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Genetic Diversity and Geographic Variation of Chromosomal races of *Nannospalax xanthodon* (Nordmann, 1840) and *Nannospalax ehrenbergi* (Nehring, 1898) from Turkey, Revealed by RAPD Analysis

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Abstract: The level of genetic polymorphism in chromosomal races of *Nannospalax xanthodon* and *Nannospalax ehrenbergi* was determined by applying random amplified polymorphic DNA (RAPDs) analysis. One hundred and fifty four mole rat samples belonging to two species *N. xanthodon* (61 populations) and *N. ehrenbergi* (3 populations) distributed in Anatolia were studied. Remarkable variations of diploid chromosome numbers were identified for *N. xanthodon* (2n=36 – 60). Eleven RAPD–PCR primers generated 160 polymorphic loci. The mean proportion of polymorphic loci was 92% (147 bands) for all chromosomal race implying high levels of genetic variability in *N. xanthodon* and *N. ehrenbergi.* Estimation of genetic diversity based on PCR amplification of RAPDs was high for both species. Results of RAPD–PCR observed within and among species were also connected with the peripatric mode of speciation. We determined that RAPD bands showed high diagnostic value between chromosomal races as they were very distinctive for each chromosomal race and absent from other forms. Genetic distance (D_s) values between chromosomal races suggest that most populations analyzed in this study may be valid biological species.

Key words: Chromosomal race, geographic variation, *Nannospalax xanthodon*, *Nannospalax ehrenbergi*, RAPD–PCR

Introduction

Blind mole rats of the genus *Nannospalax* (Palmer, 1903) has been represented by thirteen valid species including more than fifty chromosomal races (Kryštufek, Vohralik 2009, Wilson, Reeder 2005). Distribution range of *Nannospalax* species includes Balkans, steppes of Russia, Turkey, Middle East and North Africa (Savic′, Nevo 1990). However, while exhibiting this extensive distribution, their greatest diversity occurs in Anatolia, where they are a noteworthy component of the rodent fauna of the arid, semi-arid, semi-xeric regions of Turkey (Kryštufek, Vohralik 2009).

Turkish blind mole rats have high chromosomal variability, with the group currently comprising three species (*N. leucodon* Nordman 1840, *N. xanthodon* Nordman 1840, *N. ehrenbergi* Nehring 1898) and forty two chromosomal races. *N. xanthodon* (a senior synonyme of *N. nehringi*)

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with twenty eight distinctive chromosomal races (2n=36–62, NFa=64–84) includes individuals extending from the Western Anatolia to the eastern borders of Anatolia (Nevo *et al.* 1995, Sözen 2004, Sözen *et al.* 2006a, 2006b, Kankiliç *et al.* 2007a, 2007b, 2009, 2010). *N. ehrenbergi* with twelve diverse chromosomal races (2n=48–58, NFa=62–82) is noted in Southeastern Turkey (Sözen *et al.* 2006b). *N. leucodon* with two chromosomal races (2n=56, NFa=72 and 74) are distributed in Turkish Thrace (Sözen 2004, Sözen *et al.* 2006b).

The G-banding, C-Banding and Ag-NOR banding analysis of several animals show that chromosomal polymorphism in *Nannospalax* is connected with Robertsonian reorganizations, centromeric shift, inversions and also tandem fusion of middle metacentrics with formation of large metacentrics (Nevo *et al.* 1994, Ivanitskaya *et al.* 2008, Arslan *et al.* 2010a).

Taxonomic status of species in Turkey has been considered as controversial due to extreme external and craniodental morphological similarities. Thus, different chromosomal races are represented for possible valid cryptic species (Nevo *et al.* 1994, 1995). Although these chromosomal races were strikingly similar in morphological appearance, few morphological and metrical characters are available to distinguish members of this uniform group (Kivanç 1988, Kankiliç *et al.* 2006, 2009). Some differences have been observed in the several characters such as structure of upper incisors and nasal bone, number of enamel islands on molars, number of roots of molar, baculum and fallus conformation, position and structure of palatal foramen, and body size (Kivanç 1988, Kankiliç *et al.* 2006, 2009). However, the taxonomic utility of these characters may be limited due to substantial variation within chromosomal races (Nevo *et al.* 1995).

Nevo (1991) showed an evolutionary model of ecological speciation and adaptive radiation in mole rats of *N. ehrenbergi* species in Israel. *N. ehrenbergi* is represented by four chromosomal races (2n=52, 54, 58, and 60) in Israel. The restricted gene flow between chromosomal races was documented with numerous genetic markers in Israel (Nevo, Beiles 1992, Nevo *et al.* 1993). The four chromosomal races in Israel have parapatric distributions, often with narrow contact zones. Recently, it has been shown that four chromosomal races (2n=52, 54, 58, and 60) of *N. ehrenbergi* in Israel have been described as valid biological species (Nevo *et al.* 2001).

Only a limited number of genetic studies have been carried out in Turkish mole rat species. Nevo *et al.* (1995) analyzed allozyme diversity across Turkey, based on 37 allozymic loci in two species. Five (*Adh*, *Me–1*, *Pt–1*, *Mpi*, *Np*) of the 37 enzymes detected by starch-gel electrophoresis showed characteristic isoenzyme profiles between two species (*N. xanthodon* and *N. ehrenbergi*). Twenty of the thirty seven loci analyzed (54%) were monomorphic in *N. xanthodon*. This study revealed that the individuals belonging to nine different chromosomal races have distinct isoenzyme patterns.

Suzuki *et al.* (1996) found a remarkable high level of genetic diversity within five chromosomal races of *N. xanthodon* and *N. ehrenbergi* in Turkey by analyzing rDNA and mtDNA variation digested with ten restriction enzymes. It was shown that a high sequence divergence resulted from geographic distance between species, suggesting that genetic differentiation in these species are largely explained by the limited dispersal abilities of individuals rather than by the presence of long-standing ecological or geographical barriers.

Arslan *et al.* (2010b) analysed cytochrome b (*cyt b*) mitochondrial sequences of 13 samples belonging to three different chromosomal races (2n=40, 58, 60), to find and validate useful molecular markers employable for the species identification. High genetic divergences were found between chromosomal races (K2P between 8.16%±1.19 and 11.33%±1.42) and application of the 2% divergence rate to the net divergence estimates suggests their divergence about 3.84 and 5.43 Mya (95% confidence interval =1.53–8.19 Mya). Arslan *et al.* (2010b) pronounced mtDNA sequence divergence between the chromosomal races from central Anatolia, and suggest that chromosomal races from these regions should be treated as a different biological species.

