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Research Article

Chromosomal, morphological and penial variation in the blind mole rats *Nannospalax ehrenbergi* (Rodentia: Spalacidae) in Egypt

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Abstract

The blind mole rat *Nannospalax ehrenbergi* is considered a superspecies most likely containing many separate biological species. The most important reason for its taxonomic uncertainties is the remarkable morphological and chromosomal variation recorded within and between populations and species. This study presents a description and an analysis of a chromosomal, morphological (external and craniodental), and penial variation of *N. ehrenbergi* from El-Hammam, Matruh, Egypt. The results confirmed the occurrence of two karyotype forms (I and II) with different 2n, NF, and NFa number. Karyotype form I consists of 2n=60, NF=73, and NFa=70, while karyotype form II, which is newly described here in Egypt for the first time, composes of 2n=62, NF=77, NFa=74. This chromosomal variation between individuals of the two forms was accompanied by obvious differences in morphological measurements, presence or absence of supracondyloid foramen, position of hard palate in relation to the line connecting the rear edges of the alveoli of M^3, position of the nasal posterior margin relative to the line connecting the upper or lower margins of the infraorbital foramen, and number of roots of M^2 and M^3. Although individuals of the two karyotype forms were similar in penial structural characteristics, they showed clear differences in the general shape of glans penis and size of baculum. The baculum was segmented and consists of a thick basal segment or proximal baculum that starts with a broad base and a thin terminal segment or distal baculum that ends with blunt tip. Accordingly, we could suggest that an evolutionary process has likely occurred in the Egyptian *N. ehrenbergi* and led to the formation of a new putative species with different chromosomal, morphological, and penial characteristics.

Introduction

Blind mole rat of the family Spalacidae *Nannospalax ehrenbergi* (formerly *S. ehrenbergi*) distributes in a narrow coastal strip in Libya and Egypt, Syria, Jordan, Lebanon, Israel, Iraq, and Southeastern Anatolia (Lay and Nadler, 1972; Savić and Nevo, 1990; Çoşkun, 2004a and references therein; Çoşkun et al., 2006, 2016; Schlitter et al., 2008; Kryštufek and Vohralik, 2009). This palaearctic species has long been considered a superspecies presumably because it contains many separate biological species (see review by Arslan et al., 2016). The main reason for its taxonomic diversity is the remarkable morphological and chromosomal variation recorded within and between populations and species. Indeed, about 20 distinct chromosomal races or cytotypes, with a total of 7 diploid numbers, have been found so far within this species (Savić, 1982; Nevo et al., 1994a,b; Nevo, 1995; Çoşkun, 2004a; Arslan et al., 2016). Each of these cytotypes has been considered by some authors as presumptively good biological species and that some populations having identical diploid chromosome numbers have been assigned as different biological species (Nevo et al., 1994a,b, 1995, 2001). More broadly, the 2n of these cytotypes varied between 48 to 62, with fundamental number of chromosomal arms (NF) ranged between 62 to 90 and fundamental number of autosomal arms (NFa) differed between 58 to 86 (for details, see Çoşkun, 2004a; Arslan et al., 2016).

The first description of *N. ehrenbergi* has been done by Nehring (1898) in Israel. After that, many karyological (Çoşkun, 2004a; Kryštufek and Vohralik, 2009; Çoşkun et al., 2006, 2010a,b, 2014, 2016; Arslan and Zima, 2013, 2015, 2017 and references therein) as well as morphological (Nevo et al., 1998; Çoşkun, 1994, 1998, 2004a; Sözen et al., 2006a; Kryštufek and Vohralik, 2009; Çoşkun et al., 2016 and references therein) studies have been carried out along its extensive distribution range. As mentioned above, karyological examinations have approved the presence of several chromosomal forms or races with different number of 2n, NF and NFa. Morphological studies as well have shown that although the populations are morphologically very similar, they display distinct variations in the morphology of the skull and structure of teeth.

As far as known, most rodent taxonomy and phylogeny has been based upon dentition. Among dental characters, dental form has been the most striking morphological characteristic and has therefore been used as a powerful tool in many taxonomic and phylogenetic studies of extinct and living rodents (Jones, 1922; Kahmann, 1969; Knox, 1976; Bachmayer and Wilson, 1980; Carleton, 1980; Emry, 1981; Gemmeke and Niethammer, 1984; Luckett and Hartenberger, 1985; Shahin, 1999; Piras et al., 2012; Maridet and Ni, 2013; Kelly and Murphey, 2016). Alveolar patterns in the maxillae have also been considered a useful taxonomic criterion among the Muridae (Jones, 1922; Kahmann, 1969; Knox, 1976; Bachmayer and Wilson, 1980; Carleton, 1980; Gemmeke and Niethammer, 1984), Dipodidae (Bachmayer and Wilson, 1980; Shahin, 1999; Kelly and Murphey, 2016) and Spalacidae (Pavlinov and Lissovsky, 2012).

On the other hand, variation in the structure and characteristics of the penial has been observed at the inter- and intra-specific level of closely

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Figure 1 – A map showing the geographic coordinates of El-Hammam, Matruh, Egypt, where the individuals of the blind mole rats *Nannospalax ehrenbergi* were collected from two adjacent localities separated by a narrow zone of about 0.2 km.

related species, and thus, it has been assumed to be extremely useful in reconstructing the phylogeny of rodents (Lidicker, 1968; Altuna and Lessa, 1985; Bradley et al, 1989; Lessa and Cook, 1989; Simson et al., 1993). Additionally, studies on the growth and differentiation of the baculum (os penis) have demonstrated that an extensive variation and differential allometric growth versus body have been found among rodent species (Burt, 1960; Martin, 1970; Best and Schnell, 1974; Patterson, 1983; Pessôa et al., 1996; Pessôa and Strauss, 1999; Çoşkun, 2004c; Yiğit et al., 2006; Kankiliç et al., 2014). Among mole rats of the family Spalacidae, it has been concluded that the baculum structural variation is congruent with the karyotype variation, i.e., it is species-specific, and its length is not correlated significantly neither with the greatest skull length (Simson et al., 1993) nor the head and body length (Çoşkun, 2004c; Yiğit et al., 2006; Kankiliç et al., 2014).

