig.gurbanov@bilecik.edu.tr], Department of Molecular Biology and Genetics, Faculty of Science and Arts and Biotechnology Application and Research Center of Bilecik Şeyh Edebali University, Bilecik 11230, Turkey.

Использование ISSR маркеров в определении генетической связи между 2*n* = 54 и 2*n* = 60 цитотипами *Nannospalax xanthodon* (Nordmann, 1840) (Mammalia, Rodentia) из Центральной Анатолии

Т. Ягчи*, Е. Шен, Р.Р. Гурбанов

РЕЗЮМЕ. 36 образцов 2*n* = 54 и 2*n* = 60 цитотипов *Nannospalax xanthodon* из Центральной Анатолии, Турция, были впервые проанализированы с использованием ISSR маркеров. Анализ выявил 112 полос ISSR, 101 из которых были полиморфными. Из двадцати ISSR праймеров были выбраны семь: ($(AG)_8$ T, $(GGAGA)_5$, $(GACA)_4$, $(TG)_8$ A, $(CAG)_5$ GC, $(CAG)_4$ AC and $(GA)_8$ AC)). Праймеры $(AG)_8$ T и $(GA)_8$ AC создавали специфические полосы для 2*n* = 54 и 2*n* = 60 и были наиболее информативными для различения цитотипов. По результатам кластерного анализа (UPGMA) генетически близкие цитотипы образуют 3 группы, наиболее высокое разнообразие отмечено для цитотипа 2*n* = 60. Наши результаты показали, что маркеры ISSR можно использовать в качестве простого и надежного молекулярного инструмента для оценки генетического разнообразия в цитотипах *Nannospalax* при низких генетических дистанциях.

КЛЮЧЕВЫЕ СЛОВА: цитотип, ISSR, *Nannospalax xanthodon*, Турция.

* Corresponding author

Introduction

The Spalacidae family is distributed in Northeast Africa, the Balkans, Southeast Europe, Central Asia, the Middle East, and the Caucasus (Topachevskii, 1969; Savic & Nevo, 1990). Previously, Gromov & Baranova (1981) recognized two genera of this family: *Nannospalax* Palmer, 1903 and *Spalax* Guldenstaedt, 1770. The *Nannospalax* differ from *Spalax* because their karyotypes include mainly acrocentric autosomes (Arslan & Bölükbaş, 2010; Arslan *et al*., 2010; Matur *et al*., 2011; Sözen *et al*., 2011). Therefore the generic name *Nannospalax* is used in this study.

In Turkey, the genus *Nannospalax* is commonly represented by three species — *Nannospalax leucodon* (Nordmann, 1840), *N. xanthodon* (Nordmann, 1840), and *N. ehrenbergi* (Nehring, 1898). The number of diploid chromosomes (2*n*) of *N. xanthodon* ranges from 36 to 60 and the fundamental number (NF) ranges from 66 to 92 (Sözen *et al*., 2013; Kankılıç *et al*., 2015). Cytological studies revealed over 50 cytotypes (individuals with different chromosomal numbers within the species) for Turkish mole rats, and this extraordinary karyological diversity is an important taxonomic problem (Yigit *et al*., 2006; Kryštufek & Vohralík, 2009; Arslan & Zima, 2014; Sözen *et al*., 2015; Kankılıç *et al*., 2017). The taxonomic status and the geographical borders of species and cytotypes of the genus *Nannospalax* is still a matter of debate. Researchers have been seeking answers to the question of how taxonomically evaluate cytotypes using molecular techniques whether they are different species or not.

The first genetic research conducted on the genus *Nannospalax* was allozyme electrophoresis study by Nevo *et al*. (1995). In this study, it was determined that different chromosomal forms have distinct alleles in most of the studied enzyme systems. Moreover, Suzuki *et al*. (1996) investigated the differences in ribosomal DNA (rDNA) and mitochondrial DNA (mtDNA) between *N. xanthodon* and *N. ehrenbergi* species by using restriction fragment length polymorphism (RFLP) technique. In terms of genetic polymorphism, they reported discrimination between these two species at a high rate.