The mole rats of the family Spalacidae present an extraordinarily interesting model to study speciation events. Unfortunately, only little knowledge about their genetic variation and structure has been obtained so far. In this respect, it is clear that more karyological and molecular

studies are needed to resolve the taxonomic status and distribution of chromosomal races of the mole rat in Turkey. The purpose of this study was to determine chromosomal races or species-specific molecular markers, to examine the genetic differentiation and taxonomic relationships among and between *N. xanthodon* and *N. ehrenbergi* using random amplified polymorphic DNA (RAPDs), and also contribute to the knowledge of distribution of chromosomal races in Turkey.

Materials and Methods

Eleven chromosomal races of *Nannospalax* and 154 individuals were collected between 2002 and 2011 from 64 sites in Turkey. Distribution of chromosomal races and sample localities are shown in Table 1 and Fig. 1. Animals were prepared according to traditional museum standard. Tissues were preserved at –80 ºC. Skins, skulls, and tissues of the samples are housed at the Department of Biology, Faculty of Science, Ankara University. Karyological data used in this study were obtained from previous studies (Kankiliç *et al.* 2005, 2006, 2007ab, 2009, 2010).

Genomic DNA was extracted from portions of muscle or kidney tissue using the protocol specified by Doyle, Doyle (1991). DNA was stored in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) at 4 ºC. Concentration and the purity of the obtained DNA solutions were determined by Agilent 2100 Bioanalyser NanoDrop ND–1000 Spectrophotometer. The amplification reaction mixture of Kaya, Neale (1995) was modified as follows: 1 µL of DNA samples (200 ng/µL); 25 µL of buffer (750 mM Tris–HCl pH 8.8, 200 mM $(NH_4)_2SO_4$; Fermentas), 0.3 µL (1.5 units) of *Taq* DNA polymerase (Sigma); 4 µL of dNTP (200 µM of each nucleotide); 1.5 µL of 20 pmol primers.

The PCR condition included denaturation at 95 ºC for 1 min, followed by 45 cycles of denaturation at 94 ºC for 1 min, annealing at 36 ºC for 2 min, and extension at 72 ºC for 2 min. Final extension was performed at 72 ºC for 15 min. Twenty different primers, obtained from Operon Technologies, Inc. (Alameda, California), and from the University of British Columbia (Vancouver, Canada), were prescreened in order to identify primers that procedure reproducible and recordable band patterns. Eleven primers were selected for further analysis.

PCR products were separated on 1.5% agarose gels in 1 X TAE (Tris/Acetat/EDTA) buffer in an electrical field (70 mV, 4 h). The banding patterns were made visible with ethidium bromide under UV light. For each individual, RAPDs profiles were characterized in a matrix by scoring bands on agarose gel as their presence (assigned a value of 1) or absence (assigned a value of 0). The presence or absence of bands was scored for clear and reproducible bands with estimated fragment lengths of between 50 and 3000 base pairs (DNA Ladder Plus, Fermentas). Then, the data was imported into the software POPGENE v. 1.32 (YEH *et al.* 1999) for estimating parameters of genetic variation and differentiations. In order to determine genetic variability of populations, (1) mean number of observed alleles per locus (n_a), (2) mean number of effective alleles (n_e), (3) percentage of polymorphic loci at the 95% significance criterion (P_{95}), (4) expected heterozygosity (*He*), (5) Shannon's information index (*I*) (Levontin 1972) values and their standard errors for populations were calculated. The molecular differentiation between/among population pairs were quantified with total gene diversity H_T, intra sample gene diversity H_S and Nei's fixation index (Nei 1973). G_{ST} and *Nm* calculated using the software POPGENE v. 1.32. The analysis of molecular variance (AMOVA) was also used to determine the total phenotypic variance within and among populations (Excoffier *et al.* 1992). AMOVA input files were created with the use of GenAlEx v. 6.2 software program using the distance metric of Excoffier *et al.* (1992). To further examine the patterns of variation among individual samples, a principal coordinated analysis (PCO) was performed with NTSYS–pc v. 2.2 (ROHLF 2000). With a view to calculate PCO, the pairwise genetic distance was calculated using Jaccard's coefficient for binary data. The resulting matrix was double-centered using DCENTER module, and then EIGEN was used to compute principal coordinates along all axes. The results of first three axes were used to form two dimensional plot graphic with PAST v. 1.99 (Hammer *et al.* 2001). The UPGMA tree for two species was constructed based on the matrix of Nei's pairwise genetic distances. The UPGMA tree was produced using the software POPGENE v. 1.32 and manipulated using the software MEGA 4 (Tamura *et al.* 2007). Consensus network was

Table 1. Localities collecting localities of two species in Turkey. The number of sampling localities (code) corresponds to numbers given in Fig. 1, N: sample size, 2n: diploid chromosome number, NF: the fundamental number of chromosomal arms.

