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Revised classification design of the Anatolian species of *Nannospalax* (Rodentia: Spalacidae) using RFLP analysis

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Abstract: Genetic differences in 2 regions (D-loop and cytochrome *b*) of mitochondrial DNA in *Nannospalax xanthodon* and *Nannospalax ehrenbergi* were determined using restriction fragment length polymorphism (RFLP) analysis. For 2 species distributed in Anatolia, 94 specimens from 62 populations of *Nannospalax xanthodon* and 2 populations of *Nannospalax ehrenbergi* were studied. To reveal genetic differences, the D-loop (626 bp) and cytochrome *b* (500 bp) regions of mitochondrial DNA were amplified and then cut by 4 different restriction endonuclease enzymes (BamH-I, Taq-I, Alu-I, and Msp-I). RFLP analysis revealed 4 haplotypes for the D-loop region and 8 haplotypes for the cytochrome *b* region in 94 specimens. According to the results of this study, *N. nehringi*, which is distributed in eastern Anatolia, is a valid species and not a synonym of *N. xanthodon*. A total of 2 additional species (*N. nehringi* and *N. labaumei*) occur with *N. xanthodon* and *N. ehrenbergi*; thus, Anatolian blind mole rats cannot be represented by only 2 species.

Key words: *Nannospalax*, mtDNA, Turkey

1. Introduction

Representatives of the genus *Nannospalax* (=*Microspalax* of Topachevski, 1969), which are rodents that use subterranean ecological niches, were assumed to have originated in Anatolia (Savic and Nevo, 1990). Today, the genus is distributed in southeastern Europe, southwestern Russia and Ukraine around the Black Sea and Caspian Sea, Asia Minor, Caucasia, the Middle East adjacent to the Mediterranean, and northeastern Africa (Wilson and Reeder, 2005). The taxonomy of the genus *Nannospalax* is currently unstable and in need of revision. Studies based on morphology have shown that only 3 species (*N. ehrenbergi*, *N. nehringi*, and *N. leucodon*) are present in Turkey (Wilson and Reeder, 2005). However, early studies accepted that *N. leucodon* was distributed throughout Turkey except for southeastern Anatolia (*N. ehrenbergi*) (Méhely, 1909; Ognev, 1947; Corbet, 1978; Kıvanç, 1988; Harrison and Bates, 1991). Recently, Kryštufek and Vohralik (2009) reported that Anatolia contained only *N. xanthodon* and *N. ehrenbergi*. *N. nehringi* was considered a synonym of *N. xanthodon* by Kryštufek and Vohralik (2009). However, the validity of *N. xanthodon* in Anatolia is still debated.

The genus *Nannospalax* is well known for its large variation in chromosome numbers, and the diploid chromosome number (2n) varies between 36 and 62 (reviewed by Nevo et al., 2001). However, the main center of chromosomal diversity of *Nannospalax* is Anatolia, which harbors approximately 60% of the cytotype diversity (Nevo et al., 2001). Karyotypic data of genus *Nannospalax* showed that 3 cytotypes (2n = 36, 38, and 40) are unique among the mole rats of *Nannospalax* and were solely reported from western Anatolia (Nevo et al., 1995, 2001; Ivanitskaya et al., 1997; Sözen and Kıvanç, 1998; Sözen, 2004; Matur and Sözen, 2005; Sözen et al., 2006a, 2006b, 2011; Kankılıç et al., 2007a, 2007b, 2009, 2010; Matur et al., 2011).

N. leucodon has the broadest distribution in the Balkans and Thrace. *N. leucodon* has 30 cytotypes with diploid chromosome numbers and fundamental numbers of chromosomal arms in ranges of 2n = 46–58 and NFa = 72–94 (Savic and Nevo, 1990; Sözen, 2004). These species have just 2 cytotypes in Thrace: 2n = 56, NFa = 72 in Gelibolu-Eceabat (Sözen, 2004) and 2n = 56, NFa = 74 in the remaining part of Thrace (Sözen et al., 2006a).

A total of 28 different cytotypes, diploid chromosome numbers, and fundamental numbers of chromosomal arms in ranges of 2n = 36–62 and NFa = 64–84 are present in *N. xanthodon*, which is distributed largely in Anatolia (Nevo et al., 1995; Ivanitskaya et al., 1997; Sözen and Kıvanç, 1998; Sözen, 2004; Sözen et al., 2006b; Kankılıç et al., 2007a, 2007b, 2009, 2010; Matur et al., 2013; Sözen et al., 2013).

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N. ehrenbergi, which is found in Jordan, Israel, Egypt, and Turkey, had values of 2n = 48–62 and NFa = 62–86. Studies performed on Turkish mole rats revealed 12 cytotypes with different chromosomal sets (2n = 48, 52, 54, 56, 58 and NFa = 62, 64, 68, 70, 72, 76, 78, 82) (Nevo et al., 1995; Ivanitskaya et al., 1997; Coşkun et al., 2006).

Consequently, the number of described cytotypes of the 3 species in Turkey is 42 in total. Even if morphological studies could provide results concerning the taxonomical status of this species, researchers have not agreed on a cytotype classification despite recent karyological studies. Due to the high number of determined cytotypes and the similarity of morphological features, it is difficult to define Turkish cytotypes as distinct taxa. For this reason, many researchers classify the present species as a "superspecies", which is not accepted as a taxonomical category (Savic and Nevo, 1990; Nevo et al., 1995; Coşkun et al., 2010).