Recent molecular studies have provided remarkable data in the understanding of Turkish mole rat phylogeny. Although the studies based on mtDNA did not show differences in the Anatolian cytotypes at the species level, significant separations were reported between several cytotypes (Arslan *et al*., 2010; Kandemir *et al*., 2012; Kankılıç *et al*., 2013, 2015; Kankılıç & Gürpınar, 2014). In addition, Hadid *et al*. (2012) identified four clades (*leucodon, xanthodon, ehrenbergi, vasvarii*) for the Anatolian mole rats by phylogenetic analysis of five mtDNA sequences. Moreover, Kankılıç *et al*. (2013) indicated that random amplification of polymorphic DNA (RAPD) bands could be distinguishing molecular markers among chromosomal races, by using RAPD technique.

Almost all the above-mentioned studies suggest the need for more localities and more molecular data in order to resolve the taxonomic complexity of *Nannospalax*. In this framework, we aimed to determine the genetic characterization between 2*n* = 54 and 2*n* = 60 cytotypes of *N. xanthodon* distributed in the Central Anatolia region by using distinguishing markers and also to show the applicability of ISSR-PCR technique for the discrimination of these cytotypes. This molecular technique allows simple and reliable primer design without a need for sequence information for the determination of evolutionary relationships between various species (Zietkiewicz *et al*., 1994; Joshi *et al*., 2000; Bugarski-Stanojević *et al*., 2008, 2011).

Materials and methods

Animal materials and locations

This study consisted of 36 *N. xanthodon* specimens from 8 different localities in Central Anatolia region (Fig. 1). Liver tissues were preserved at –80 ºC at the Department of Molecular Biology and Genetics, Bilecik Şeyh Edebali University, Bilecik, Turkey. Karyological data were obtained from the previous study (Yagci, 2010).

DNA extraction and ISSR-PCR amplification

DNA was isolated from liver tissues of 36 individuals of *N. xanthodon* by using DNA extraction kit (GeneJET Genomic DNA Purification Kit, Thermo Scientific, USA). DNA purity was determined by NanoDrop and the degree of DNA degradation was measured using 0.1% agarose gels with 0.5 µg/ml ethidium bromide. ISSR-PCR amplification was performed from the obtained genomic DNA. 18 primers (UBC807, 809, 811, 813, 818, 826, 827, 828, 830, 841, 842, 843, 864, 866, 868, 873, 876, and 880) were randomly chosen from UBC (British Columbia University) primer set #9 and tested in ISSR-PCR analysis. In addition, primers 52 $(CAG)_5$GC 32 and 52 $(CAG)_4$AC 32 were custom designed (Sentegen) and tested, since they were previously found as appropriate primers for rodent species (Bugarski-Stanojević *et al*., 2011). Only 7 primers (UBC807, 811, 828, 873, 880, $(CAG)_5$ GC and $(CAG)_4$ AC producing clear and reproducible bands were chosen for ISSR analysis (Tab. 1). PCR amplifications were performed for a total volume of 25 uL in the Bio-Rad T100™ Thermal cycler by using 0.3 ul Taq DNA polymerase enzyme (5u/ul, Thermo Scientific, USA), 2.5 ul 10x PCR Buffer, 1.5uL $MgCl_2$ (25 mM), 0.4 uL dNTP (10 mM), 1 u1 DNA (30 ng), 1 ul primer and 16.8 ul ddH_2O. The PCR program started with an initial phase of 5 min at 94 ºC, followed by 40 cycles of 45 s at 94 ºC, 45 s at 40 ºC, 2 min at 72 ºC and 10 min final elongation at 72 ºC. ISSR amplification products were stained with ethidium bromide and eluted at 90 V with 1X TBE buffer on 1% agarose gel electrophoresis.