Code	Localities	N	2n	Altitude	Latitude	Longitude
Loc 1	Aydın-Koçarlı	N=7	2n=36 NF=68	35 m	37°44'N	27°34'E
Loc 2	Aydın-Magnesia -Ortaklar	N=2	2n=36 NF=68	128 m	37°44'N	28°06'E
Loc 3	İzmir-Foça-Bağarası	N=4	2n=38 NF=74	100 m	38°40'N	26°45'E
Loc 4	Manisa-Akhisar	N=2	2n=38 NF=74	132 m	38°55'N	27°51'E
Loc 5	Balıkesir-Kepsut	N=3	2n=38 NF=74	80 m	39°41'N	28°10'E
Loc 6	Konya-Beyşehir-Yeşildağ	N=3	2n=40 NF=72	1130 m	37°32'N	31°28'E
Loc 7	Isparta-Yenişarbademli	N=6	2n=40 NF=72	1210 m	37°42'N	31°23'E
Loc 8	Kars-Susuz	N=4	2n=50 NF=70	1930 m	40°46'N	43°20E
Loc 9	Kars-Selim	N=2	2n=50 NF=70	1955 m	40°27'N	42°47E
Loc 10	Ardahan-Göle	N=4	2n=50 NF=70	2196 m	40°47'N	42°36E
Loc 11	Ardahan-City Center	N=2	2n=50 NF=70	1881 m	41°06'N	42°42E
Loc 12	Erzurum 20 Km E	N=2	2n=50 NF=72	2376 m	40°01'N	41°27E
Loc 13	Bayburt-Demirözü	N=3	2n=50 NF=72	1744 m	40°09N	39°53E
Loc 14	Rize-Ovid Mountain	N=4	2n=50 NF=72	1012 m	40°47'N	40°32E
Loc 15	Giresun-Eğribel Mountain pass	N=2	2n=50 NF=72	2174 m	40°26'N	38°24E
Loc 16	Mersin-Sebil Plateau	N=5	2n=52 NF=72 South	1080 m	37°07'N	34°33'E
Loc 17	Bolu-Yeniçağa	N=2	2n=52 NF=70 North	1000 m	40°46'N	32°01'E
Loc 18	Bolu-Mudurnu	N=1	2n=52 NF=70 North	880 m	40°27'N	31°12'E
Loc 19	Bolu-Mengen-Demirciler	N=3	2n=52 NF=70 North	690 m	40°57'N	32°05'E
Loc 20	Bolu-Gerede	N=1	2n=52 NF=70 North	1326 m	40°47'N	32°11'E
Loc 21	Bolu-Seben	N=2	2n=52 NF=70 North	761 m	40°24'N	31°34'E
Loc 22	Bolu-Abant	N=1	2n=52 NF=70 North	1326 m	40°47'N	32°11'E
Loc 23	Kırıkkale-Keskin	N=2	2n=54 NF=74	1144 m	39°40'N	33°36'E
Loc 24	Kırıkkale 10 Km E	N=2	2n=54 NF=74	773 m	39°51'N	33°35'E
Loc 25	Kırıkkale 5 Km E	N=3	2n=54 NF=74	744 m	39°50'N	33°33'E
Loc 26	Kırşehir-Seyfe Lake	N=2	2n=54 NF=74	1135 m	39°11'N	34°20'E
Loc 27	Mersin-Gülek-Elmalı Village	N=4	2n=56 NF=72 South	1835 m	37°22'N	38°45'E
Loc 28	Isparta-Aksu-Yılanlı Village	N=2	2n=56 NF=72 West	1227 m	37°47'N	30°59'E
Loc 29	Manisa-Kula	N=2	2n=56 NF=72 West	661 m	38°32'N	28°38'E
Loc 30	Uşak-Gediz Turning Point	N=1	2n=56 NF=72 West	691 m	38°40'N	29°14'E
Loc 31	Niğde-Ulukışla-Madenköy	N=9	2n=58 NF=72	1717 m	37°26'N	34°37'E
Loc 32	Manisa-Selendi	N=1	2n=60 NF=74	446 m	38°44'N	28°52'E
Loc 33	Afyon-10 Km NE	N=2	2n=60 NF=82	1009 m	38°48'N	30°32'E
Loc 34	Afyon-Eber Lake	N=1	2n=60 NF=82	1009 m	38°35'N	31°07'E
Loc 35	Afyon-Çay-Çayırpınar Village	N=1	2n=60 NF=82	985 m	38°35'N	31°05'E
Loc 36	Isparta-Atabey	N=1	2n=60 NF=78	1037 m	37°57'N	30°38'E
Loc 37	Isparta-Gönen	N=1	2n=60 NF=78	1063 m	37°57'N	30°30'E
Loc 38	Isparta-Gelendost-Madenli Village	N=1	2n=60 NF=78	1032 m	38°11'N	31°06'E
Loc 39	Burdur 5 Km S	N=2	2n=60 NF=84	985 m	37°42'N	30°16'E
Loc 40	Burdur-Yeşilova-Harmanlı Village	N=4	2n=60 NF=84	1085 m	37°35'N	29°54'E
Loc 41	Denizli-Çameli-Bıçaklı Village	N=2	2n=60 NF=84	1300 m	37°04'N	29°20'E
Loc 42	Denizli-Acıpayam	N=1	2n=60 NF=84	926 m	37°25'N	29°21'E

Table 1. Continued.

Code	Localities	N	2n	Altitude	Latitude	Longitude
Loc 43	Antalya-Akseki-Salamut Plateau	N=2	2n=60 NF=78	1252 m	37°02'N	31°46'E
Loc 44	Konya-Beyşehir-Hüyük-Kıreli	N=1	2n=60 NF=76	1150 m	37°55'N	31°32'E
Loc 45	Konya-Cihanbeyli	N=1	2n=60 NF=80	973 m	38°39'N	32°55'E
Loc 46	Kütahya-Hava Tugayı	N=2	2n=60 NF=76	929 m	39°24'N	30°01'E
Loc 47	Kütahya-Simav-Küplüce	N=2	2n=60 NF=76	1132 m	38°59'N	29°03'E
Loc 48	Kütahya-Emet	N=2	2n=60 NF=76	904 m	39°20'N	29°15'E
Loc 49	Bilecik-Bozüyük	N=2	2n=60 NF=76	754 m	39°54'N	30°02'E
Loc 50	Bilecik-Söğüt	N=3	2n=60 NF=76	818 m	39°05'N	28°58'E
Loc 51	Eskişehir-Sivrihisar-Günyüzü	N=3	2n=60 NF=76	888 m	39°23'N	31°48'E
Loc 52	Ankara-Bala	N=1	2n=60 NF=80	1324 m	39°33'N	33°07'E
Loc 53	Ankara-Çeltikli	N=2	2n=60 NF=78	688 m	40°07'N	32°03'E
Loc 54	Aksaray-Şereflikoçhisar	N=1	2n=60 NF=76	1095 m	39°00'N	35°93'E
Loc 55	Kahramanmaraş-Göksun	N=3	2n=60 NF=78	1341 m	38°01'N	36°29'E
Loc 56	Samsun-Kavak	N=2	2n=60 NF=78	610 m	41°04'N	36°02'E
Loc 57	Yozgat-Saraykent	N=2	2n=60 NF=80	1316 m	39°39'N	35°52'E
Loc 58	Sivas-Yıldızeli	N=1	2n=60 NF=80	1401 m	39°51'N	36°35'E
Loc 59	Sivas-İmranlı	N=1	2n=60 NF=80	1611 m	39°52'N	38°06'E
Loc 60	Erzincan-Refahiye-Gemecik	N=1	2n=60 NF=80	1883 m	39°53'N	38°26'E
Loc 61	Erzincan-Tercan-Yollarüstü	N=1	2n=60 NF=80	1389 m	39°37'N	40°10'E
Loc 62	Mersin-Tarsus-Özbek Village	N=9	2n=56 NF=72	46 m	36°58'N	34°56'E
Loc 63	Adana-Yüreğir-Şeyhmurat	N=2	2n=56 NF=72	11 m	36°51'N	35°25'E
Loc 64	Kilis 10 Km E	N=1	2n=56 NF=72	664 m	36°43'N	37°08'E