Although Turkey is the genetic source of blind mole rats, studies on Turkish mole rats to date have mainly focused on chromosomes and morphology. Only a limited number of genetic studies have been carried out on Turkish mole rat cytotypes. Phylogenetic studies using data from allozymes (Nevo et al., 1995), randomly amplified polymorphic DNA (Kankılıç et al., 2013), mitochondrial sequences (Arslan et al., 2010; Hadid et al., 2012; Kandemir et al., 2012; Kryštufek et al., 2012), and cytogenetics (Ivanitskaya et al., 2008; Arslan et al. 2011) have been conducted in an attempt to understand relationships within *Nannospalax*. These molecular studies with limited cytotype sampling supported the monophyly of the genus and recognized 4 major clades (*vasvarii*, *leucodon*, *xanthodon*, and *ehrenbergi*) (Hadid et al., 2012). The clade *vasvarii* represents the cytotypes (2n = 62 and 60) from the Central Anatolian Plateau. None of the previous molecular analyses included all of the cytotypes that have been recognized from Turkey. In particular, the *nehringi* group (2n = 48 and 50) and some cytotypes of the *vasvarii* group (2n=52, 56, and 58) were also missing in previous molecular studies.

Currently, in light of all the information that we know about Turkish mole rats, it can easily be seen that the taxonomic status of Turkish mole rat species and subspecies remains uncertain as some cytotypes have genetic differences on the species level (Nevo et al., 1995; Arslan et al., 2010; Kandemir et al., 2012). Therefore, the validity of the taxonomic status of all species and subspecies previously described from Turkey should be investigated to form a foundation for understanding taxonomic relationships.

The purpose of this study was to ascertain the diversity of cytotypes in a range of species to help in clarifying species relationships and to provide a general picture of taxonomic relationships of the most prevalent cytotypes in Turkey.

2. Materials and methods

2.1. Collection and preparation of material

A total of 94 mole rat specimens were collected in 62 localities for restriction fragment length polymorphism (RFLP) analysis (Table 1; Figure 1). A total of 12 specimens

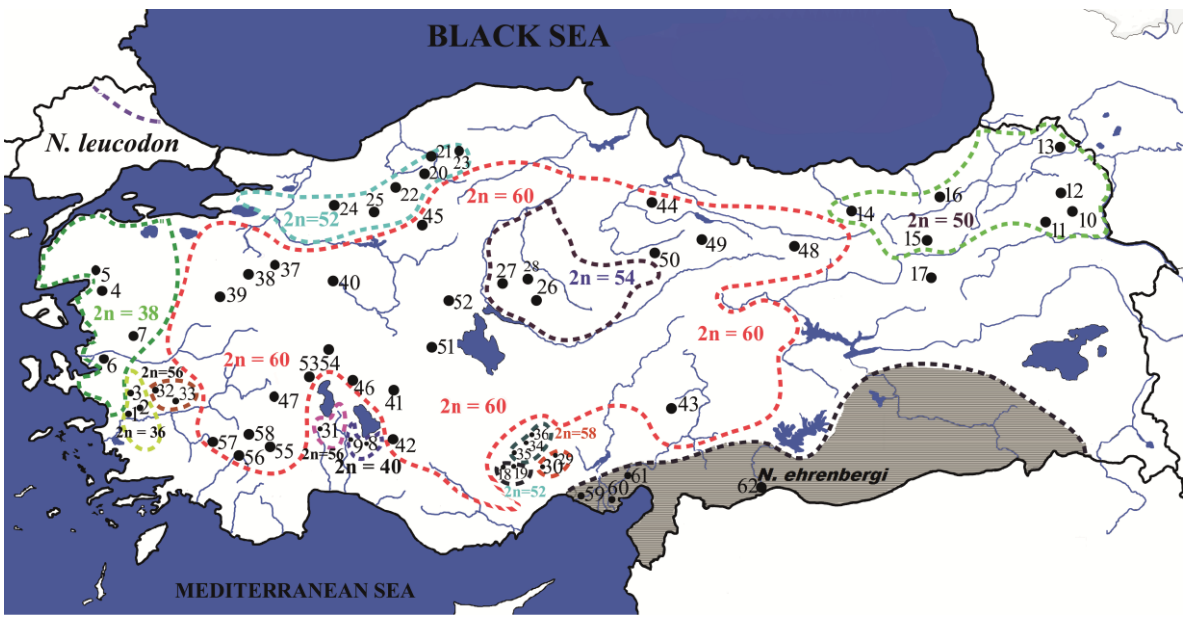

Figure 1. Sampling localities of *Nannospalax xanthodon* and *Nannospalax ehrenbergi* from Turkey for molecular studies. Names for numbered localities indicated in Table 1.

Table 1. Laboratory registration numbers and locations of the samples of blind mole rats analyzed in Turkey. MN: Map number (see Figure 1).