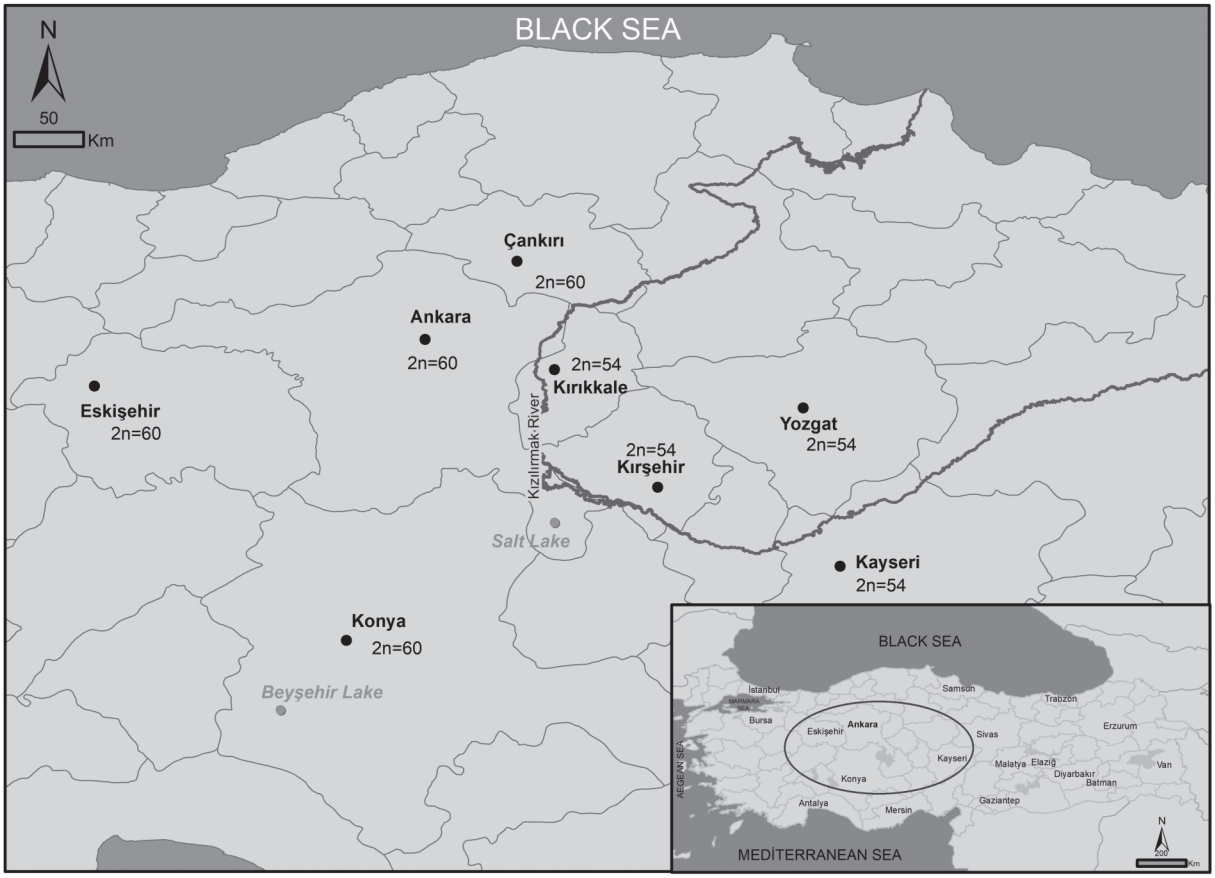

Fig. 1. Localities and sample sizes of ISSR analysed *N. xanthodon* individuals. 2*n* — diploid chromosome number, *n* — specimens number.

Table 1. List of ISSR primers used to detect polymorphism.

