Pakistan J. Zool., vol. 50(5), pp 1671-1680, 2018. **DOI**: http://dx.doi.org/10.17582/journal.pjz/2018.50.5.1671.1680

Genetic Differentiation and Relationship between Two Karyotype Forms of *Nannospalax ehrenbergi* (Rodentia: Spalacidae) in Egypt

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Article Information
Received 22 November 2017
Revised 30 December 2017
Accepted 27 January 2018
Available online 31 July 2018

Authors' Contribution
AABS conceived and designed the study and collected and examined the samples. AMMM performed the electrophoretic techniques. AABS analyzed the data and wrote the article.

Key words
Electrophoresis, Genetic differentiation, *Nannospalax ehrenbergi*, Protein variation, Spalacidae.

ABSTRACT

Genetic differentiation between the two karyotype forms I (2n = 60) and II (2n = 62), which have been recently recognized in the Egyptian *N. ehrenbergi*, was examined at 17 structural genetic loci and interrelationship was discussed. Of these 17 genetic loci, 10 (58.8%) loci were monomorphic with the same allele fixed in all individuals from both karyotype forms and only 7 (41.2%) loci were polymorphic with two different alleles. Levels of genetic variability between the two forms were relatively low and are comparatively either within the range or quite different from those of the same species occurring elsewhere. The means of expected (H_e) and observed (H_o) heterozygosity were 0.175 ± 0.503 and 0.124 ± 0.496, respectively, the mean percentage of polymorphic loci (P) was 29.42%, while the mean number of alleles per locus (A) was 1.30 ± 0.11. In addition, the mean levels of genetic identity (I) and genetic distance (D) were 0.932 and 0.070, respectively, indicating that the two forms were genetically highly similar. Thus, the present results are concordant with that obtained from the chromosomal, morphological and penial study and support the recent hypothesis of occurrence of new species in *N. ehrenbergi*. Divergence between the two species would have occurred during Pleistocene (ca 1.26 million years ago).

INTRODUCTION

Blind mole rats of the family Spalacidae are strictly subterranean rodents with specific various morphological, physiological and behavioral features that emphasize their adaptation to underground life (Topachevskii, 1969; Nevo, 1979; Savić, 1982a; Savić and Nevo, 1990). Although the family Spalacidae is currently represented by nine living species in the recent taxonomic checklists (Musser and Carleton, 2005; Kryštufek and Vohralik, 2009), its taxonomy has long been the subject of controversial discussion. While some authors treated the family as monogeneric with a single recognized genus, *Spalax* (Savić and Nevo, 1990; Musser and Carleton, 2005; Kryštufek and Vohralik, 2009), some others distinguished two genera namely *Spalax* and *Microspalax* or *Nannospalax* (Topachevskii, 1969; Gromov and Baranova, 1981; Savić, 1982a, b; Savić and Soldatović, 1984; Musser and Carleton, 1993; Németh *et al*., 2009, 2013). In addition, fossil record (Topachevskii, 1976), chromosomal variation (Lyapunova *et al*., 1974) and mitochondrial sequences (Hadid *et al*., 2012) have suggested a major cladogenesis of the subfamily Spalacinae into two genera *Spalax* and *Nannospalax*. Moreover, Chişamera *et al*. (2014) demonstrated that the division of the extant species of the subfamily Spalacinae into two distinct genera, *Spalax* and *Nannospalax*, is justified and congruent with the pattern of phylogenetic divergence.

Basically, *Nannospalax* differs from *Spalax* by the presence of supracondyloid foramina above the occipital condyles of the skull, two enamel islands on the chewing surface of the third upper molar (M^3), three rooted upper molars, and the anterior surface of the upper incisors has two longitudinal ridges (Nehring, 1898; Ellerman, 1940; Ellerman and Morrison-Scott, 1951; Topachevskii, 1969; Mursaloğlu, 1979; Nevo *et al*., 1988; Çoşkun, 1994). Karyotypically, however, *Nannospalax* has both low diploid (2n) and fundamental (NF) numbers and acrocentric chromosomes, while *Spalax*, on the contrary, has high 2n and NF and no acrocentric chromosomes (Topachevskii, 1969; Lyapunova *et al*., 1974; Savić and Soldatović, 1984; Savić and Nevo, 1990; Zima and Kral, 1984; Çoşkun *et al*., 2012a, b; Arslan *et al*., 2016).