Although the occurrence of *N. ehrenbergi* is known in Egypt (Topachevskii, 1969; Lay and Nadler, 1972; Nevo, 1991; Nevo et al., 1999), its morphological, genetical, behavioral, biological, molecular and karyological peculiarities unfortunately have not yet been documented in detail and should be subjected to further investigation. Thus, the present study was undertaken to detail for the first time the variation in the karyotype, morphological (external and craniodental), and penial characteristics of *N. ehrenbergi* in Egypt to make a contribution to its karyological, morphological and penial data known so far.

Materials and methods

Sampling and study area

A total of 14 adult individuals (10 males and 4 females) of the blind mole rat *Nannospalax ehrenbergi* (Nehring, 1898) were captured alive between 2014 and 2015 from two adjacent localities separated by a narrow zone of about 0.2 km in El-Hammam (30°50′34.53″ N, 29°23′37.59″ E), Matruh (Fig. 1), by digging burrow systems according to the method described by (Sözen, 2004; Sözen et al., 2006a).

El-Hammam locality has a semi-arid Mediterranean climate that is characterized by a brief, mild, rainy winter and long warm summer months (May to September) of clear sky, high radiation, and no rain. Topographically, this region is extremely flat and its soil is composed of white, loose carbonate sands and covered in most regions with alluvial loam deposits mixed with calcareous sand. It has an elevation of about 82 m and receives an average annual rainfall of approximately 140 mm/yr. The average humidity percentage is around 61.3% and 75.6% during the year and the average daily temperature generally does not exceed 30.5 °C in the summer months and does not go below 7 °C in winter months.

Laboratory techniques

External morphological examination

The animals were sexed, weighed, aged into young adult, old, and oldest (Fig. 6, a–f) based upon molars enamel folds patterns and wear stages (Çoşkun et al., 2016), and two external morphological measurements (head and body length (HBL) and hind foot length (HFL) were taken by using a digital caliper (RUPAC, Italy) and values were approximated to the nearest 0.1 mm.

Conventional technique and karyotype preparation

The animals were intraperitoneally injected with 0.05% colchicine solution (0.01 ml/g body weight). After one hour, the animals were anesthetized and femurs were dissected out and bone marrow cells were obtained as explained by Abu Shnaf (2014). Chromosome spreads were conventionally prepared by using the air-drying technique according to the method of Yosida (1973), with slight modification as described by Shahin and Ata (2001). About 30 to 50 metaphase cells from each animal were examined at 100× magnification and well-spread chromosomes of five to ten metaphase cells were recorded and photographed using Olympus BX51 microscope with a C-4040 zoom digital camera. Definition of the shapes of chromosomes was established according to the system of nomenclature proposed by Levan et al. (1964). The karyotype was determined on the basis of the well-spread metaphase chromosomes. Chromosomes lengths were measured under the microscope using the Soft Imaging System (SIS) analysis program (version 3.0) edited in 1999 by Soft Imaging System GmbH, Germany, and then arranged into descending series according to their sizes. Both the fundamental (NF) and autosomal (NFa) number of chromosome arms were computed by counting the bi-armed autosomes as two arms and acrocentric autosomes as one arm.

Craniodental examination

Skulls of all individuals were skinned, prepared for craniodental examination, and photographed using KYOWA dissecting stereomicroscope provided with digital camera. Thirty-seven linear measurements were taken by using a digital caliper (RUPAC, Italy) following Nevo et al. (1998); Çoşkun (2004a); Sözen et al. (2006a). The craniodental variables included: condylobasal length (CBL), condylonasal length (CNL), occipitonasal length (ONL), basal length (BL), nasal length (NL), nasal width (NW), maximum skull height (SKH), auditory meatus diameter (AMD), upper molars crown length (UCL), upper molars alveolai length (UAL), diastema length (DL), facial region length (FRL), supraoccipital length (SOL), zygomatic breadth (ZB), tympanic bullae length (TBL), tympanic bullae width (TBW), foramina incisiva length (FIL), interorbital constriction (IOC), rostrum width (RW), hind palatal length (HPL), palatal length (PAL), sagittal crest length (SCL), parietal length (PL), parietal width on lambdoid crest (PW), infraorbital foramen width (IFW), infraorbital foramen height (IFH), upper incisors alveolai width (UAW), upper incisors width (UIW), M^2 crown width (MCW), lower incisors width (LIW), lower molars crown length (LCL), lower molars alveoli length (LAL), mandible height (MH), coronoid process height (CPH), angular length (AL), articular length (ARL), alveolar process length (APL).

Statistical analysis

A non-parametric multiple analysis of variance (NP-MANOVA in PAST with 10000 permutations, *p*-values Bonferroni corrected) for the 39 external morphological and craniodental measurements was performed on the Euclidean distances in PAST in order to test significant differences between the two karyotype forms I and II. Additionally, Principal Component Analysis (PCA) for the log-transformed data of morphometric variables was conducted. All variables were transformed into logarithms to eliminate the biased effect of large measurements in multivariate analysis (D'Elia and Pardinas, 2004). PCA is based upon the variance-covariance matrix of log-transformed variables. Statistical analyses were performed by using PAST 1.81 (Hammer et al., 2001) and Statistica version 13 (StatSoft, Inc., http://www.statsoft.com).

Penial examination

Phalli of all males were dissected out and fixed in 70% ethanol for 1 week. Later on, they were cleared in 4% KOH for as many as 4 days, and then stained with 0.003% Alizarin Red S for 3 days following Lidicker (1968) and as described by Simson et al. (1993). This was followed by dehydrating the specimens successively in 25, 50, and finally

100% glycerin for 1 day each. Five linear measurements of baculum characters, including baculum length (BL), baculum width (BW), baculum height (BH), baculum tip width (BTW), and baculum base height (BAH), in addition to the relative baculum/greatest skull length, were measured for five males with fully extended bacula following Simson et al. (1993). Photographs of the bacula were taken with KYOWA dissecting stereo-microscope provided with digital camera and measurements were done on the digital images by using the QuickPhoto Micro 3.1 imaging software (Promicra s.r.o., Prague, Czech Republic).

The slides and voucher specimens were kept at the Department of Zoology, Faculty of Science, Minia University, El Minia, Egypt.