Primer Sequence	Number of bands	Number of polymorphic bands	Polymorphism, %
UBC 807 $(AG)_8$ T	20	20	100
UBC 811 $(GA)_8$ AC	14	14	100
UBC 828 $(TC)_8$ A	9	7	78
UBC 873 $(GACA)_4$	14	12	86
UBC 880 $(GGAGA)_5$	17	15	88
Custom primer $(CAG)_5$ GC	19	16	84
Custom primer $(CAG)_4$ AC	19	17	89
ISSR total	112	101	90

Molecular sizes of amplified products were estimated using 100-10000 bp DNA ladder marker (GeneRuler DNA Ladder Mix, Thermo Scientific, USA).

Data analysis

DNA band profiles were scored based on the DNA ladder from gel photographs. Scoring was done by recording in the presence of a band (1) and in the absence of band (0). The genetic distance matrix created in the presence and absence of bands is calculated according to Nei (1978). To calculate genetic diversity, Multivariate Statistical Package (MVSP) v. 3.22 and POPGENE v. 1.32 (Population Genetic Analysis) were used (Yeh *et al*., 1999). This was done by the UPGMA analysis, which was calculated based on the dendrogram derived from the Jaccard coefficient (Fig. 2).

Results

In this study, 7 ISSR primers which gave the best amplification from total 20 primers were selected for the analysis of *N. xanthodon* cytotypes. These 7 primers amplified a total of 101 polymorphic loci, of estimated sizes from 150 to 2200 bp. The numbers of

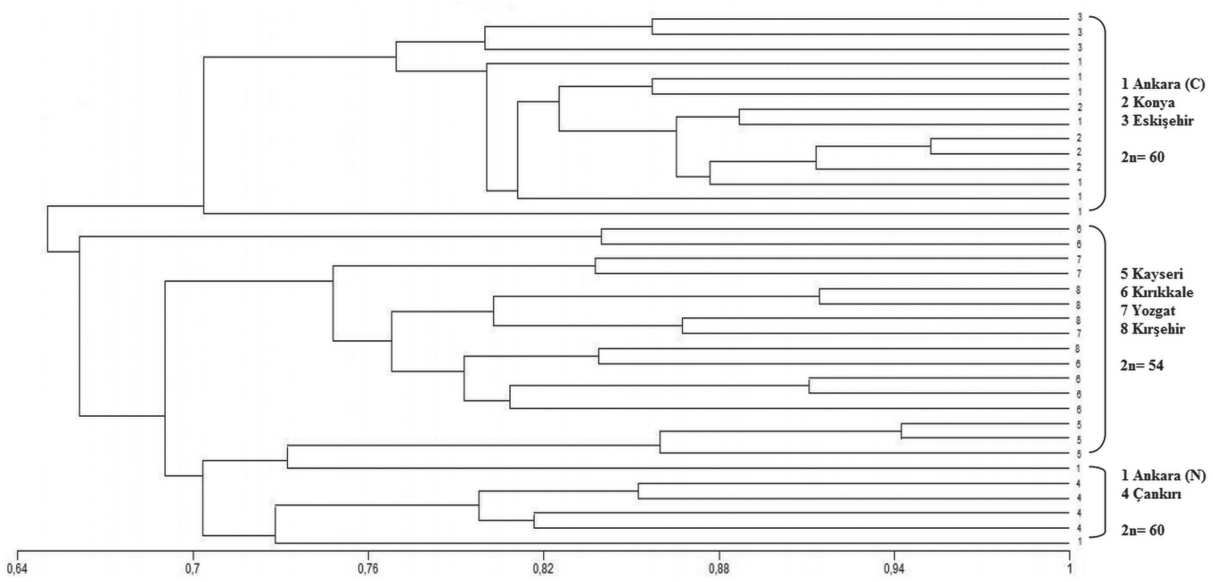

Fig. 2. Dendrogram of genetic similarity among 36 *N. xanthodon* specimens obtained from ISSR markers using the UPGMA method.