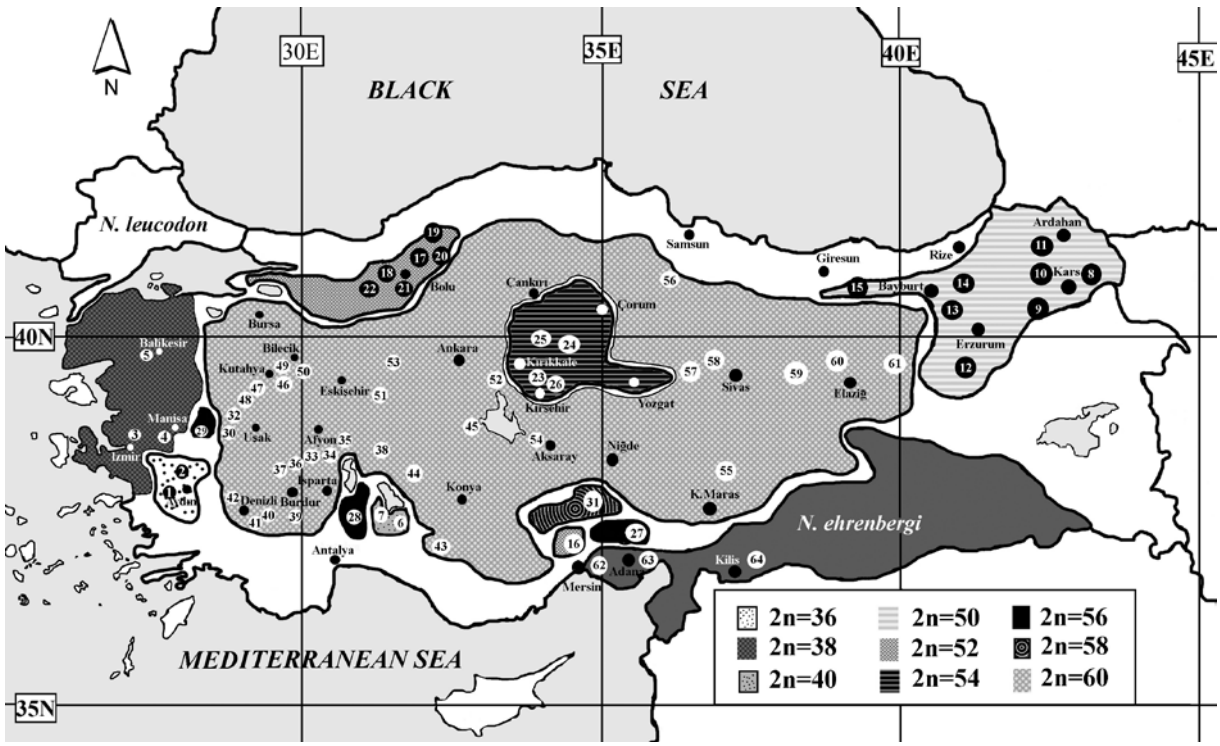

Fig 1. Map of sampling sites for populations of the subterranean mole rats species group in Turkey. Place names are given in Table 1 for each number.

constructed based on the matrix of pairwise G_{ST} genetic diversity using the software SplitsTree v. 4. 10 (Huson, Bryant 2006).

Results

Description of RAPD patterns between populations

Eleven random primers were applied to find out possible differences between *N. ehrenbergi* and *N. xanthodon* populations. Among 11 random primers, two primers (OPD–11 BC–471) were the most noteworthy for distinguishing from *N. ehrenbergi* populations to *N. xanthodon* populations, producing unique DNA patterns for *N. ehrenbergi*. Band patterns among Turkish subterranean mole rat populations were illustrated in Table 2. The selected 11 primers yielded a total of 160 clear and reproducible bands, and 92% (147 bands) of which were polymorphic for *N. xanthodon* and *N. ehrenbergi* populations. The total number of bands were scored per primer ranged from 11 (Primer B–03 and B–05) to 23 (Primer BC–461) with an average of 14.5 bands. For two species, a total 160 bands scored and 104 of which were present in both species. Off these, 52 bands were unique to *N. xanthodon* (loc. 1–61) and 4 bands were unique to *N. ehrenbergi* (loc. 62–64).

Genetic polymorphism within population

RAPD–PCR analysis has demonstrated a high level of genetic diversity of the mole rats from the collecting localities examined in Table 3 (H_e = 0.00 to 0.232, total sample H_e = 0.125). The highest heterozygosity was observed in 2n=52 North (loc. 17–22), and the lowest heterozygosity value was detected in 2n=60 NF=74 (loc. 32). Shannon's index similarly showed a high level of genetic diversity (0.068 to 0.343). Both of these indices also indicated that genetic diversity of *N. xanthodon* was higher than that of *N. ehrenbergi*. The mean number of observed alleles (n_a) was rather low (0.93) and varied from 0.394 (2n= 60 NF=74) to 1.369 (2n=52 North). In accordance with other measurements, the effective number of alleles (n_e) was characterized by low values (2n=60 NF=74, 1.00 to 2n=52 North, 1.397). The level of genetic polymorphism (P_{95}) of *N. xanthodon* (93.13%) was slightly higher than that of *N. ehrenbergi* (43.13%). The highest levels of polymorphism were detected in 2n=52 North chromosomal race (61.88%).

Genetic differentiation and gene flow between populations

To determine genetic relationships among chromosomal races, principal coordinate analysis (PCO) was carried out. The first three axes accounted for 18.85%, 12.72% and 7.2% of the total variation (cumulative value 38.8%), respectively (Fig. 2). PCO provided a better graphical illustration and a clear separation between *N. xanthodon* and *N. ehrenbergi*. However, some of the chromosomal race (2n=60, 2n=58, 2n=56 South) in *N. xanthodon* showed a certain degree of genetic similarity. PCO showed that chromosomal races of *N. xanthodon* are basically separated into three main groups by the first three

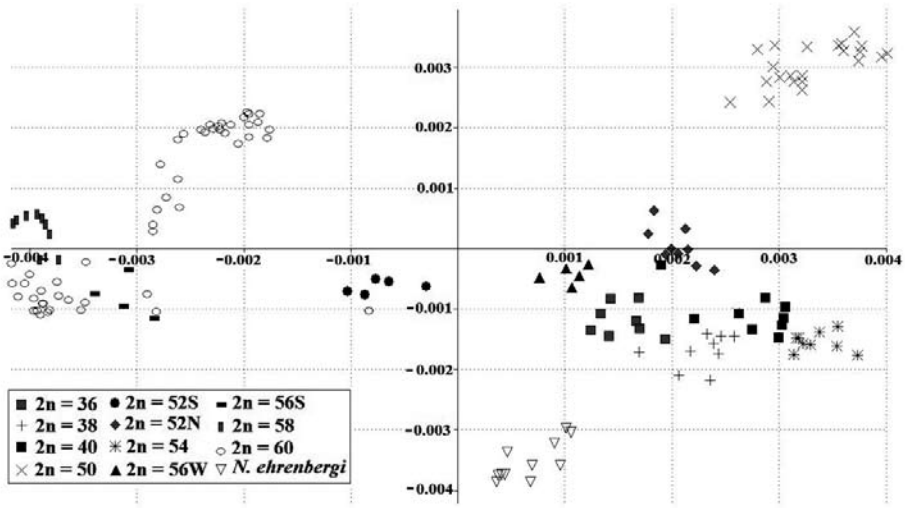

Fig. 2. Two dimensional plot graphic showing genetic relationships among chromosomal races of *N. xanthodon* and *N. ehrenbergi*, based on the first three axes extracted PCO.

Table 2. RAPD primers yielding inter and intra-specific PCR profiles in populations examined, N: sample size, 2n: diploid chromosome number, NF: the fundamental number of chromosomal arms.