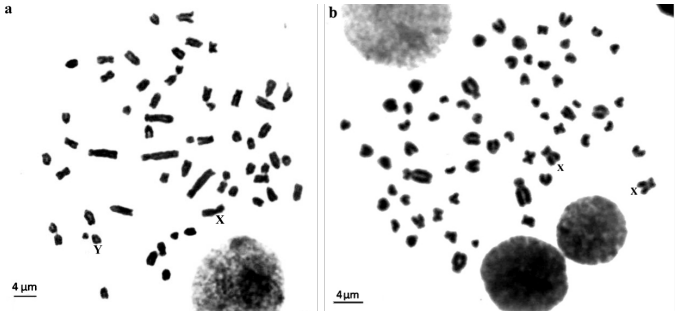

Figure 2 – Photographs showing the chromosomal set of karyotype form I consisting of 2n=60 chromosomes of the blind mole rats *Nannospalax ehrenbergi* a) metaphase cell from a male; b) metaphase cell from a female.

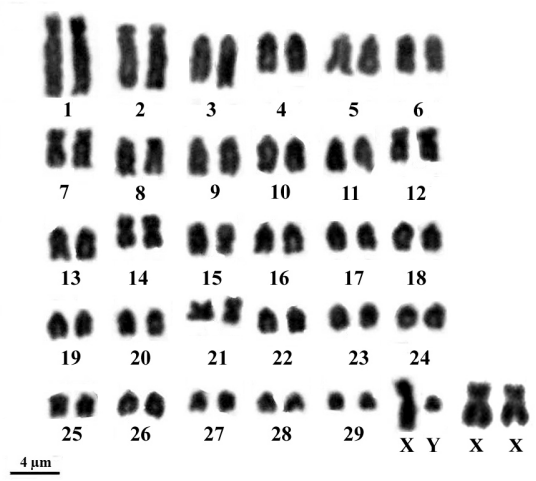

Figure 3 – A karyotype of the male metaphase cell given in Figure 2a showing the chromosomal set of karyotype form I consisting of 2n=60 chromosomes of the blind mole rats *Nannospalax ehrenbergi,* in addition to the female XX chromosomes..

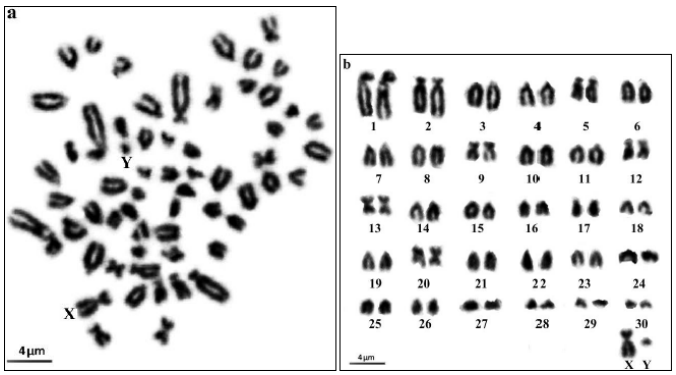

Figure 4 – Photographs showing the chromosomal set of karyotype form II consisting of 2n=62 chromosomes of the blind mole rats *Nannospalax ehrenbergi.* a) metaphase cell from a male; b) karyotype of the male metaphase cell given in a).

Results

Chromosomal variation

As a rule, two karyotype forms (I and II) with different diploid (2n), fundamental (NF) and autosomal (NFa) numbers were recognized in the 14 individuals examined. The chromosome complements in both forms were variable in size and are largely forming a graded series, and so, they were arranged according to length into five groups (Figs. 2,3 and 4). Although the sex chromosomes in both karyotypes have a similar morphology, they showed relative variation in size. Basically, the X chromosome was medium-sized submetacentric, while the Y was small-sized acrocentric (Figs. 2, 3 and 4). A detailed description of the morphology of the autosomes in each of the two karyotype forms is as follows:

Karyotype form I

This karyotype was recorded in a total of 12 (8 males and 4 females) individuals and consists of 2n=60, NF=74 in females and 73 in males, and NFa=70. The autosomal chromosome set includes two subtelocentrics (pairs no. 1 and 2), two submetacentrics (pairs no. 7 and 12), two metacentrics (pairs no. 13 and 20), and 23 telocentrics (acrocentrics) (pairs no. 3–6, 8–10, 11, 14–19, and 21–29). The morphology the chromosome complement is seen in Figs. 2 and 3.

Table 1 – Comparison of the 39 external morphological and craniodental measurements (in mm) between individuals of the two karyotype forms of the blind mole rats *Nannospalax ehrenbergi.* Values are approximated to the nearest 0.1 mm.