polymorphic bands were between 7 and 20. The average number of polymorphic bands per primer was calculated as 14.42 (Tab. 1). The polymorphism rates of the primers range from 78% to 100% (Fig. 3). Total polymorphism rate was recorded as 90%. According to these data, primers $(AG)_8$ T and $(GA)_8$ AC are excellent primers for discriminating cytotypes (100%), since they produced high and specific bands. Nei's original genetic distances were calculated independently for the specimens separated on the basis of all 8 localities and *N. xanthodon* cytotypes ($2n$ = 54, 60). In the $2n$ = 54 and $2n$ = 60 cytotypes, the average similarity was calculated as 89% using 7 ISSR markers and this high similarity is because of their common ancestry (Tabs 2, 3). When locality samples were evaluated, the highest similarity was 91% in Ankara and Konya populations, because Ankara and Konya's populations share the same number of diploid chromosomes ($2n$ = 60). The highest average distance (35%) was seen in Konya and Yozgat populations since these geographic patterns are separated by diploid chromosome numbers ($2n$ = 60, $2n$ = 54, respectively).

Table 2. Nei's original measures average of genetic identity and genetic distance between *N. xanthodon* cytotypes ($2n$ = 54 and $2n$ = 60).

Cytotypes	$2n$ = 60	$2n$ = 54
$2n$ = 60	****	0.8984
$2n$ = 54	0.1071	****

The genetic polymorphism between populations and cytotypes of *N. xanthodon* was calculated to present possible differences in between a total of 36 *N. xanthodon* individuals in 8 provinces of Central Anatolia and between two cytotypes. The highest genetic diversity index as locality was found in Ankara populations (h=0.216 and I=0328), whereas Konya populations have the lowest genetic diversity (h=0.059, I=0.089). When the samples separated by cytotypes are taken into account, the percentage of polymorphic loci in $2n$ = 60 is found higher than $2n$ = 54 (Tabs 4, 5).

The genetic distance matrix was calculated using the polymorphism ratios determined by the markers indicated in Table 1. In the UPGMA analysis, which was calculated based on the Jaccard coefficient; two main groups were revealed significantly. Group I, consisted of samples with diploid chromosome numbers $2n$ = 60 and the samples from the localities with boundaries between each other (Eskişehir, Ankara, Konya). Group II was separated from Group I with regard to all of the $2n$ = 54 species (Kırıkkale, Kırşehir, Yozgat, Kayseri) inside the Kızılırmak basin. These two groups were separated from each other both chromosomally and geographically by Kızılırmak River. A small population of 6 samples isolated from $2n$ = 54, which we can refer as Group III, contains $2n$ = 60 samples taken from the north of Central Anatolia, Çankırı, and north of Ankara (Figs 1–2).

Discussion

Wahrman *et al.* (1969) proposed that the appearance of ancestral *Spalax* could be any place in Southeastern Europe or Anatolia. The oldest spalacid *Heramys eviensis* living in early Miocene epoch was re-