N	MN	Museum Number	Locality	2n, NF	Altitude (m)	Latitude (N)	Longitude (E)
1	1	6256♀	Koçarlı (Aydın)	2n = 36 NF = 68	35	37°44′	27°34′
2	1	6250♀	Koçarlı (Aydın)	2n = 36 NF = 68	35	37°44′	27°34′
3	2	6207♀	Koçarlı (Aydın)	2n = 36 NF = 68	26	37°44′	27°33′
4	2	6209♂	Koçarlı (Aydın)	2n = 36 NF = 68	26	37°44′	27°33′
5	3	6197♀	Koçarlı (Aydın)	2n = 36 NF = 68	41	37°45′	27°34′
6	4	6161♀	Çömlekçi (Balıkesir)	2n = 38 NF = 74	417	39°29′	28°02′
7	5	6160♀	Kepsut (Balıkesir)	2n = 38 NF = 74	80	39°41′	28°10′
8	6	6132♀	Foça-Bağarası (İzmir)	2n = 38 NF = 74	100	38°40′	26°45′
9	6	6165♂	Foça-Bağarası (İzmir)	2n = 38 NF = 74	100	38°40′	26°45′
10	7	6131♂	Akhisar (Manisa)	2n = 38 NF = 74	132	38°55′	27°51′
11	7	6153♀	Akhisar (Manisa)	2n = 38 NF = 74	132	38°55′	27°51′
12	8	6267♀	Yeşildağ (Konya)	2n = 40 NF = 72	1130	37°32′	31°28′
13	8	6263♀	Yeşildağ (Konya)	2n = 40 NF = 72	1130	37°32′	31°28′
14	8	6268♀	Yeşildağ (Konya)	2n = 40 NF = 72	1130	37°32′	31°28′
15	9	6225♀	Yenişarbademli (Isparta)	2n = 40 NF = 72	1210	37°42′	31°23′
16	9	6254♂	Yenişarbademli (Isparta)	2n = 40 NF = 72	1210	37°42′	31°23′
17	9	6262♀	Yenişarbademli (Isparta)	2n = 40 NF = 72	1210	37°42′	31°23′
18	10	4887♀	Susuz (Kars)	2n = 50 NF = 70	1930	40°46′	43°20′
19	11	4880♂	Selim (Kars)	2n = 50 NF = 70	1955	40°27′	42°47′
20	12	5306♀	Göle (Ardahan)	2n = 50 NF = 70	2196	40°47′	42°36′
21	12	5286♀	Göle (Ardahan)	2n = 50 NF = 70	2196	40°47′	42°36′
22	13	3369♀	City Center (Ardahan)	2n = 50 NF = 70	1881	41°06′	42°42′
23	13	3322♀	City Center (Ardahan)	2n = 50 NF = 70	1881	41°06′	42°42′
24	14	4642♂	Şebinkarahisar (Giresun)	2n = 50 NF = 72	2174	40°26′	38°24′
25	14	4645♂	Şebinkarahisar (Giresun)	2n = 50 NF = 72	2174	40°26′	38°24′
26	15	4671♀	Demirözü (Bayburt)	2n = 50 NF = 72	1744	40°09′	39°53′
27	15	4661♂	Demirözü (Bayburt)	2n = 50 NF = 72	1744	40°09′	39°53′
28	16	4669♀	Ovid Mountain (Rize)	2n = 50 NF = 72	1012	40°47′	40°32′
29	16	4648♂	Ovid Mountain (Rize)	2n = 50 NF = 72	1012	40°47′	40°32′
30	17	3324♂	Erzurum, 20 km E	2n = 50 NF = 72	2376	40°01′	41°27′
31	18	2169♀	Sebil Plateau (Mersin)	2n = 52 NF = 72 South	1080	37°07′	34°33′
32	18	2165♂	Sebil Plateau (Mersin)	2n = 52 NF = 72 South	1080	37°07′	34°33′
33	18	2166♀	Sebil Plateau (Mersin)	2n = 52 NF = 72 South	1080	37°07′	34°33′
34	19	3851♀	Çamlıyayla (Mersin)	2n = 52 NF = 72 South	2024	37°16′	34°33′
35	19	2658♂	Çamlıyayla (Mersin)	2n = 52 NF = 72 South	2024	37°16′	34°33′
36	20	4009♂	Yeniçağa (Bolu)	2n = 52 NF = 70 North	1000	40°46′	32°01′
37	21	3985♂	Demirciler-Mengen (Bolu)	2n = 52 NF = 70 North	690	40°57′	32°05′
38	22	4849♀	Seben (Bolu)	2n = 52 NF = 70 North	761	40°24′	31°34′
39	23	4830♂	Gerede (Bolu)	2n = 52 NF = 70 North	1326	40°47′	32°11′
40	24	4201♂	Abant (Bolu)	2n = 52 NF = 70 North	868	40°43′	31°32′
41	25	4203♀	Mudurnu (Bolu)	2n = 52 NF = 70 North	880	40°27′	31°12′
42	26	4572♀	Seyfe Lake (Kırşehir)	2n = 54 NF = 74	1135	39°11′	34°20′
43	26	4571♀	Seyfe Lake (Kırşehir)	2n = 54 NF = 74	1135	39°11′	34°20′
44	27	5170♀	Kırıkkale, 5 km E	2n = 54 NF = 74	744	39°50′	33°33′
45	27	5172♂	Kırıkkale, 5 km E	2n = 54 NF = 74	744	39°50′	33°33′
46	28	5452♂	Kırıkkale, 10 km E	2n = 54 NF = 74	773	39°51′	33°35′
47	29	3499♂	Elmalı-Gülek (Mersin)	2n = 56 NF = 72 South	1835	37°22′	38°45′
48	29	3497♂	Elmalı-Gülek (Mersin)	2n = 56 NF = 72 South	1140	37°15′	34°46′
49	30	3500♀	Karboğazı-Gülek (Mersin)	2n = 56 NF = 72 South	1305	37°18′	34°43′
50	31	4793♀	Aksu (Isparta)	2n = 56 NF = 72 West	1227	37°47′	30°59′

Table 1. Continued.