Variable	Karyotype form I (2n=60) No. of individuals=12		Karyotype form II (2n=62) No. of individuals=2	
	Range	Mean ± SD	Range	Mean± SD
HBL	190.0–220.0	204.3 ±9.5	221.9–222.1	222.0 ±0.1
HFL	45.0–55.0	50.3 ±3.2	56.9–57.3	57.1 ±30.3
CBL	38.3–43.2	41.3 ±1.5	44.2–44.8	44.5 ±0.4
CNL	38.1–41.5	40.10±1.04	44.3–45.1	44.7 ±0.6
ONL	38.4–41.6	40.0 ±1.0	43.2–43.8	43.5 ±0.4
BL	34.6–39.1	37.4 ±1.4	41.4–42.0	41.7 ±0.4
NL	15.2–17.0	15.9 ±0.6	19.7–20.0	19.9 ±0.2
NW	4.8–6.5	5.3 ±0.5	5.8–6.2	6.0 ±0.3
SKH	13.8–15.9	14.9 ±0.6	16.8–17.1	17.0 ±0.2
AMD	2.2–3.7	2.7 ±0.5	3.8–4.4	4.1 ±0.4
UCL	6.4–7.0	6.8 ±0.2	6.9–7.2	7.1 ±0.2
UAL	7.5–8.5	8.0 ±0.3	8.2–8.8	8.5 ±0.4
DL	12.4–15.6	13.4 ±0.8	16.5–19.9	16.7 ±0.3
FRL	24.7–29.8	27.1 ±1.2	30.0–30.3	30.2 ±0.2
SOL	7.3–9.8	8.4 ±0.9	10.4–10.5	10.5 ±0.1
ZB	26.9–31.3	29.0 ±1.4	31.7–32.0	31.9 ±0.2
TBL	7.5–9.3	8.5 ±0.4	9.0–9.2	9.1 ±0.1
TBW	9.5–7.0	6.6 ±0.3	7.2–7.8	7.5 ±0.4
FIL	3.0–3.8	3.4 ±0.3	3.1–3.8	3.5 ±0.5
IOC	6.8–7.5	7.2 ±0.2	7.3–7.5	7.4 ±0.1
RW	7.3–8.3	7.8 ±0.2	8.5–8.9	8.7 ±0.3
HPL	10.6–12.7	11.8 ±0.6	12.8–13.1	13.0 ±0.2
PAL	7.4–8.5	8.0 ±0.4	8.4–8.9	8.7 ±0.4
SCL	15.1–16.6	16.2 ±0.5	17.0–17.2	17.1 ±0.1
PL	6.0–7.8	6.8 ±0.5	6.7–6.9	6.8 ±0.1
PW	10.0–12.5	11.2 ±0.8	11.2–11.3	11.3 ±0.1
IFW	2.4–3.4	2.9 ±0.3	3.5–3.6	3.6 ±0.1
IFH	5.1–6.0	5.5 ±0.3	6.7–6.9	6.8 ±0.1
UAW	4.8–6.8	6.0 ±0.6	6.0–6.4	6.2 ±0.3
UIW	1.7–2.0	1.9 ±0.1	1.95–2.4	2.2 ±0.3
MCW	2.0–2.4	2.2 ±0.1	2.3–2.9	2.6 ±0.4
LIW	1.7–2.0	1.9 ±0.1	2.1–2.3	2.2 ±0.1
LCL	6.4–7.0	6.6 ±0.2	6.8–6.9	6.9 ±0.1
LAL	6.5–8.0	7.0 ±0.5	7.1–7.4	7.3 ±0.2
MH	6.7–7.7	7.2 ±0.4	7.5–7.9	7.7 ±0.3
CPH	12.2–18.0	15.9 ±1.4	17.2–17.9	17.6 ±0.5
AL	19.5–23.2	21.3 ±1.1	22.5–22.8	22.7 ±0.2
ARL	21.6–25.6	23.7 ±1.4	23.4–23.8	23.6 ±0.3
APL	22.0–25.0	23.2 ±1.1	26.6–27.0	26.8 ±0.3

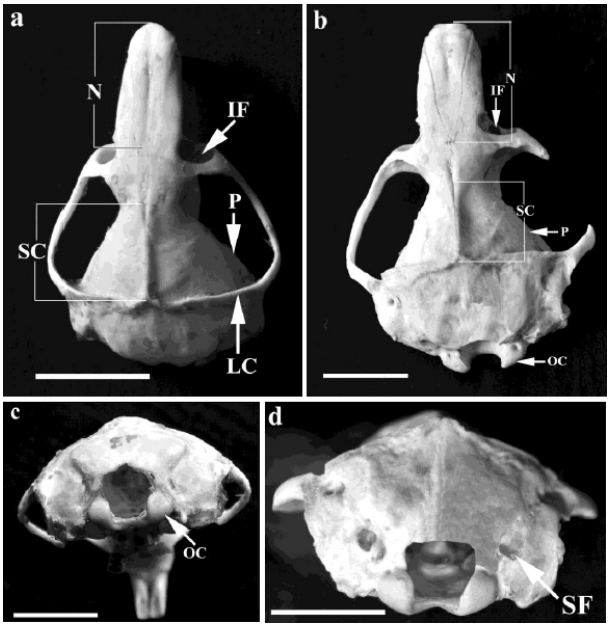

Figure 5 – Photographs of skull morphology in the two karyotype forms I (a, c) and II (b, d) of the blind mole rats *Nannospalax ehrenbergi* showing that the lambdoid (LC) and sagittal (SC) crests are well developed, parietal (P) length is less than its width, nasal (N) length is longer than the sagittal crest length (a, b), but its posterior margin either reaches the line connecting the upper (a) or the lower (b) margins of the infraorbital foramen (IF) on both sides, and absence (c) or presence (d) of the supracondyloid foramen (SF) above the occipital condyles (OC) on both sides. Scale bar=1.15 cm.

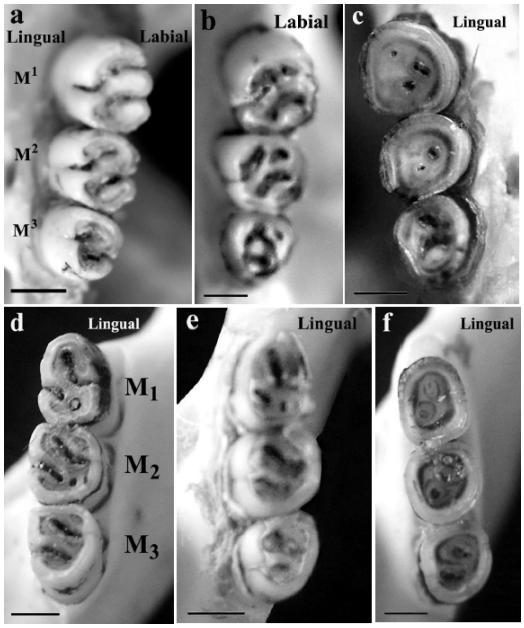

Figure 6 – Photographs of enamel folds on the occlusal or chewing surface of upper (a-c) and lower (d-f) molars in the two karyotype forms of the blind mole rats *Nannospalax ehrenbergi* showing that in young adult individual (a, d), M^1 has one lingual and two labial folds, M^2 has a deep intruding fold on each side and an additional inlet on the antero-labial side giving the chewing surface an inverse "S" shape, and M^3 has two converging folds that give the chewing surface the form of a horseshoe shape (a); M_1 , M_2 and M_3 have a similar enamel pattern on the chewing surface where they have one labial and one lingual intruding fold giving the chewing surface the form of S-shape (d). In old individuals where the crown is eroded, the enamel folds become one, two, three, or four separate islands (b, e); in oldest individuals with advanced crown erosion, the islands are fragmented into separate islets, which completely disappeared in M^3 and the crown becomes more round in shape (c, f). Scale bar=1.15 cm.