Specifically, *Nannospalax* is formerly accepted as a valid generic name for the blind mole rats in Turkey, where three superspecies (*N. leucodon*, *N. xanthodon* (formerly

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0030-9923/2018/0005-1671 $ 9.00/0
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N. nehringi) and *N. ehrenbergi*) have been recognized (Topachevskii, 1969; Zima and Kral, 1984; Kryštufek and Vohralik, 2005, 2009; Yiğit *et al*., 2006; Kandemir *et al*., 2012; Arslan and Zima, 2013; Çoşkun *et al*., 2014). Of these three superspecies, *N. ehrenbergi* (formerly *S. ehrenbergi*) inhabits 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; Çoşkun *et al*., 2006, 2016; Schlitter *et al*., 2008; Kryštufek and Vohralík, 2009). This species has long been considered a superspecies presumably because it contains many separate biological species. The main reason for this taxonomic diversity is the remarkable morphological and chromosomal variation recorded within and between populations and species (for review, see Arslan *et al*., 2016). In addition, it 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) and Nevo *et al*. (1991) 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 (Savić and Soldatović, 1984; Yüksel, 1984; Gülkaç and Yüksel, 1989; Nevo, 1991; Yüksel and Gülkaç, 1992; Nevo *et al*., 1994a, b, 1995; Kılıç, 1995; Çoşkun, 1997, 1998, 1999, 2004a, b; Ivanitskaya *et al*., 1997; Çoşkun *et al*., 2006; 2010a, b, 2012a, b, 2014, 2015, 2016; Arslan and Zima, 2015, 2017). Moreover, two karyotype forms (I and II) corresponding to two species, with different 2n, NF, and NFa number, have been recently recognized in Egypt by Shahin *et al*. (2018). Karyotype form I consists of 2n = 60, NF = 73, and NFa = 70, while karyotype form II, which is described in Egypt for the first time, composes of 2n = 62, NF = 77, NFa = 74.

Gel electrophoresis of proteins (Shahin, 2003) and genomic DNA (Kök, 2017; Liu et al., 2018) has been proven as a powerful tool for examining the genetic relationships and inferring the taxonomic status of many assemblages within and among species. The development of this technique for demonstrating allelic variation at genetic loci controlling the structure of enzymes and proteins facilitated the estimation of the degrees of genetic similarity and divergence between populations, species, and consequently, between genera within family. This is efficiently performed based on the assumption that homology in electrophoretic migration is equal to genetic identity and that the structural genes sampled are representative of the genome (for review, see Shahin, 2003 and references therein).

As described by Nevo *et al*. (1984), allozyme polymorphism in mammals appears to be largely correlated with, and predictable by, ecological parameters and at least in part, seems to involve natural selection. According to the literature, few studies on allozyme variation in the blind mole rats have been carried out along their geographical range (Nevo *et al*., 1990, 1994a, 1995, 2000; Kankılıç *et al*., 2005, 2015). In Turkey, Nevo *et al*. (1995) analyzed allozyme diversity in two species, *N. xanthodon* and *N. ehrenbergi* based on 37 allozymic loci. This study revealed that the individuals belonging to nine different chromosomal races have distinct isoenzyme patterns. Also, Nevo *et al*. (2000) examined the chromosome and allozyme diversities, encoded by 32 loci, in 12 populations of the *S. ehrenbergi* superspecies in Jordan and identified four new putative biological species. In addition, Kankılıç *et al*. (2015) studied allozyme variation in Anatolian mole rats and distinguished four species, *viz*., *N. xanthodon*, *N. ehrenbergi*, *N. cilicicus*, and *N. nehringi*.

Following this approach, it is apparent that the Egyptian mole rat, namely *N. ehrenbergi*, is poorly studied from the point of view of biochemical and molecular aspects. Therefore, it was found urgent to conduct further biochemical investigation on this species in Egypt, particularly after the recent recognition of two karyotype forms (I and II) corresponding to two species, with different 2n, NF, and NFa number by Shahin *et al*. (2018). The major objectives of the present study were to: 1) describe the patterns of protein variation, estimate the amount of genetic variation, and assess levels of genetic divergence between the two karyotype forms or species and 2) compare the present data and interrelationship between the two forms with that derived from the previous chromosomal, morphological and penial study carried out by Shahin *et al*. (2018).

MATERIALS AND METHODS

Sampling

This study was conducted only on the same individuals (14 adult individuals; 10 ♂, 4 ♀) of *Nannospalax ehrenbergi* (Nehring, 1898), which have been previously examined in terms of chromosomal, morphological (external and craniodental) and penial variation by Shahin *et al*. (2018). The animals have been previously 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, by digging burrow systems according to the method described by Sözen (2004) and Sözen *et al*. (2006). As previously described by Shahin *et al*. (2018), 12 of these 14 individuals were assigned having karyotype form I (2n = 60) and only two individuals have karyotype form II (2n = 62).

Laboratory and electrophoretic techniques

The animals were sacrificed and blood samples were collected from the heart and centrifuged at 13,000 rpm for 5 min to separate plasma from cells. Liver, heart, kidney and part of skeletal body muscles were also rapidly dissected out. The sera and organs were immediately frozen and kept at -86 °C until the time of electrophoretic analysis.

Tissue samples, 75 mg, were thawed and homogenized in 0.5 ml of ice-cold bi-distilled water in ground glass homogenizer (Model Glas-Col, GKH, USA) for 60 sec. The homogenates were quickly frozen and stored at -86 °C for 5 to 10 days prior to analysis. Immediately before electrophoresis, the extracts were thawed and centrifuged (Model VSMC-13 mini-Centrifuge, Shelton Scientific Mfg. Inc., USA) at 13,000 rpm for 5 min to separate debris from supernatant. Then, the clear supernatant was removed for analysis. At the time of electrophoresis 50 µl of the clear supernatant was mixed with 20 µl of 20 % sucrose solution and 20 µl of protein dye (naphthalene black B 0.03 %). For each slot, caused by the comb in the gel, 20 µl of this mixed sample was applied.