N	MN	Museum Number	Locality	2n, NF	Altitude (m)	Latitude (N)	Longitude (E)
51	31	6270♀	Aksu (Isparta)	2n = 56 NF = 72 West	1227	37°47′	30°59′
52	32	6141♀	Kula (Manisa)	2n = 56 NF = 72 West	661	38°32′	28°38′
53	32	6135♂	Kula (Manisa)	2n = 56 NF = 72 West	661	38°32′	28°38′
54	33	6224♀	Gediz Sapağı (Uşak)	2n = 56 NF = 72 West	691	38°40′	29°14′
55	34	2220♂	Madenköy (Niğde)	2n = 58 NF = 72	1717	37°26′	34°37′
56	34	2433♀	Madenköy (Niğde)	2n = 58 NF = 72	1717	37°26′	34°37′
57	35	2337♂	Karagöl (Niğde)	2n = 58 NF = 72	2588	37°24′	34°33′
58	35	2333♂	Karagöl (Niğde)	2n = 58 NF = 72	2588	37°24′	34°33′
59	36	2656♂	Alpu (Adana)	2n = 58 NF = 72	1060	37°28′	34°52′
60	37	6148♂	Bozüyük (Bilecik)	2n = 60 NF = 76	754	39°54′	30°02′
61	38	6145♂	Küplüce (Kütahya)	2n = 60 NF = 76	1132	38°59′	29°03′
62	39	5848♂	Emet (Kütahya)	2n = 60 NF = 76	904	39°20′	29°15′
63	40	5944♂	Günyüzü (Eskişehir)	2n = 60 NF = 76	888	39°23′	31°48′
64	41	4310♂	Kıreli (Konya)	2n = 60 NF = 76	1150	37°55′	31°32′
65	42	3396♀	Akseki (Antalya)	2n = 60 NF = 78	1252	37°02′	31°46′
66	43	3619♂	Göksun (Maraş)	2n = 60 NF = 78	1341	38°01′	36°29′
67	44	5049♀	Kavak (Samsun)	2n = 60 NF = 78	610	41°04′	36°02′
68	45	4835♂	Çeltikli (Ankara)	2n = 60 NF = 78	688	40°07′	32°03′
69	46	4790♂	Madenli (Isparta)	2n = 60 NF = 78	1032	38°11′	31°06′
70	47	6231♀	Atabey (Isparta)	2n = 60 NF = 78	1037	37°57′	30°38′
71	48	4657♂	İmranlı (Sivas)	2n = 60 NF = 80	1611	39°52′	38°06′
72	49	4644♀	Yıldızeli (Sivas)	2n = 60 NF = 80	1401	39°51′	36°35′
73	50	5289♀	Saraykent (Yozgat)	2n = 60 NF = 80	1316	39°39′	35°52′
74	51	4541♂	Cihanbeyli (Konya)	2n = 60 NF = 80	973	38°39′	32°55′
75	52	4534♂	Bala (Ankara)	2n = 60 NF = 80	1324	39°33′	33°07′
76	53	2504♂	Afyon, 10 km NE	2n = 60 NF = 82	1009	38°48′	30°32′
77	53	6214♂	Afyon, 10 km NE	2n = 60 NF = 82	1009	38°48′	30°32′
78	54	5563♀	Eber Gölü (Afyon)	2n = 60 NF = 82	1009	38°35′	31°07′
79	55	2516♀	Burdur, 5 km S	2n = 60 NF = 84	985	37°42′	30°16′
80	56	6234♀	Çameli (Denizli)	2n = 60 NF = 84	1300	37°04′	29°20′
81	57	6198♂	Acıpayam (Denizli)	2n = 60 NF = 84	926	37°25′	29°21′
82	58	6253♂	Yeşilova (Burdur)	2n = 60 NF = 84	1085	37°35′	29°54′
83	59	3850♀	Özbek Village (Tarsus)	2n = 56 NF = 72 *ehrenbergi*	46	36°58′	34°56′
84	59	3849♀	Özbek Village (Tarsus)	2n = 56 NF = 72 *ehrenbergi*	46	36°58′	34°56′
85	59	2081♀	Özbek Village (Tarsus)	2n = 56 NF = 72 *ehrenbergi*	46	36°58′	34°56′
86	59	2090♂	Özbek Village (Tarsus)	2n = 56 NF = 72 *ehrenbergi*	46	36°58′	34°56′
87	59	2935♂	Özbek Village (Tarsus)	2n = 56 NF = 72 *ehrenbergi*	46	36°58′	34°56′
88	59	2936♀	Özbek Village (Tarsus)	2n = 56 NF = 72 *ehrenbergi*	46	36°58′	34°56′
89	59	2106♀	Özbek Village (Tarsus)	2n = 56 NF = 72 *ehrenbergi*	46	36°58′	34°56′
90	60	2089♂	Tarsus, 5 km NE	2n = 56 NF = 72 *ehrenbergi*	15	36°55′	34°54′
91	60	2104♂	Tarsus, 5 km NE	2n = 56 NF = 72 *ehrenbergi*	15	36°55′	34°54′
92	61	2085♂	Yüreğir (Adana)	2n = 56 NF = 72 *ehrenbergi*	11	36°51′	35°25′
93	61	2091♀	Yüreğir (Adana)	2n = 56 NF = 72 *ehrenbergi*	11	36°51′	35°25′
94	62	2167♂	Kilis, 10 km E	2n = 56 NF = 72 *ehrenbergi*	664	36°43′	37°08′

of a single cytotype (2n = 56) of *N. ehrenbergi* and 82 specimens of 9 different cytotypes (2n = 36, 38, 40, 50, 52, 54, 56, 58, and 60) of *N. xanthodon* were evaluated; diploid chromosome numbers were previously determined by Kankılıç et al. (2007a, 2007b, 2009, 2010).