Karyotype form II

This karyotype was found in 2 males only and consists of 2n=62, NF=77, NFa=74. The autosomal set, likewise karyotype form I, comprises two subtelocentrics (pairs no. 1 and 2), two metacentrics (pairs no. 13 and 20), and 23 telocentrics (pairs no. 3, 4, 6–8, 10, 11, 14–19, and 21–30), but it contains three submetacentrics (pairs no. 5, 9, and 12) instead of two (pairs no. 7 and 12) in karyotype form I. The morphology of the chromosome complement is seen in Fig. 4.

Morphological variation

Morphometric comparison of the mean values of the 39 external and craniodental measurements showed clear distinction between individuals of the two karyotype forms I and II (for details, see Tab. 1).

In addition, comparison of the similarities (Fig. 6 a–f) as well as differences (Figs. 5, 7 and 8) in the craniodental characters between individuals of the two karyotype forms clearly demonstrated that they displayed the following diagnostic characteristics:

In all individuals from karyotype form I, the skull has no supracondyloid foramen above the occipital condyles on both sides (Fig. 5c). The nasal length was longer than the sagittal crest length (for details, see Tab. 1) and its posterior margin reaches the line connecting the upper margins of the infraorbital foramen on both sides (Fig. 5a). In young adult individuals, the hard palate terminates at about the line connecting the rear edges of the alveoli of M^3 and its posterior margin ends with a median sharp pointed styloid process (Fig. 7a). However, in old and oldest individuals it ends definitely at some distance above the line connecting the rear edges of the alveoli of M^3 and its posterior margin ends straight, i.e., without styloid process (Fig. 7b and c).

M^1 has three roots in all skulls, while M^2 has either three roots in eight skulls or four roots in four skulls (Fig. 8a, b, c and e). However, M^3 exhibited either two roots in only one skull, three roots in ten skulls, or four roots in just one skull (Fig. 8a, b, c and e).

On the contrary, the skull in the individuals from karyotype form II has a supracondyloid foramen above the occipital condyles on both sides (Fig. 5d). The nasal length was also longer than the sagittal crest length, but its posterior margin reaches the line connecting the lower margins of the infraorbital foramen on both sides (Fig. 5b). In young adult individuals, likewise those of form I, the hard palate extends at about the line connecting the rear edges of the alveoli of M^3 and its posterior margin ends with a median sharp pointed styloid process (Fig. 7a). However, in old and oldest individuals, it terminates markedly behind the rear edges of the alveoli of M^3 and its posterior margin obviously has no styloid process (Fig. 7d). In all individuals, both the M^1 and M^3 have three roots, while the M^2 has four roots (Fig. 8f).

Non-parametric multiple analysis of variance (NP-MANOVA) for the 39 external morphological and craniodental measurements in the 14 individuals examined revealed that there were significant differences in the multivariate means for the two karyotype forms I and II ($F=5.556$; $p=0.0107$). Also, Bonferroni corrected pairwise comparisons demonstrated significant differences between the two forms ($p<0.001$). In addition, PCA clearly showed differentiation of the 14 individuals into two distinct morphometric groups I and II (Fig. 9). Group I comprised individuals of karyotype from I (2n=60), while group II included individuals of form II (2n=62). The two PCs 1 and 2 were collectively accounted for 64.07% of the total variation between the 39 morphometric variables, where 53.74% of which was explained by PC1 and 10.33% by PC2.

Table 2 – Comparison of the five linear bacular measurements (in mm), in addition to the relative baculum/greatest skull length, between males of the two karyotype forms of the blind mole rats *Nannospalax ehrenbergi*. Values are approximated to the nearest 0.1 mm.

Variable	Karyotype form I (2n=60) No. of males=4		Karyotype form II (2n=62) No. of males=1
	Range	Mean±SD	Value
BL	5.1–5.7	5.2 ± 0.3	5.2
BW	0.8–1.4	1.0 ± 0.3	1.0
BH	0.8–1.2	0.9 ± 0.2	0.9
BTW	0.5–0.7	0.6 ± 0.1	0.5
BAH	0.9–1.3	1.0 ± 0.2	1.2
Relative baculum/ greatest skull length	0.1–0.1	0.1 ± 0.0	0.1

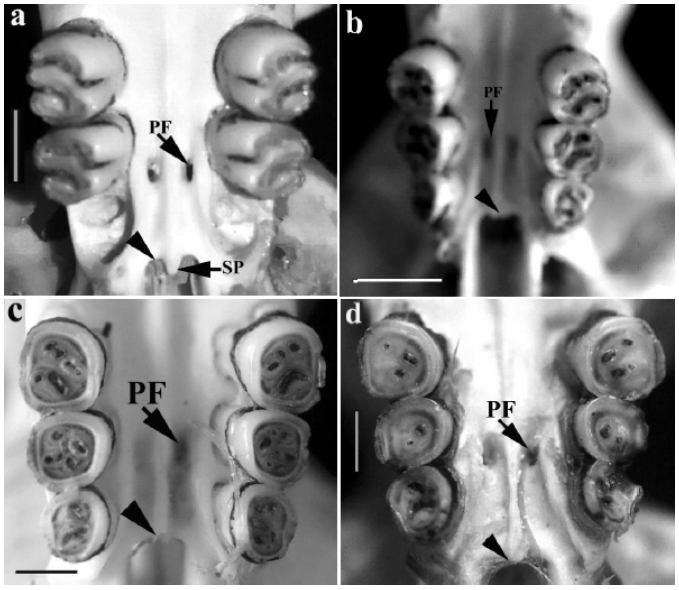

Figure 7 – Photographs of the hard palate structure (arrow head) in the two karyotype forms of the blind mole rats *Nannospalax ehrenbergi* showing that the palatal foramen (PF) lies on both sides above the line separating between the M^2 and M^3 (a-d). In young adult individuals from both karyotypes (a), the hard palate terminates at about the line connecting the rear edges of the alveoli of M^3 and its posterior margin ends with a median sharp pointed styloid process (SP) and in old and oldest individuals from karyotype I, it ends definitely at some distance above the line connecting the rear edges of the alveoli of M^3 and its posterior margin ends shows no styloid process (b, c), however, it extends markedly without styloid process behind the rear edges of the alveoli of M^3 in those individuals from karyotype form II (7d). Scale bar=1.15 cm.