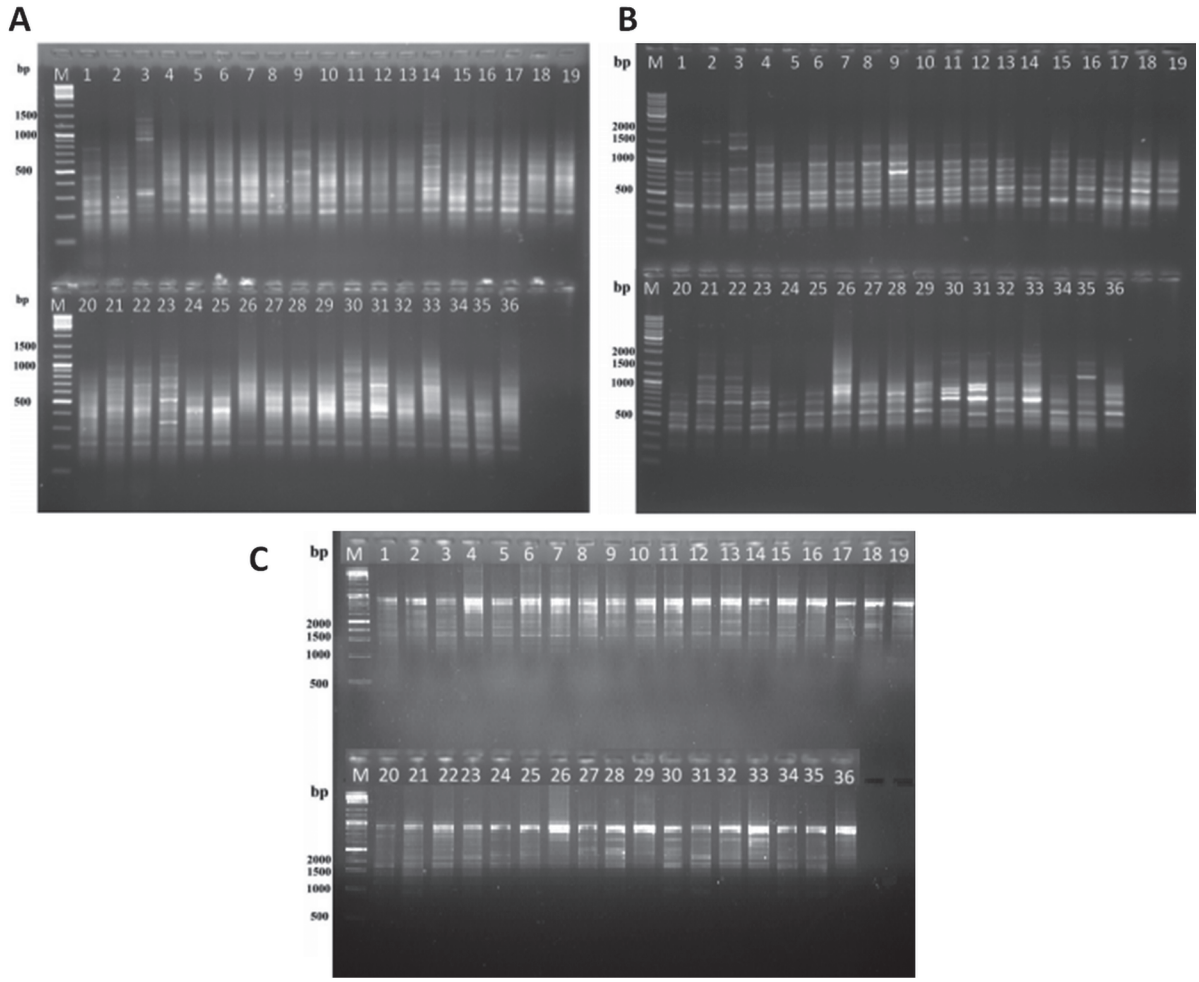

Fig. 3. Gel electrophoresis profiles of PCR products by using ISSR primers for total 36 *N. xanthodon* specimens. Primers showing the highest polymorphism: A — UBC 807, B — UBC 811. Primers showing the lowest polymorphism: C — UBC 828. M — DNA marker; 2*n* = 60 (1–19), 2*n* = 54 (20–36).

Table 3. Nei's original measures average of genetic identity and genetic distance between geographic samples of *N. xanthodon*.

Samples	1	2	3	4	5	6	7	8
1	****	0.9126	0.8750	0.8412	0.8141	0.8422	0.7713	0.7861
2	0.0914	****	0.8711	0.7527	0.7812	0.7672	0.6992	0.7765
3	0.1335	0.1380	****	0.7995	0.8172	0.7798	0.7439	0.7906
4	0.1729	0.2840	0.2237	****	0.7875	0.8242	0.7999	0.7931
5	0.2057	0.2469	0.2019	0.2388	****	0.8225	0.8140	0.8319
6	0.1717	0.2650	0.2487	0.1934	0.1954	****	0.8549	0.8815
7	0.2597	0.3578	0.2958	0.2232	0.2058	0.1568	****	0.8667
8	0.2407	0.2530	0.2350	0.2319	0.1841	0.1261	0.1430	****

1 — Ankara, 2 — Konya, 3 — Eskişehir, 4 — Çankırı, 5 — Kayseri, 6 — Kırıkkale, 7 — Yozgat, 8 — Kırşehir.