2.2. DNA isolation and polymerase chain reaction

Genomic DNA isolated from kidney and liver tissues using CTAB were modified according to Doyle and Doyle's (1991) protocol. D-loop and cytochrome *b* regions were amplified using polymerase chain reaction. The universal primers 1FR and 3RV (Reyes et al., 2003) were used in the D-loop reactions, and RL14724 (Suzuki et al., 2000) and UH15154 (Shinohara et al., 2003) were used in the cytochrome *b* reactions. Approximately 626 bp for the linear part of the D-loop region and 500 bp for the cytochrome *b* region were amplified.

The amplification reaction mixture of Kaya and 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), and 1.5 µL of 20 pmol primers. The PCR conditions included a denaturing step at 95 °C for 1 min, followed by 45 cycles of denaturing at 94 °C for 1 min, annealing at 36 °C for 2 min, and extension at 72 °C for 2 min. Final extension was performed at 72 °C for 15 min. The PCR product was digested by 4 restriction enzymes (Alu-I, BamH-I, Msp-I, and Taq-I) for the D-loop region and by 3 restriction enzymes (BamH-I, Msp-I, and Taq-I) for the cytochrome *b* region. The PCR product was incubated at 37 °C for 3–4 h until completely digested.

2.3. Statistical analysis

The PCR products were separated on 1.5% agarose gels in a 1X TAE (Tris-acetic acid-EDTA) buffer in an electrical field (70 mV, 4 h). For each sample, RFLP profiles were characterized in a matrix by scoring bands on agarose gel as present (assigned a value of 1) or absent (assigned a value of 0). The presence or absence of bands was scored for clear and reproducible bands with estimated fragment lengths of between 100 and 3000 bp (DNA Ladder Plus, Fermentas). The data were then imported into the software GenAlEx v. 6.2 (Peakall and Smouse, 2006) to estimate the parameters of genetic variation and differentiation. To determine the genetic variability of populations, the mean number of observed alleles per locus (n_a), the mean number of effective alleles (n_e), the percentage of polymorphic loci at the 95% significance criterion (P_{95}), and Shannon's information index (I) values and their standard errors were calculated. An analysis of molecular variance (AMOVA) was used to determine the total phenotypic variance within and among populations. The AMOVA input files were created with GenAlEx v. 6.2 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 NTSYSpc v. 2.2 (Rohlf, 2000); the pairwise genetic distance was calculated using Jaccard's coefficient for binary data. The resulting matrix was double-centered using the DCENTER module, and EIGEN was used to compute principal coordinates along all axes. The results of the first 2 axes were used to form a 2-dimensional plot with PAST v. 1.99 (Hammer et al., 2001). The neighbor-joining tree for chromosomal forms was constructed based on the matrix of Nei's pairwise genetic distances. The NJ tree was produced using the software POPGENE v. 1.32 and manipulated using the software MEGA 4 (Tamura et al., 2007).

3. Results

3.1. Results of RFLP analysis of mitochondrial DNA control region

After PCR amplification, a polymorphism in a part of the D-loop region of approximately 626 bp long was detected by cutting Alu-I, BamH-I, Msp-I, and Taq-I. A total of 8 restriction sites were detected. RFLP analysis of the mitochondrial DNA control region in 94 individuals from Anatolia yielded 4 haplotypes (Table 2). Only 2 (Alu-I and Msp-I) of the 4 restriction endonucleases produced these haplotypes. All other enzymes produced monomorphic restriction fragment patterns among all individuals.

D-loops of all specimens, excluding the 2n = 40 and 2n = 52 cytotypes from northern Turkey, were cut in only a single region by restriction endonuclease enzyme Alu-I. Two DNA bands with lengths of 210 and 416 bp were observed after cutting near this enzyme; thus, the 2n = 40 and 2n = 52 cytotypes were separated from the other cytotypes.

The Msp-I restriction endonuclease enzyme was not digested for all specimens of 2n = 52 from southern Turkey, 2n = 56 from eastern Turkey, 2n = 58, and 2n = 60; the enzyme was digested for only 2 specimens of the 2n = 36 cytotype in the D-loop region. Therefore, only 626 bp of DNA bands were observed on the agarose gel. Apart from these cytotypes, all specimens of the 2n = 36, 2n = 38, 2n = 40, 2n = 50, 2n = 52 N, 2n = 54, and 2n = 56 cytotypes from southern Turkey were digested, and DNA bands of 200 bp and 426 bp in length were detected. The BamH-I and Taq-I restriction endonuclease enzymes did not cut the D-loop region in any sample, and only a DNA band of 626 bp was obtained.

3.2. Results of RFLP analysis of cytochrome *b* region of mitochondrial DNA

A total of 500 bp of the cytochrome *b* locus in the mitochondrial DNA were amplified by PCR. The polymorphism was analyzed by cutting 3 restriction endonuclease enzymes (BamH-I, Msp-I, and Taq-I); a

Table 2. Restriction patterns for haplotypes in the mtDNA (D-loop and cytochrome *b*) fragment from 12 chromosomal races from Anatolia.