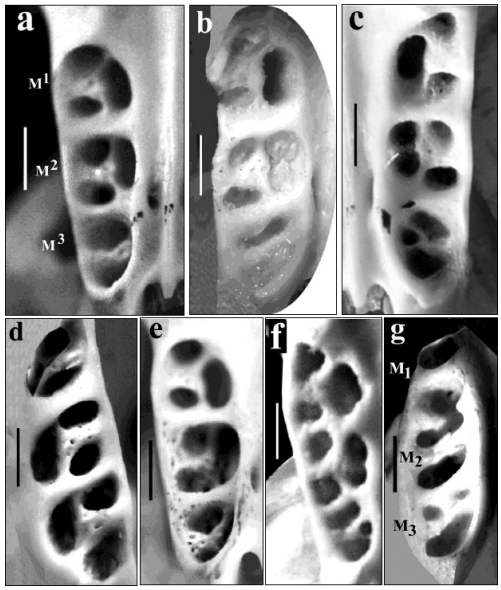

Figure 8 – Photographs of the alveolar patterns of the upper (a-f) and lower (g) molars roots in the two karyotype forms of the blind mole rats *Nannospalax ehrenbergi* showing that the upper molars M^1, M^2 and M^3 roots exhibited five alveolar patterns 3/3/2, 3/4/2, 3/4/3, 3/3/3 and 3/3/4 (a-e, respectively) in those individuals from karyotype form I and only one alveolar pattern 3/4/3 (f) in those individuals from karyotype form II. In all individuals from both karyotypes, the lower molars M_1, M_2 and M_3 showed only one alveolar pattern 2/2/3 (g). Scale bar=1.15 cm.

Penial variation

As a rule, the phalli of all males examined exhibited a basic similar structure, but they were relatively different in the general shape of the glans penis and size of the baculum (Figs. 10 and 11). The urethral opening was surrounded by three lobes: one dorsal and two lateral lobes. The lobes were large and prominent in the phalli of all males, but they were thick in the phallus of only one male from karyotype form II (11a and b), making its general shape apparently different from those of the other four males from karyotype form I (Fig. 10a and b). In all males, the baculum lies dorsal to the urethra and consists of two segments: a thick basal segment or proximal baculum that starts with a broad base and a thin terminal segment or distal baculum that ends with blunt tip (Fig. 10 and 11). The overall length of the baculum ranges between 4.58 mm to 5.74 mm, with about 36–39% of which belongs to the proximal baculum that has a base of about 2.0 to 2.3 times wider.

Morphometric comparison of the five linear measurements of the baculum characters, in addition to the relative baculum/greatest skull length, between the five males of both karyotype forms showed no clear distinction between them, in spite of their relative variation in the size. Also, there was no relation between the baculum length and greatest skull length. The five linear measurements, in addition to the relative baculum/greatest skull length, of bacula characters from the five males are shown in Tab. 2.

Discussion

Nannospalax ehrenbergi has been recognized for the first time by Nehring (1898) in Israel and its karyological peculiarities have been described by Wahrman et al. (1969a,b) where four different chromosomal forms are recorded in Israel with diploid number of 2n=52, 54, 58 and 60. Subsequently, Lay and Nadler (1972) confirmed the diploid number of 2n=60 chromosomes in the Egyptian specimens. Afterwards, several karyotype studies have been carried out across its distribution range and revealed obvious chromosomal polymorphisms as well as several different karyotypes (for details, see Kryštufek and Vohralik, 2009; review by Arslan et al., 2016). Studies on the chromosomes of natural populations of small mammals including rodents have revealed a relatively large amount of karyotypic variation within and between individuals, populations, species, and higher taxa (for review, see Zima, 2000). Definitely, these studies have concluded that the driving forces of karyotype evolution may be found either in selection or drift acting at the organismal level, or in the internal processes occurring within the cell. The forces acting at the organismal level are based either on negative heterosis of chromosomal rearrangements or on the altered pattern of gene expression resulting from karyotypic repatterning. Additionally, chromosomal differentiation in the mole rats from Jordan, Israel and Turkey has been explained in connection with speciation and adaptation to different environmental conditions, primarily to different climatic regions varying in aridity and temperature regimes (Wahrman et al., 1969a,b, 1985; Nevo, 1985, 1991, 2000; Nevo et al., 1994a, 1995).

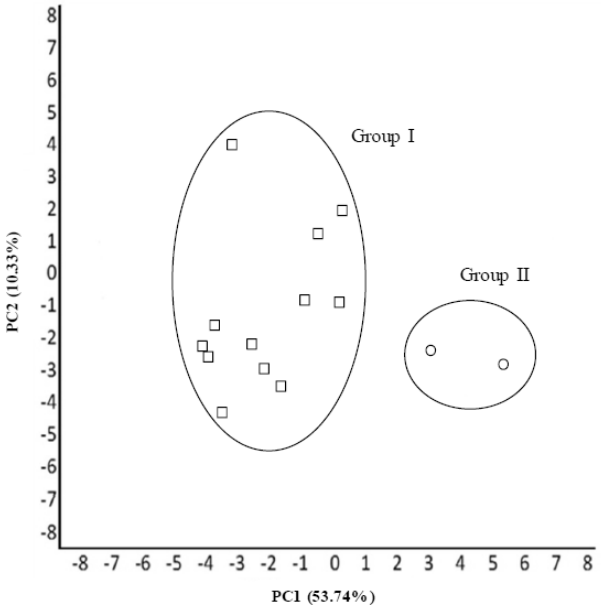

Figure 9 – Results of the first (PC1) and second (PC2) principal component analysis for the 39 external morphological and craniodental measurements analyzed in the 14 individuals of *Nannospalax ehrenbergi* showing two groups I and II corresponding to the two karyotype forms I and II, respectively (□:individuals of karyotype form I (2n=60); ○: individuals of karyotype form II (2n=62).