Banding pattern among chromosomal races of *N. xanthodon*					
Chromosomal races	No. Band	No. Band Freq. ≥ 5%	Private Bands	No. Loc. Comm. Band ≤ 25%	No. Loc. Comm. Band. ≤ 50%
2n=36	114	114	OPB-11$_{300}$	10	23
2n=38	101	101	BC-461$_{950}$	5	16
2n= 40	101	101	BC-471$_{100}$	5	19
2n=50 NF=70	90	90	0	2	11
2n=50 NF=72	90	90	0	4	12
2n=52 South	106	106	0	4	17
2n=52 North	120	120	BC-461$_{475}$, BC-461$_{600}$	7	25
2n=54	106	106	BC-461$_{75}$, BC-461$_{150}$, BC-461$_{1031}$, OPA-02$_{125}$	5	19
2n=56 West	80	80	BC-471$_{900}$	2	9
2n=56 South	84	84	0	2	4
2n=58	67	67	0	1	2
2n=60 NF=74	63	63	0	0	3
2n=60 NF=76	97	97	0	0	6
2n=60 NF=78	102	102	0	3	11
2n=60 NF=80	101	101	0	1	8
2n=60 NF=82	92	92	0	1	5
2n=60 NF=84	97	97	0	2	6
Banding pattern between *N. xanthodon* and *N. ehrenbergi*					
Species	No. Band	No. Band Freq. ≥ 5%	Private Bands	No. Loc. Comm. Band ≤ 25%	No. Loc. Comm. Band ≤ 50%
			OPA-02$_{100, 350, 750, 850}$; OPA-04$_{850}$; OPB-05$_{350}$		
			OPA-08$_{75, 300, 880}$; OPB-03$_{300, 880, 900}$		
			OPB-11$_{500, 530, 560, 590, 900}$; OPD-11$_{75, 100, 700}$		
N. xanthodon	151	136	BC-441$_{100, 700, 730, 900, 930}$; BC-471$_{320, 450, 680, 700, 930}$	0	0
			BC-591$_{50, 250, 275, 350, 450, 500, 750, 820, 850, 950, 1031}$		
			BC-461$_{50, 100, 400, 700, 750, 850, 920, 950, 1031}$		
N. ehrenbergi	102	102	OPD-11$_{930,}$ BC-471$_{150,}$ BC-471$_{300}$ BC-471$_{500}$	7	19
No. Bands= No. Of Different Bands					
No. Bands Freq. ≥ 5%= No. Of Different Bands with a Frequency ≥ 5%					
Private Bands= Bands Unique to a Single Population					
No. L. Comm Bands (≤ 25%)= No. Of Locally Common Bands (Freq. ≥ 5%) Found in 25% or Fewer Populations					
No. L. Comm Bands (≤ 50%)= No. Of Locally Common Bands (Freq. ≥ 5%) Found in 50% or Fewer Populations					

Table 3. Genetic variability within *N. xanthodon* and *N. ehrenbergi* species. *% P*, percentage of polymorphic loci; H_e, expected heterozygosity; *I*, Shannon's diversity; n_e, effective number of alleles; n_a, average allele number per locus, and N, sample size.

Chromosomal races		N	n_a	n_e	*I*	H_e	UH_e	*%P*
2n=36 NF=68	Mean	9	1.15	1.276	0.232	0.157	0.166	43.75
	SE	-	0.066	0.03	0.023	0.016	0.017	-
2n=38 NF=74	Mean	9	0.913	1.187	0.156	0.106	0.112	28.13
	SE	-	0.064	0.027	0.021	0.014	0.015	-
2n=40 NF=72	Mean	9	1.00	1.262	0.212	0.146	0.154	36.88
	SE	-	0.068	0.03	0.023	0.016	0.017	-
2n=50 NF=70	Mean	12	0.825	1.186	0.152	0.104	0.109	26.25
	SE	-	0.065	0.027	0.021	0.015	0.015	-
2n=50 NF=72	Mean	11	0.875	1.202	0.171	0.115	0.121	31.25
	SE	-	0.068	0.027	0.021	0.015	0.015	-
2n=52 NF=72 South	Mean	5	1.044	1.233	0.198	0.132	0.147	38.13
	SE	-	0.067	0.029	0.022	0.015	0.017	-
2n=52 NF=70 North	Mean	10	1.369	1.397	0.343	0.232	0.244	61.88
	SE	-	0.068	0.029	0.023	0.016	0.017	-
2n=54 NF=74	Mean	9	0.963	1.202	0.167	0.114	0.12	30
	SE	-	0.063	0.028	0.021	0.015	0.016	-
2n=56 NF=72 West	Mean	5	0.838	1.206	0.169	0.114	0.127	31.25
	SE	-	0.069	0.029	0.021	0.015	0.017	-
2n=56 NF=72 South	Mean	4	0.656	1.108	0.089	0.06	0.069	15.63
	SE	-	0.058	0.022	0.017	0.012	0.013	-
2n=58 NF=72	Mean	9	0.531	1.086	0.068	0.047	0.05	11.25
	SE	-	0.055	0.02	0.015	0.011	0.011	-
2n=60 NF=74	Mean	1	0.394	1.00	0.00	0.00	0.00	0.00
	SE	-	0.039	0.00	0.00	0.00	0.00	-
2n=60 NF=76	Mean	16	1.031	1.284	0.237	0.161	0.166	42.5
	SE	-	0.072	0.03	0.023	0.016	0.017	-
2n=60 NF=78	Mean	12	1.106	1.286	0.249	0.167	0.174	46.88
	SE	-	0.072	0.029	0.023	0.016	0.016	-
2n=60 NF=80	Mean	8	1.044	1.249	0.219	0.146	0.156	41.25
	SE	-	0.07	0.028	0.022	0.015	0.016	-
2n=60 NF=82	Mean	4	0.90	1.236	0.194	0.134	0.153	32.5
	SE	-	0.068	0.029	0.023	0.016	0.018	-
2n=60 NF=84	Mean	9	1.025	1.285	0.236	0.161	0.17	41.88
	SE	-	0.071	0.031	0.023	0.016	0.017	-
N. ehrenbergi	Mean	12	1.069	1.257	0.223	0.149	0.156	43.13
	SE	-	0.07	0.029	0.022	0.015	0.016	-
Total	Mean	-	0.93	1.219	0.184	0.125	0.125	33.47
	SE	-	0.016	0.007	0.005	0.004	0.004	3.37

principal coordinate. In fact, this separation was in accordance with the geographical regions and ecological structure of Turkey. The first group which inhabits mostly in western part has chromosome forms with low diploid numbers. The second group contains chromosome forms with high diploid numbers, which distributed in mostly Central Anatolia. The other group has 2n=50, and showed insular distributional area in the eastern part of Turkey.