Restriction pattern for D-loop region				
Population	Alu-I	Msp-I	BamH-I	Taq-I
2n = 36	210, 416	200, 426, 626 (2)	626	626
2n = 38	210, 416	200, 426	626	626
2n = 40	626	200, 426	626	626
2n = 50	210, 416	200, 426	626	626
2n = 52 South	210, 416	626	626	626
2n = 52 North	626	200, 426	626	626
2n = 54	210, 416	200, 426	626	626
2n = 56 South	210, 416	200, 426	626	626
2n = 56 West	210, 416	626	626	626
2n = 58	210, 416	626	626	626
2n = 60	210, 416	626	626	626
N. ehrenbergi	210, 416, 626 (1)	200, 426	626	626
Restriction pattern for Cytochrome b region				
Population		Msp-I	BamH-I	Taq-I
2n = 36	-	200, 300, 500 (1)	500	230, 270
2n = 38	-	200, 300, 500 (2)	500	230, 270
2n = 40	-	200, 300, 500 (2)	500	230, 270
2n = 50	-	200, 300	500	200, 300, 500 (2)
2n = 52 South	-	500	500	500
2n = 52 North	-	200, 300, 500 (3)	500	230, 270
2n = 54	-	200, 300	500	200, 300
2n = 56 South	-	500	110, 390	200, 300
2n = 56 West	-	500	500	500
2n = 58	-	500	500	500
2n = 60	-	500	110 (3), 390 (3), 500	200, 300
N. ehrenbergi	-	200, 300	110, 130, 260	200, 300

total of 13 restriction sites were detected. RFLP analysis of the mitochondrial DNA cytochrome *b* region (500 bp) in 94 individuals from Anatolia yielded 8 haplotypes (Table 2). All restriction endonuclease enzymes produced these haplotypes.

The Msp-I restriction endonuclease enzyme digested the cytochrome *b* loci of *N. ehrenbergi* and those of some specimens of *N. xanthodon* (2n = 36, 4 specimens of 2n = 38, 4 specimens of 2n = 40, 2n = 50, 3 specimens of 2n = 52, and 2n=54) in 1 region; thus, 2 bands (200 bp and 300 bp in length) were defined on the agarose gel, although other cytotypes belonging to *N. xanthodon* (2n = 52 from southern Turkey, 2n = 56, 2n = 58, and 2n = 60) were not cut after the application of this enzyme.

The BamH-I restriction endonuclease enzyme digested the cytochrome *b* loci of the *N. xanthodon* cytotypes in 1 region and those of *N. ehrenbergi* in 2 regions. In the *N. xanthodon* cytotypes, only 2n = 56 S, 2n = 60, NF = 76 (1 sample) and 2n = 60, NF = 80 (2 specimens) were digested in 1 region. A total of 2 bands occurred with

lengths of 110 bp and 390 bp. Except for these cytotypes, other restriction sites for BamH-I were not detected in the remaining cytotypes of *N. xanthodon*. The cytochrome *b* region in *N. ehrenbergi* was digested in 2 sites producing 3 DNA bands, approximately 110 bp, 130 bp, and 260 bp in length. Different restriction patterns of BamH-I separated *N. xanthodon* from *N. ehrenbergi*.

Restriction sites of Taq-I in the cytochrome *b* region were not observed in 2 specimens of the 2n = 50 cytotype and in all specimens of the 2n = 52 S, 2n = 56 S, 2n = 58, and 2n = 60 cytotypes. When these specimens were excluded, Taq-I digested the cytochrome *b* in 1 region in all specimens. In the cytochrome *b* regions of the 2n = 56 S and 2n = 54 cytotypes of *N. xanthodon*, 11 specimens of the 2n = 50 cytotypes and all specimens of *N. ehrenbergi* were cut by Taq-I, and 2 bands were obtained with lengths of 200 and 300 bp. Additionally, the 2n = 36, 2n = 38, 2n = 40, and 2n = 52 cytotypes of *N. xanthodon* from northern Turkey had only 1 Taq-I restriction site. However, lengths of obtained DNA bands were 230 bp and 270 bp, distinct from other *N. xanthodon* cytotypes.

Estimates of Nei's unbiased genetic diversity demonstrated a very low level of genetic diversity (h = 0.00 to 0.104) for the populations shown in Table 3. The average number of alleles per locus (N_a) in the specimens from different populations was rather low and varied from 0.391 (2n = 52 S, 56, 58, and 60) to 0.826 (2n = 36). Similar to the previous estimate, the effective number of alleles (N_e) was characterized by low values (1 to 1.182). Average numbers of observed alleles, average numbers of effective alleles, genetic diversity, and the highest percentage of polymorphic loci were found in the 2n = 36 (Aydın) populations. The lowest values were observed in the 2n = 56, 2n = 58 (Niğde), and 2n = 60 cytotypes distributed in central Anatolia (Table 3).

The AMOVA showed significant differences among and between populations ($P < 0.01$). According to the AMOVA, the degree of differentiation was detected at 10% among individuals and at 90% between populations.

Results of the PCO analysis showed that geographically close populations tend to cluster together. Total variation (a cumulative value of 81%) was explained by the first 3

Table 3. Gene diversity in chromosomal races of *Nannospalax* (N_a = number of different alleles, N_e = number of effective alleles, I = Shannon's information index, h = Nei's unbiased genetic diversity, P% = percentage of polymorphic loci).