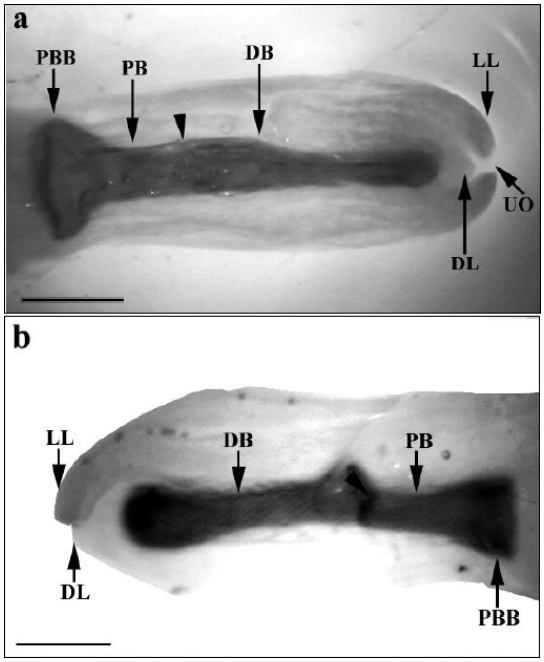

Figure 10 – Photographs of the phallus in males from karyotype form I of the blind mole rats *Nannospalax ehrenbergi* showing that the urethral opening (UO), as clearly appears in (a), is surrounded by three lobes: one dorsal (DL) and two lateral lobes (LL) and the baculum lies dorsal to the urethra and consists of two segments: a thick basal segment or proximal baculum (PB) that starts with a broad base (PPB) and a thin terminal segment or distal baculum (DB) that ends with blunt tip (a, b). (a) ventral view; (b) ventrolateral view; Arrow head in (a) and (b) refers to the site of segmentation. Scale bar=1.2 mm.

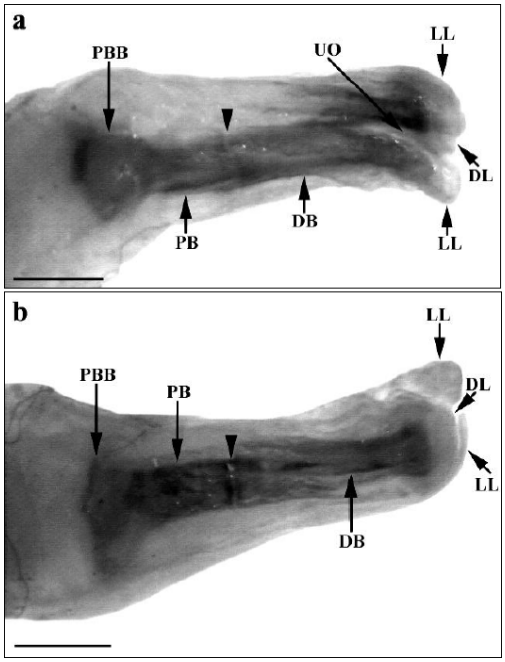

Figure 11 – Photographs of the phallus in males from karyotype form II of the blind mole rats *Nannospalax ehrenbergi* showing that the urethral opening (UO), as clearly appears in (a), is surrounded by three lobes: one dorsal (DL) and two lateral lobes (LL) and the baculum lies dorsal to the urethra and consists of two segments: a thick basal segment or proximal baculum (PB) that starts with a broad base (PPB) and a thin terminal segment or distal baculum (DB) that ends with blunt tip (b). (a) ventrolateral view; (b) ventral view; Arrow head in (a) and (b) refers to the site of segmentation. Scale bar=1.2 mm.

Moreover, Nevo (1985) determined that the main trend in chromosomal evolution of *Spalax* is the process of Robertsonian fission that leads to increase in the number of 2n with aridity. This meant that *Spalax* has an adaptive strategy to occupy arid and unpredictable climatic regimes through increase in chromosome number that maximizes the opportunity to have high recombination phenomena, and thus, higher genetic variability. Nevo et al. (1994a) confirmed as well that the chromosome number shows a positive correlation with the environmental conditions due to the restrictions of the biotic and climatic factors such as dryness and temperature. Thus, they concluded that the diploid number of Anatolian *Spalax* increases from the rainy and warm coastal regions to the dry and harsh climatic zone of middle Anatolia. Importantly, it has been found that the increase of acrocentrics occurs by Robertsonian fission and the variations in the number of the chromosome arms frequently occur by centromeric translocation (Yüksel, 1984; Nevo et al., 1994a; Savić and Soldatović, 1979). In addition, it has been assumed that the major initial mechanism of speciation in Spalacidae is chromosomal that primarily occurs through Robertsonian rearrangements and the variation in chromosome morphology is caused by pericentric inversions (Wahrman et al., 1969a,b, 1985; Lay and Nadler, 1972; Nevo et al., 2000) and/or centromeric shifts (Nevo, 1991; Nevo et al., 1994b, 1995, 2000; Arslan et al., 2011b).

In the present investigation, two karyotype forms I and II, with different chromosomal 2n, NF and NFa numbers were recognized. Karyotype form I displayed 2n=60, NF=74 in females and 73 in males, and NFa=70, while form II exhibited 2n=62, NF=77, NFa=74 due to the presence of an extra submetacentric pair. The presence of 2n=60 is consistent with the findings of Wahrman et al. (1969a,b); Lay and Nadler (1972); Nevo (1991); Nevo et al. (1991, 1994a, 2000). But, the finding of 2n=62 in *N. ehrenbergi* is described herein for the first time. As depicted by Arslan and Bölükbaş (2010), the 2n=60 karyotype apparently occupies the largest range among mole rat karyotypes in Anatolia and this distributional pattern may indicate its ancestral position among other karyotypes described. In addition, Nevo (1991, 1995) stated that this karyotype should be considered to be derived in relation to the hypothesis of environmental stress. However, it is fairly important to mention that the 2n=62 has previously been described in *Spalax leucodon* from Turkey (Nevo et al., 1994b, 1995; Tez et al., 2001), *S. ehrenbergi* from Jordan (Nevo et al., 2000) and *N. ehrenbergi* from Madaba, Jordan, with different NF and NFa numbers (for details, see Nevo et al., 2000; Arslan et al., 2016). Interestingly, Nevo et al. (1994a) and Nevo (1995) recognized that the 2n values and heterozygosity (*H*) increase toward the ecologically harsh, arid, and climatically unpredictable and geologically young central Anatolian Plateau from the west, north, south, and east. However, Ivanitskaya et al. (2008) indicated that the determination of some chromosomal forms such as 2n=62 is due to the small B chromosomes. So, Sözen et al. (2011) assumed that the 2n=62 forms should be eliminated from the list of Turkish mole rats. Also, Arslan and Bölükbaş (2010); Arslan et al. (2011a) pointed out that the extent of variation in the number of autosomal arms may be broad and additional centromeric shifts in other autosomes are apparently involved. Moreover, molecular analyses of the cytochrome *b* sequences have suggested that associations between genetic and chromosomal variation are not widespread and common in mole rats, and therefore, they have refuted the generalization of a "cytotype-equals-species" approach (Kryštufek et al., 2012; Kandemir et al., 2012). Accordingly, it is clear that the differences in chromosomes morphology between the individuals examined herein from El-Hammam were affected by centromeric translocation. However, the increase in 2n from 60 to 62, which was presumably occurred as a result of Robertsonian fission, i.e., metacentric fission (Nevo et al., 2000), could be explained in terms of speciation and adaptation to environmental conditions, particularity the aridity and relatively high temperature characterizing this region.