Table 4. Genetic diversity according to locality distribution of *N. xanthodon*.

Localities and coordinates	2*n*	NF	N	n_a	n_e	*I*	*h*	*P%*
Yenimahalle, Ankara 39°57'N, 32°47'E Altındağ, Ankara 40°02'N, 32°56'E	60	82	9	1.687	1.368	0.328	0.216	68.75
Kulu, Konya 39°11'N, 33°08'E Cihanbeyli, Konya 38°40'N, 32°55'E	60	80	4	1.160	1.102	0.089	0.059	16.07
Sarıcakaya, Eskişehir 40°05'N, 30°36'E Mihalgazi, Eski°ehir 40°02'N, 30°36'E	60	80	3	1.285	1.197	0.165	0.112	28.57
Kızılırmak, Çankırı 40°24'N, 34°07'E Eldivan, Çankırı 40°32'N, 33°29'E	60	78	4	1.348	1.244	0.200	0.137	34.82
Bayramhacı, Kayseri 38°49'N, 35°01'E Yuvalı, Kayseri 38°49'N, 35°05'E	54	74	3	1.160	1.111	0.093	0.063	16.07
Çelebi, Kırıkkale 39°27'N, 33°31'E Yahşihan, Kırıkkale 39°50'N, 33°27'E	54	74	6	1.410	1.248	0.218	0.145	41.07
Yerköy, Yozgat 39°40'N, 34°37'E Şefaatli, Yozgat 39°29'N, 34°44'E	54	74	3	1.258	1.190	0.154	0.106	25.89
Akpınar, Kırşehir 39°38'N, 33°54'E Bayramözü, Kırşehir 39°23'N, 33°35'E	54	74	4	1.330	1.198	0.175	0.116	33.04

2*n* — cytotypes, NF — fundamental number, N — sample size, n_a — average allele number per locus, n_e — the effective number of alleles, *I* — Shannon's diversity, *h* — Nei's gene diversity, *P*% — the percentage of polymorphic loci.

Table 5. Genetic diversity of *N. xanthodon* cytotypes (2*n* = 60 and 2*n* = 54).

Cytotypes	N	n_a	n_e	*I*	*h*	*P%*
2*n* = 60	20	1.803	1.402	0.367	0.240	80.36
2*n* = 54	16	1.687	1.358	0.321	0.211	68.75

N — sample size, n_a — average allele number per locus, n_e — the effective number of alleles, *I* — Shannon's diversity, *h* — Nei's gene diversity, *P%* — the percentage of polymorphic loci.

corded by Klein Hofmeijer & de Bruijn (1985) in Greece (Nevo, 1991). The latest studies have presented the facts that Anatolian Spalacidae originating from muroid-cricetoid stock have evolved 20-30 million years ago during the Oligocene epoch and distributed across the Northern Africa, Middle East, Russia, and Balkans. Spalacidae species distributed in Europe have an Asian origin. Before the early Pleistocene, blind mole rat populations have crossed the Balkan Peninsula and after the formation of Bosphorus and Dardanelles straits they were separated and speciatiated independently (Yüksel & Gülkaç, 1990). The original factors affecting the distribution of rodents in Turkey are vegetation structure, climate, and altitude (Yiğit *et al*., 1999).