Populations	N	*Na*	*Ne*	*I*	*h*	*P%*
2n = 36	5	0.826	1.182	0.153	0.104	26.09
2n = 38	6	0.652	1.104	0.083	0.058	13.04
2n = 40	6	0.609	1.104	0.083	0.058	13.04
2n = 50	13	0.826	1.077	0.093	0.057	21.74
2n = 52 South	5	0.391	1	0	0	0.00
2n = 52 North	6	0.609	1.13	0.09	0.065	13.04
2n = 54	5	0.522	1	0	0	0.00
2n = 56 South	3	0.522	1	0	0	0.00
2n = 56 West	5	0.391	1	0	0	0.00
2n = 58	5	0.391	1	0	0	0.00
2n = 60 NF = 76	5	0.609	1.061	0.065	0.042	13.04
2n = 60 NF = 78	6	0.391	1	0	0	0.00
2n = 60 NF = 80	5	0.391	1	0	0	0.00
2n = 60 NF = 82	3	0.609	1.104	0.083	0.058	13.04
2n = 60 NF = 84	4	0.391	1	0	0	0.00
N. ehrenbergi	12	0.783	1.024	0.037	0.02	13.04
Total	94	0.557	1.049	0.043	0.104	7.88

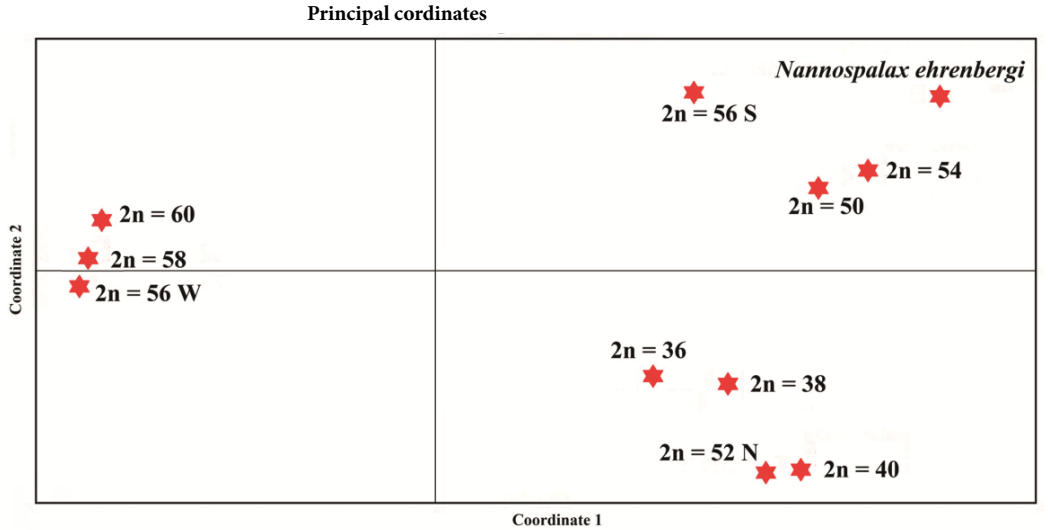

Figure 2. Principal coordinate analysis plot of the chromosomal races of *Nannospalax*.

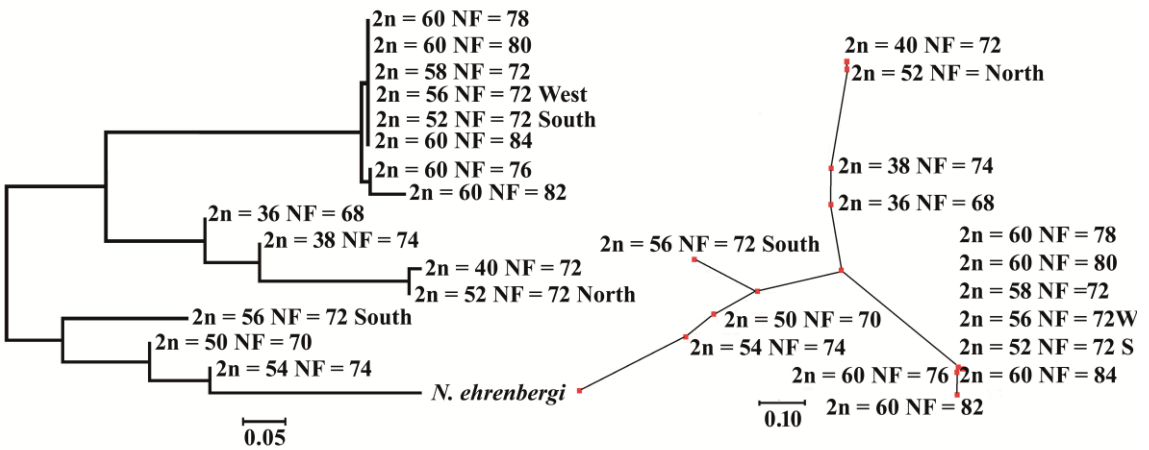

Figure 3. Neighbor-joining and span tree showing genetic relationships among populations, based on Nei's genetic distance measure.

eigenvectors. The first vector, with an eigenvalue of 22.33, represented 50.19% of total variation. The second and third vectors, with eigenvalues of 9.12 and 4.58, accounted for the total variation by 20.49% and 10.29%, respectively. Studied populations were clustered into 4 main groups by the PCO analysis. The first group included the 2n = 60 population that is widely distributed in central Anatolia and the 2n = 52, 2n = 56 W, and 2n = 58 populations found in the south near the distribution border of 2n = 60. The second group generally contained populations with lower diploid chromosome values (2n = 36, 2n = 38, 2n = 40, and 2n = 52 N) from western Anatolia. Populations from eastern Anatolia (2n = 50, 54, and 56 S) composed the third group. The last group consisted of populations of *N. ehrenbergi* (Figure 2).

The neighbor-joining and span trees revealed similar results to those obtained by PCO; geographically close populations clustered together in both analyses. However, the neighbor-joining analysis created 3 main clades distinct from PCO. *N. ehrenbergi* clustered with the geographically adjacent 2n = 56 S and 2n = 50 populations (Figure 3).