On the other hand, morphological studies have shown that although the populations of *Nannospalax* are morphologically very similar, they display distinct craniodental variation along their geographical range (for details, see Çoşkun et al., 2016 and references therein). Of these studies, Çoşkun et al. (2016) comprehensively reported about nine taxonomic peculiarities of the body and skull, which are distinctive to the North-Iraq populations. These characters can be enumerated as follows: 1) the presence of supracondyloid foramen above each occipital condyle, 2) the presence of two longitudinal ridges on the anterior surface of the upper incisors, 3) the appearance of two enamel islands on

the chewing surface of M^3, 4) the palatines do not extend behind the line connecting the rare edges of the alveoli of upper molars in young samples, but extend or pass through the back in mature and old specimens, 5) the foramen post palatines is present in front of the line between M^2 and M^3, 6) the palate ends with a long and weak styloid process, 7) the pattern of the occlusal surface changes according to the erosion of teeth, 8) the upper molars have three roots, and 9) the lower molars have two roots. In fact, the first two of these nine characters are the most important diagnostic characters for *N. ehrenbergi* and they have been solely confirmed in many previous works (see Çoşkun, 1994, 2004a and references therein).

In the present study, distinct variation in the 39 external and craniodental measurements was found between individuals of the two karyotype forms. This variation was confirmed by significant differences in the multivariate means for the two karyotype forms I and II (F=5.556; p=0.0107). Also, PCA for the 39 measurements clearly revealed differentiation of the individuals into two distinct morphometric groups I and II corresponding to the two karyotype forms I and II, respectively. Alongside this variation, there was an obvious variation in four craniodental characters that can be used effectively to discriminate between individuals from the two karyotype forms. These characters are: 1) the presence of supracondyloid foramen above the occipital condyles on both sides only in those individuals from karyotype form II, 2) the location of hard palate in old and oldest individuals that either ends above the line connecting the rear edges of the alveoli of M^3 in those from karyotype form I or markedly extends behind them in those from form II, 3) the posterior margin of nasals that either reaches the line connecting the upper margins of the infraorbital foramen in those from form I, or it reaches the line connecting the lower margins of the infraorbital foramen in those from form II, and 4) the M^2 has either three roots in eight specimens from form I or four roots in four specimens from form I and two specimens from form II, while the M^3 has either two roots in only two specimens from form I, three roots in nine specimens from form I and two specimens from form II, or four roots in only one specimen from form I. Nevertheless, the M^1 has three roots in all individuals from both karyotypes. This is consistent with the findings of Çoşkun (2004a,c) and Çoşkun et al. (2016). Accordingly, we have recognized five root formulae: 3/3/3, 3/3/4, 3/3/2, 3/4/2, and 3/4/3 in those individuals from karyotype form I and just one formula: 3/4/3 in those from form II for M^1, M^2, and M^3, respectively. Similar or differential results have been found separately in *N. ehrenbergi* (Çoşkun, 2004a; Çoşkun et al., 2016), *N. nehringi* (Çoşkun, 2003 and references therein), *N. tuncelicus* and *N. munzuri sp. n.* (Çoşkun, 2004c) and *S. leucodon* (Sözen et al., 2006a).

Regarding the structure and characteristics of the phallus and baculum, it was found that the urethral opening in all males was surrounded by three lobes: one dorsal and two laterals. Nevertheless, the lateral lobes in the males from karyotype form II were thicker than those found in males from form I, and thus, they gave the glans penis of those from form II a distinct general shape. This agrees in some respects with the findings of Simson et al. (1993); Çoşkun (2004c); Yiğit et al. (2006); Kankiliç et al. (2014) who pointed out that the phallus and baculum distinctly differ between the karyotype forms of *S. ehrenbergi* and *N. ehrenbergi* examined from Israel, Egypt and Turkey. This meant that the baculum is species specific and its length is not correlated neither with the greatest skull length (Simson et al., 1993) nor the head and body length (Çoşkun, 2004c; Yiğit et al., 2006; Kankiliç et al., 2014). In addition, although relative measuring variation at the five bacular characters was observed between the males from both karyotype forms, the mean values showed no clear distinction between these forms (Tab. 2). Also, as previously described by Simson et al. (1993), no relation was found herein between the baculum length and greatest skull length.

In conclusion, our results confirm the occurrence of two karyotype forms (I and II) with different 2n, NF, and NFa number in *N. ehrenbergi* from El-Hammam. Karyotype form I consists of 2n=60, NF=73, and NFa=70, while karyotype form II, which is described herein for the first time, composes of 2n=62, NF=77, NFa=74. This chromosomal variation was associated by differences in both morphological (external and craniodental) and penial characters. Therefore, it seems reasonable to suggest that this variation may represent clear evidence for occurrence of evolutionary process in *N. ehrenbergi*, which led to the formation of a new putative biological species with different chromosomal, morphological, and penial characters. This species, which is not yet formally named, its status as a distinct biological species could be justified due to its 1) distribution parapatrically with the other species (formerly described by Lay and Nadler, 1972), separated by a narrow zone of about 0.2 km, 2) chromosomal, morphological and penial characteristics differences, and 3) biochemical variation at seven polymorphic genetic loci (Shahin et al., 2018). Confirmation of this supposition, however, needs to carry out further studies involving examination of more samples along the species distribution range as well as conducting additional morphological, biochemical, cytogenetical, physiological, ecological, behavioral, and molecular investigations. In addition, it is not yet known whether this new putative karyotype consisting of 2n=62 chromosomes has evolved from the ancestral local karyotype having 2n=60 chromosomes by a specific kind of chromosomal rearrangements other than Robertsonian fission. This could be clarified by means of various banding techniques which are currently under investigation.

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