The total gene diversity (H_T) and gene diversity within populations (H_S) were 0.3050 and 0.1338, respectively. The coefficient of overall genetic differentiation (G_{ST}) among populations was 0.5617. The degree of population genetic subdivision measured by gene fixation index revealed that a high level of genetic differentiation was found among the populations of *N. xanthodon* and *N. ehrenbergi* (0.44 to 0.63). For the chromosomal races of *N. xanthodon*, the highest G_{ST} values were observed between 2n=60 NF=74 and the chromosome forms with low diploid numbers (2n=36, 38, 40, 50, 52 South, 54, 56 West, 58), ranged from 0.61 to 0.83). The lowest G_{ST} was detected among 2n=60 with the exception of 2n=60 NF=74 (0.07 to 0.14).

Nm values for examined populations were detected as 0.3901. The pairwise *Nm* value between *N. xanthodon* and *N. ehrenbergi* ranged from 0.29 to 0.98. The pairwise *Nm* values between chromosomal races of *N. xanthodon* ranged from 0.10 (2n=60 NF=74 and 2n=56 West) to 1.36 (2n=36 and 2n=52 South). *Nm* values for only 10 pairs (observed between different NF values of 2n=60 with the exception of 2n=60 NF=74) out of entire population pairs were estimated to be larger than 2, so the genetic differentiation among populations was small.

Consensus network constructed among chromosomal races based on pairwise G_{ST} genetic diversity was illustrated in Fig. 3. There was clear separation of the four major groups. These groups were the central, the western, the eastern and *N. ehrenbergi*.

AMOVA was used to examine genetic variation of subterranean mole rats in Turkey within and between populations of *N. xanthodon* and *N. ehrenbergi*. The result of AMOVA indicated that 55% of total variation was attributable to the differences among populations while the remaining 45% was due to the variation within populations. The result of a random permutation test indicated that two variance component were both highly significant (P<0.001).

Phylogenetic reconstruction

Genetic distances between populations ranged from 0.03 to 0.46, with a mean of 0.237. The genetic distances between *N. xanthodon* and *N. ehrenbergi* populations were strongly different, varying from 0.22 to 0.38. The genetic distances within the 2n= 60 dramatically decreased (0.03 to 0.17), while genetic distances between 2n=60 NF=74 and the other populations strongly increased (0.11 to 0.46).

UPGMA dendrogram of the chromosomal races of *N. xanthodon* and *N. ehrenbergi* based on Nei's unbiased genetic distance were showed in Fig. 4. Blind mole rat populations were grouped in six major clusters. Chromosomal races with low diploid number (2n=36, 2n=38, 2n=40, 2n=54) inhabited from mostly western part of Turkey formed a monophyletic group. On the other hand, these chromosomal races were well separated from each other and the distance between each group was significant.

Chromosomal races (2n=50 NF=70 and NF=72) from eastern part of Turkey formed a distinct monophyletic group. The chromosome forms with high diploid number (2n=56 South, 2n=58, 2n=60) distributed in central Anatolia clustered in the third monophyletic group. The highest similarity was obtained in this group between 2n=60 and 2n=58; 2n=60 and 2n=56 South. Chromosomal races (2n=52 North and 2n=52 South) distributed in peripheral central Anatolia of *N. xanthodon* clustered a distinct monophyletic group and joined with the group clustering the chromosomal races with high diploid number. *N. ehrenbergi* was well separated from the eastern Anatolian and chromosomal races with low diploid number. It was found closely related with chromosomal races with high diploid number and 2n=56 West form more than each other.

Discussion

Although the subterranean mole rats originated from Anatolia according to fossil (Hofmeijr, De Bruijn 1985) and genomic records (Catzeflis *et al.* 1989), only a few studies have been carried out on genetics of Turkish mole rat species. Furthermore, taxonomic

status and phylogeny of different *Nannospalax* chromosomal races distributed in Turkey are unclear. To date, morphologic (Kivanç 1988, Kankiliç *et al.* 2006), cytogenetic (Sözen *et al.* 2006a, Ivanitskaya *et al.* 2008, Kankiliç *et al.* 2007a, Arslan *et al.* 2010a), allozyme (NEVO *et al.* 1994, 1995, Kankiliç *et al.* 2005), rRNA and mtDNA (Nevo, Beiles 1992, Nevo *et al.* 1993, Arslan *et al.* 2010b) variations has been investigated between and within two species and some of chromosomal races from Turkey for dealing with this taxonomic complexity. So far, about 40 chromosomal race of *N. xanthodon* have been described by karyological studies. 2n=62 chromosomal races were only determined by Nevo *et al.* (1995) and Suzuki *et al.* (1996). Recent studies have shown that 2n=62 are not a valid chromosomal race for Turkish mole-rats and should be excluded from chromosomal race list of Turkish *N. xanthodon* (Ivanitskaya *et al.* 2008). Population in all localities shown with 2n=62 by previous studies (Nevo *et al.* 1995, Suzuki *et al.* 1996) were detected with 2n=60 as a result of this study. Similar finding were observed for Bolu population, although Nevo *et al.* (1995), Suzuki *et al.* (1996) mentioned Bolu population with 2n=54, our results revealed that Bolu population has 2n=52. Thus, 2n=60 and 2n=52 North are respectively considered instead of 2n=62 (central Anatolia) and 2n=54 (Bolu).

Species discrimination by RAPD-PCR

RAPD markers were employed in order to determine differentiation of subterranean mole rat populations in Turkey. Five enzymatic loci (*Adh*, *Me–1*, *Pt–1*, *Mpi*, *Np*) fixed different alleles between species from Turkey were shown by an allozyme study (Nevo *et al.* 1995). Similar differences were found in this study and 151 polymorphic bands for *N. xanthodon* and 102 polymorphic bands for *N. ehrenbergi* were determined. Of the total 160 bands scored, 52 for *N. xanthodon* and 4 for *N. ehrenbergi* were considered as diagnostic bands.