The idea that the climatic properties are effective in chromosomal distribution of Spalacidae family in Turkey is supported by the distribution of 2*n* = 38 in the humid Aegean region and 2*n* = 60 in the arid Central Anatolia region (Nevo *et al*., 1994, 1995). In this study, 8 populations from the Central Anatolian region with an arid clime have the same climatic characteristics. However, as they consist of two different cytotypes, it seems that the effect of drought is open to different and contradictory interpretations. In this respect, even our findings do not clearly reveal that there is a precise rule for this issue. In the further studies, not only the precipitation characteristics but also the use of the drought with other climatic characteristics (i.e. the geological structure of localities, water holding feature of soil, slope, elevation and etc.) can be evaluated for the localities in the same climate zone.

Ivanitskaya *et al.* (2008), recorded different populations of 2*n* = 60 between Northern Anatolia and Central Anatolia using molecular cytogenetic techniques. Kandemir *et al*. (2012) identified a different clade of Northern Anatolia and differentiated them from other popu-

lations by characterizing genetic variations of cytochrome *b* gene sequences. According to Matur *et al*. (2011), there is an independent evolutionary pathway for Northern clade identified as ancestral karyotype 2*n* = 60R. Although, the diploid chromosome numbers (2*n* = 60) were same in our study, the samples belonging to North were separated as a different group (Group III) and differentiated from Central Anatolian samples. Kankılıç & Gürpınar (2014) emphasized that cytotype 2*n* = 60 has the highest number of common alleles in Central Anatolia for *N. xanthodon* and *N. ehrenbergi* based on mtDNA analysis, which was also supported in our study.

According to several studies, a generalization of a "cytotypes – equals – species" opinion is not appropriate, based on low genetic deviations especially in cytotypes of *N. leucodon* (Kryštufek *et al*., 2012). Another opinion is that several cytotypes of *N. xanthodon* such as 2*n* = 54 and 2*n* = 60 should be considered as different biological species (Arslan *et al*., 2010, Kankılıç *et al*., 2013). In our study, 2*n* = 54, 2*n* = 60C (Central) and 2*n* = 60N (Northern) cytotypes were separated at a low genetic distance from each other with UPGMA dendrogram, but this differentiation is not enough to consider them as separate species. According to our results, 2*n* = 60C and 2*n* = 60N populations might have different origins. Since, 2*n* = 54 population is genetically more close to 2*n* = 60N than 2*n* = 60C, 2*n* = 54 can be originated from 2*n* = 60N. Genetic differences among the cytotypes of *Nannospalax* genus in Turkey have not yet been clarified. All the authors reported that the collection of further molecular data is necessary to better evaluate the phylogenetic relationship. In addition, they specify the importance of intrapopulation variations in this respect. In short, putting out the phylogenetic and taxonomic results for this species seems to be extremely hard, since they possess remarkable chromosomal diversities even at very close localities in line with morphological similarities. However, the detection of very small distinction and/or similarity between cytotypes and individuals is important for the explanation of the genetic relationship between mole rats during their speciation processes in evolution, which is accepted as a common consensus between scientists. Evaluation of molecular studies together with karyological and morphological studies is recommended to illuminate the taxonomic classification of Spalacidae. Further cytogenetic studies should be performed between cytotypes of *Nannospalax* by taking into account of Robertsonian translocations, B chromosomes and individual variations causing chromosomal diversity.

ISSR marker systems are widely used DNA markers to predict and characterize the genetic diversities in natural populations. Numerous studies have been used ISSR markers for the determination of relationship degrees in sibling, cryptic, sympatric animals and plant species. In these studies, the reliability of ISSR markers were also confirmed by the use of different marker such as cytochrome *b* gene analysis of mtDNA, isoenzymes, ISSR and RAPD PCR (Bugarski-Stanojević *et al*., 2008; Jojić *et al*., 2014; Zhigileva *et al*., 2014; Buczkowska *et al*., 2016; Styan *et al*., 2017; Zhigileva & Gorbacheva, 2017). Similarly, in our study, ISSR markers were functional and convenient for the estimation of genetic diversity and relations in *N. xanthodon* cytotypes (2*n* = 54, 2*n* = 60).

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