4. Discussion

Until recently, only morphological observation was used as the main criterion to classify Turkish blind mole rats, which have been classified into 3 morphospecies (*N. leucodon*, *N. ehrenbergi*, and *N. xanthodon*) (Kryštufek and Vohralik, 2009). More recently, Hadid et al. (2012) analyzed the phylogenetic relationships among samples of *Nannospalax* using mitochondrial DNA genes, and they

recognized 4 major clades (*vasvarii*, *leucodon*, *xanthodon*, and *ehrenbergi*).

Studies performed to date have accepted that *N. ehrenbergi* in southeastern Anatolia and *N. leucodon* in Thrace are valid species (Kryštufek and Vohralik, 2009; Hadid et al., 2012; Kryštufek et al., 2012). However, many researchers have indicated that 2 other species (*N. xanthodon* and *N. nehringi*) described from different geographical parts of Turkey were conspecific (Arslan et al., 2010; Kryštufek et al., 2012). Therefore, each of these species was considered as geographically widespread taxa in Anatolia by researchers during different periods of time (Ognev, 1947; Topachevski, 1969; Kıvanç, 1988; Savic and Nevo, 1990; Wilson and Reeder, 2005; Kryštufek and Vohralik, 2009).

The present data support the classification of *N. ehrenbergi* in southeastern Anatolia as a valid species but do not support the opinion that 1 of 3 species (*N. xanthodon*, *N. leucodon*, or *N. nehringi*) is the most widespread species in the remaining part of Anatolia. Furthermore, high genetic differences are observed between ecologically different forms, such as cytotypes in western Anatolia (2n = 36, 2n = 38, 2n = 40, and 2n = 52 N) and cytotypes in central Anatolia (2n = 60, 58, 56, and 52 S). The results of this study indicate a clear assignment of these species into 3 clades, and this is consistent with other studies (Hadid et al. 2012; Kryštufek et al., 2012). Our analysis suggests a primary division between a clade (*vasvarii*) including the central Anatolian populations that have higher diploid chromosome numbers and another clade (*xanthodon*) encompassing the western Anatolian populations with lower diploid chromosome numbers. Similar results were also suggested by Arslan et al. (2010) and Kandemir et al. (2012), in which the western Anatolian and the central Anatolian populations formed 2 monophyletic groups. However, this result is not consistent with current taxonomic treatments based on morphological comparisons. There is no provision in the classical morphospecies for the clade "*vasvarii*" distributed in the Central Anatolian Plateau. However, within the latter clade, this case was not present among taxa, and *N. nehringi* (2n = 50) formed a group with *N. ehrenbergi*.

Because *N. xanthodon* is not distributed throughout Anatolia and has a type locality near İzmir, this species is accepted as the most widespread species in İzmir, Manisa, Balıkesir, and in the surrounding areas. Similarly, the type locality of *N. nehringi* is near Kars-Kazıkoparan; therefore, it is not a synonym of *N. xanthodon* and should be considered a distinct species. *N. nehringi* is the most widespread species in eastern Anatolia where the 2n = 50 cytotype is found. Neither *N. xanthodon* nor *N. nehringi* is found in the Central Anatolian Plateau. When the central Anatolian populations are taken into account, the 2n = 60 cytotype and its descendant cytotypes (2n = 58, 2n = 56, 2n = 54, and 2n = 52 S) were not significantly different, and they should be collected into a separate species. *N. labaumei*, defined from Eskişehir for the first time by Matschie (1919), should be considered as a valid species that represents the current populations in central Anatolia. In this case, 5 mole rat species are living in Anatolia: *N. leucodon* in Thrace, *N. ehrenbergi* in southeastern Anatolia, *N. nehringi* in eastern Anatolia, *N. xanthodon* primarily in western Anatolia, and *N. labaumei* primarily in central Anatolia.

The RFLP analysis in this study showed that the cytotypes with the maximum number of common alleles are the 2n = 60 cytotype in central Anatolia, the 2n = 36 and 2n = 38 cytotypes in western Anatolia, the 2n = 50 cytotype in eastern Anatolia, and the species *N. ehrenbergi* in southeastern Anatolia. By considering the opinion stated by Zima (2000) that the chromosomal forms with larger distribution areas are the ancestral forms, it can be inferred that the southern cytotypes 2n = 52 S, 2n = 56, 2n = 58, and 2n = 52 evolved from 2n = 60. However, it is currently accepted that 2n = 36, 2n = 40, and 2n = 52 N originated from 2n = 38.

Although none of the cytotypes in Anatolia has differences that could divide them on a species level, there are obvious differences among certain cytotypes. The 2n = 38 cytotype of *N. xanthodon* is located in İzmir, the 2n = 36 cytotype is found close to populations with 2n = 38 in Aydın, the 2n = 40 cytotype is located in a small and highly isolated population in Beyşehir, the 2n = 52 N cytotype is largely distributed in Bolu and the surrounding areas; these populations are found to be highly genetically different. Data from this study and accumulated data from other studies (Arslan et al., 2010; Kandemir et al., 2012) indicate that some cytotypes in *N. xanthodon* would best be treated as distinct species. Although results from this study suggest that biological species diversity in *Nannospalax* is considerably higher than currently recognized taxonomically, the hypothesis that all of the cytotypes are separate species, which was suggested by Nevo et al. (1994, 1995), was not supported.

Differences found among these cytotypes and between these forms and present species should be analyzed by further molecular studies, and the taxonomical status of these species should be reevaluated.

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