Nevo *et al.* (1995) noticed considerable allozyme differences in alleles shared between chromosomal races, and therefore they can be considered as different species. High polymorphism level was observed within species, resembling results of allozyme study and was also found in chromosomal races in this study. Numbers of polymorphic band ranged from 67 (2n=58) to 120 (2n=52 North) in RAPD analysis. One diagnostic band was found for 2n=36, 2n=38, 2n=40 and 2n=56 West, two for 2n=52 North and four for 2n=54. Ribosomal DNA and mitochondrial DNA variations for three

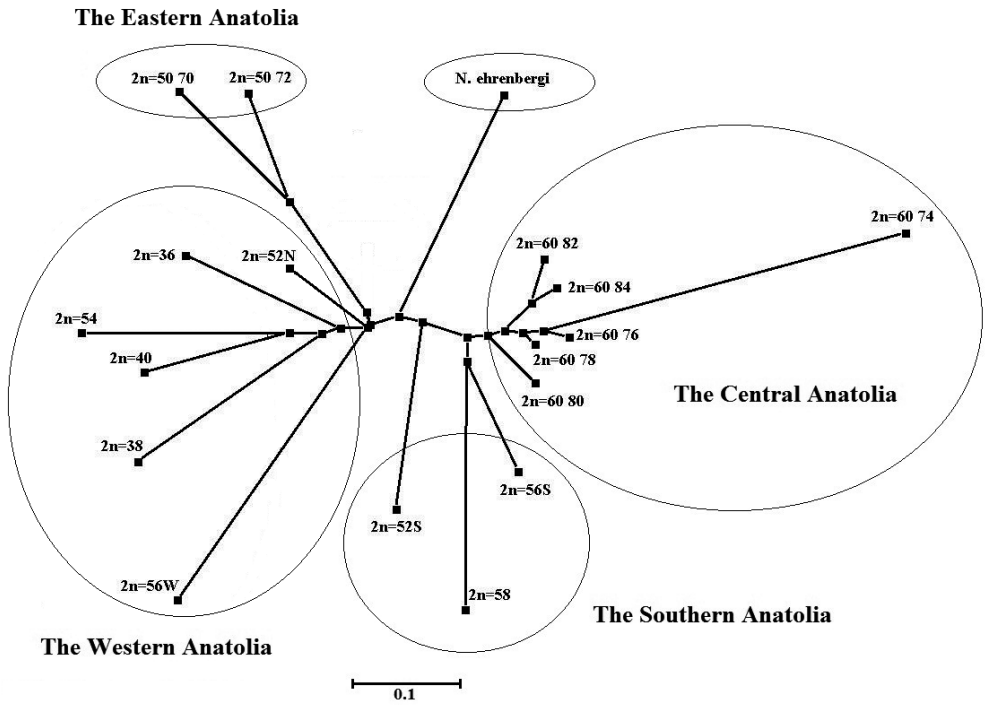

Fig. 3. Consensus network among chromosomal races of *N. xanthodon* and *N. ehrenbergi* based on pairwise G_{ST} genetic diversity.

chromosomal races (2n=38, 54, 62) in *N. xanthodon* and two (2n=52, 58) in *N. ehrenbergi* were investigated in the genus *Nannospalax* from Turkey (Suzuki *et al.* 1996). While variation within each species was found about 4%, as a results of the rDNA and mtDNA analysis, sequence divergence of rDNA spacers between members of species was calculated as approximately 8%. Furthermore, the sequence divergence of mtDNA among haplotypes of *N. ehrenbergi* and *N. xanthodon* was determined as 10% and 12%, respectively. When entire results of allozyme (Nevo *et al.* 1995), rDNA–mtDNA (Suzuki *et al.* 1996, Arslan *et al.* 2010b) and present study focused on Turkish mole–rats are evaluated, it was shown that most of these chromosomal races of both species show differences in the species level. Moreover other studies based on cytogenetic techniques for Turkish mole rats showed that populations with different NF values within chromosomal races had even considerable differences (Ivanitskaya *et al.* 2008, Arslan *et al.* 2010a). The pairwise value of gene fixation index (G_{ST}) among some chromosomal races is almost in similar that of between populations of two species. Particularly, the pairwise value of gene fixation index (G_{ST}) between 2n=60 and four chromosomal races (2n=36, 2n=38, 2n=40, 2n=56 West) is as high as between species. Populations with 2n=52 North diploid chromosome number from Bolu (loc. 17-22) are the most polymorphic chromosomal race in terms of Shannon's diversity index (I=0.343), numbers of effective allele (n_e=1.397) and estimated heterozgoty (He=0.232). This result is consistent with Suzuki *et al.* (1996) who found that the Bolu population was distinct haplotypes determined by mtDNA.

Geographic distribution of populations and genetic distance

To date, studies on the genus *Nannospalax* have

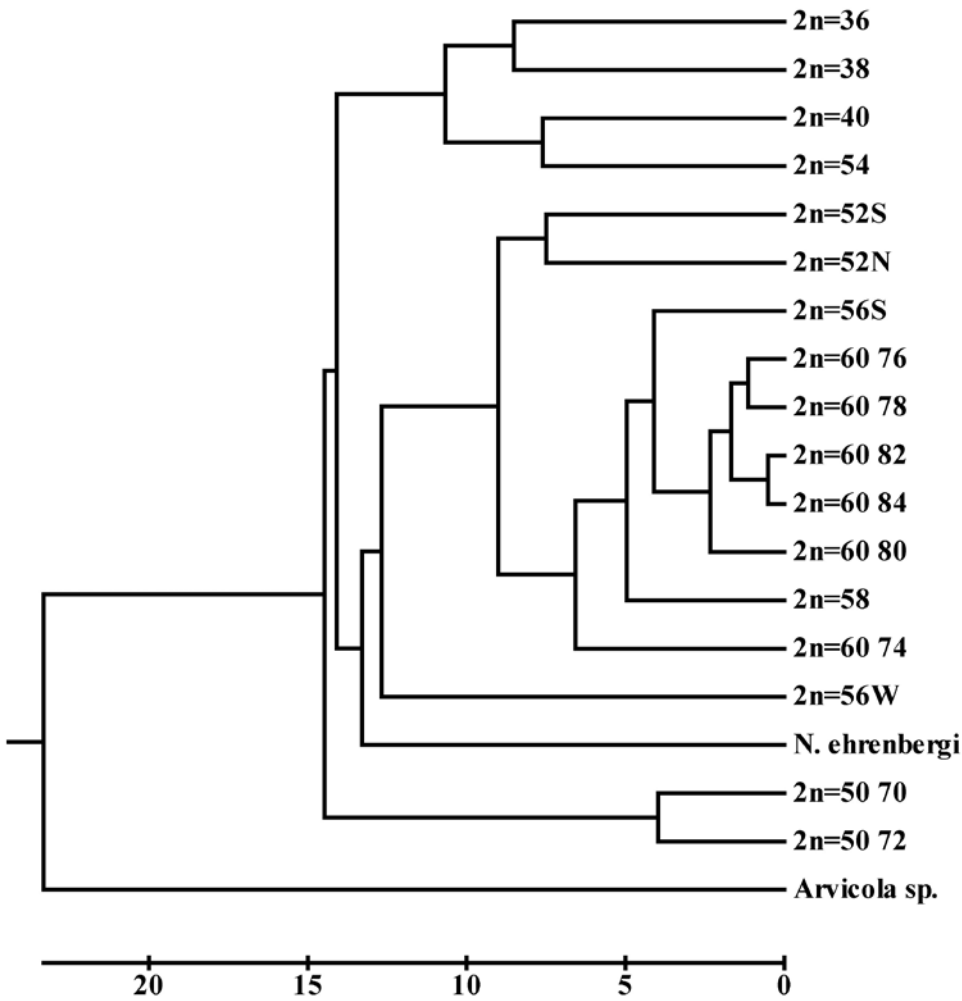

Fig. 4. UPGMA dendrogram showing genetic relationships among populations, based on Nei's genetic distance measure.

especially focused on mole rats of Israel. Increased in diploid chromosome number and heterozygosity values observed in this species were positively correlated with increasing steppe conditions. These values were tend to increase from 2n=52 to 2n=60 through to south desert conditions. Following these finding, Nevo *et al.* (1995) focused on Turkey to find out whether this situation in Israel is an exception or a general tendency in all distribution areas of *Nannospalax*. It has been shown that diploid chromosome number of Turkish *Nannospalax* was positively correlated with increasing steppe conditions, as it was in Israel. Nevo *et al.* (1995) suggested that heterozygoty value arisen from allozyme as well as diploid chromosome number tends to increase in central Anatolia plateau which was ecologically arid and hard, climatically variable and geologically newly established. However, our findings indicated that diploid chromosome number and heterozygoty value did not increase in such conditions and these values alter even in some populations distributed as closely as three or five km in same area. A good example for explaining this situation was observed in 2n=40 (loc. 6) and 2n=60 (loc. 44) populations. Samples belonging to these two populations were collected from two different localities that are not isolated geographically and being only 5 km away from each other. The gene flow between populations sharing the same ecological conditions was mostly higher than pairs of population lived in the different geographical conditions (Fig. 1 and Fig. 3).

Turkey has variable geographic structure at even close area, and comprises blind mole rats exhibiting high levels of genetic diversity even at a local scale. These conditions are an important factor leading to speciation in mole rats. Mole rats have adapted to subterranean life and rarely appear in surface, and slowly move under the ground. Thus, habitat structure, altitude, climatic conditions along with geographical, and ecological factors play role on speciation of mole rats. Therefore, mole rats differ without complete geographical barrier due to its limited dispersal abilities. Present geographical barriers have also accelerating genetic differentiation among chromosomal races. Southern Anatolia mountain chain (Taurus Mountain) separate absolutely two species, and chromosomal races found at the west and east of *N. xanthodon* are isolated by Anatolia diagonal. When geographical structure and distribution areas of main chromosomal race are considered together, it was clearly shown that populations with different chromosome number were completely isolated from each other by some geographic barriers and clustered as four main groups (Fig. 2 and 4). The first group includes 2n=60 with different NF values widely distributed in Central Anatolia. The second group contained 2n=52 South, 56 South, and 58 consisting of small isolated populations adapted different ecological conditions in southern borders of distribution areas of 2n=60. The third group is composed of the chromosomal races with lower diploid chromosome number (2n=36, 38, 40, 52 North, 54, 56 West), living at the edge of river, lake and being nearer costal area in western Turkey. The fourth group contains 2n=50 found between Caucasia geographical region and plateaus with high altitude of Central Anatolia. This chromosomal race is separated by Anatolia diagonal extending from north to south from both 2n=60 and its peripheral derivatives distributed in Central Anatolia. Geographically separating in four different groups was accurately seen in 2 dimensional plot obtained from PCO analysis based on RAPD scores, UPGMA dendrogram and network constructed on the basis of pairwise G_{ST} (Fig. 2 and 4). Distinct chromosomal races within these four groups are also clustered in different regions and separate from each others. 2n=60 widely distributed in Central Anatolia, are separated from other chromosomal races found in western Anatolia by Boz Mountain, Aydın Mountains, Gediz River and Menderes River. Köroğlu and Ilgaz Mountains, Sakarya and Kızılırmak rivers separate 2n=60 from 2n=52 in northern. Kızılırmak River seems to be a geographic barrier between 2n=60 and 2n=54. 2n= 60 chromosomal race differ from 2n=50 as a result of the isolation caused by East Toros Mountains, Yeşilırmak and Fırat River. Finding of this study showed that same isolation mechanisms also works on four main groups living in Central, Eastern, Western and Northern Anatolia. For example 2n=36 and 2n=38 are separated by Aydın Mountains and Menderes River, 2n=40 and 2n=56West are separated by Dedegöl Mountains. Pairwise genetic distance values (D_s=0.18; 0.40; 0.28, respectively) among these chromosomal races (2n=36–2n=38, 2n=60–2-n=54, 2n=40–2n=56W) has showed that these populations are strongly isolated from each other.

Peripheral speciation and small isolate populations

In chromosomal speciation, a differentiation takes place in peripheral populations as a consequence of Robertsonian fusion first occurring and/or tandem fusion (King 1981, White 1978) and lead to beginning of isolation from main population. Since these small populations are not differing from main population genetically, a small hybrid zone occurs between isolated population and their contact with main population continuous. Genetic and morphological differences appear among populations having chromosomal differences by time and newly splitting populations disperse into new areas or habitats (White 1978). Peripatric speciation mechanism in Peripheral small isolates observed in other rodent species (King 1985) were described in allozyme level (Nevo 1989), nuclear and mitochondrial DNA levels (Nevo, Beiles 1992, Nevo *et al.* 1993) for mole-rats of Israel. In present study, it was determined that small isolated populations with a fixed different chromosome number (2n=52 South, 2n=56, 2n=58) found in periphery of the main populations (2n=60) having larger distribution. However, no hybrid zone among Turkish mole-rats has been found so far. Turkish mole rats which include ancestor populations highly differentiated from each other and no longer can make up hybrids. It has been shown that existence of 2n=60 chromosomal race which has longer distribution and has small peripheral populations supporting peripheral speciation. The results of UPGMA, network, PCO and Nei's genetic distance value indicate that small populations (2n=52 South, 2n=56, 2n=58) are different from 2n=60. A similar pattern was shown between 2n=38 chromosomal race having wide distribution in western Anatolia and peripheral populations living in its distribution borders (2n=36, 2n=40, 2n=52 North). Also, 2n=52 living in North and 2n=52 found in South are differently clustered by the results of network and PCO due to having different ancestors. Therefore, forms with the same diploid chromosome number living in different geographical areas should be evaluated as distinct populations.

Consequently, we have shown that subterranean mole rats in Turkey exhibiting high levels of genetic diversity even at a local scale. Genetic distance (D_s) indicates that most populations analyzed in this study may be considered as valid biological species, because chromosomal races showed as high as D_s value existed between *N. ehrenbergi* and *N. xanthodon*. Some chromosomal races such as 2n=60 and 2n=54 in Central Anatolia, 2n=36 and 2n=38 from Western Anatolia, 2n=52N from Northern Anatolia, 2n=40 from Southwest Anatolia, and 2n=50 from Eastern Anatolia could be considered valid biological species based on D_s. This study provides baseline genetic information for future studies. In the future, all chromosomal races should be analyzed using other genetic markers such as microsatellite and mitochondrial DNA to clarify taxonomic status of chromosomal races as well as their phylogeographic analyses in Turkey. Place names are given in Table 1 for each number.

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