Vertical polyacrylamide gel electrophoresis (Model Mini Vertical 2 # 400000, Semi-Dry Apelex, France) was used to fractionate liver, kidney, muscle and serum enzymatic and non-enzymatic proteins by 2 systems: continuous (Stegemann, 1977) and discontinuous (Maurer, 1968) gel electrophoresis. Enzymes fractionated from liver homogenate included: glutamate dehydrogenase (E.C. 1.4.1.3; Gdh), glutamate-oxaloacetate transaminase (E.C. 2.6.1.1; Got) and esterases (E.C. 3.1.1.1; Est-1, Est-2, Est-3 and Est-4). Fractions from heart included: Isocitrate dehydrogenase (E.C. 1.1.1. 42; Idh), Malate dehydrogenase (E.C. 1.1.1.37; Mdh) and Malic enzyme (E.C. 1.1.1.40; Me). Fractions from muscle included: lactate dehydrogenases (E.C. 1.1.1.27; Ldh-1, Ldh-2, Ldh-3, Ldh-4 and Ldh-5). Non-enzymatic proteins fractionated from liver included: Pre-albumin (Pal), Albumin (Alb) and Transferrin (Trf).

Standard techniques of gel electrophoresis and protein staining were applied following the methods of Shahin (1993) and protein bands were designated according to the system of nomenclature proposed by Allendorf and Utter (1978). Alphabetical designation for alleles was determined according to their relative mobility on a specified buffer system, with the assumption of homology between individuals. The allele producing fast anodally migrating band was designated as "*a*", and alleles corresponding to slow migrating bands were assigned letters proportional to their respective mobility. The allelic frequencies were calculated according to formula given by Ferguson (1980).

Data analysis

Genetic variability of individuals, estimated by the mean number of alleles per locus (A), the percentage of polymorphic loci (P), and the expected and observed mean heterozygosity (H_o and H_e), was determined by use of BIOSYS-1 program (Swofford and Selander, 1981). Coefficients of Nei's (1972) genetic distance (D) and identity (I) were calculated based on allelic frequencies at the 17 loci for all pair-wise comparisons or OTUs using BIOSYS-1 program. Divergence times based on fast and slow evolving loci were estimated from Nei's (1972) genetic distance (D) (see Gardenal *et al*., 1990). Wright's (1978) hierarchical analysis of *F*-statistics was used to partition the total variation into intrapopulation and interpopulation variation at the specific and subspecific levels.

RESULTS

Protein variation

Seventeen loci encoding 7 enzymatic and 3 non-enzymatic proteins were compared between individuals of the two karyotype forms or populations collected from El-Hammam, Matruh, Egypt. Of these 17 genetic loci, 10 (58.8 %) loci (Ldh-1, Ldh-2, Ldh-3, Ldh-4, Ldh-5, Mdh-1, Me-1, Gdh-1, Est-1 and Est-4) were monomorphic with the same allele fixed in all individuals of the two forms, *i.e.* they exhibited no interpopulation variation, and 7 (41.2 %) loci (Idh-1, Got-1, Est-2, Est-3, Pal-1, Alb-1 and Trf-1) were polymorphic with different alleles. The maximum number of allele observed at a single locus in a population was 2. Details of variation in allele patterns and frequencies observed at the 17 loci among individuals of the two karyotype forms are shown in Table I.

Genetic variability and similarity

A summary of genetic data on the individuals of the two karyotype forms I (2n = 60) and II (2n = 62) is given in Table II. None of the individuals within a karyotype form or population exhibited a unique allele at any of the 7 polymorphic loci. Since the larger mean number of alleles per locus (A) and the larger percentage of polymorphic loci (P) are in the smaller samples, dependence on sample size is ruled out as a cause of the observed differences in diversity. Thus, it is possible to use these two parameters, in addition to the expected (H_e) and observed (H_o) heterozygosity, for comparison between the two karyotype forms. In views of A and P, individuals of karyotype form I were genetically more variable (A = 1.41 ± 0.12 and *P* = 41.18) than those of form II (A= 1.18 ± 0.10 and *P* = 17.65), but those of the latter showed more heterozygotes (H_e = 0.176 ± 0.950 and H_o = 0.176 ± 0.950) than those of

the former (H_e = 0.174 ± 0.055 and H_o = 0.072 ± 0.041) (Table II).

Table I.- Allozyme variation between the two karyotype forms of the blind mole rat *Nannospalax ehrenbergi*. Allele frequencies at variable loci in a given form are in parentheses. Alleles of monomorphic loci (Ldh-1, Ldh-2, Ldh-3, Ldh-4, Ldh-5, Mdh-1, Me-1, Gdh-1, Est-1, Est-2, Est-3, Est-4, Pal-1, Alb-1, and Trf-1) are fixed. 2n = diploid chromosome number.

Locus	Karyotype form (2n)	
	Form I (60)	Form II (62)
Ldh-1	a	a
Ldh-2	a	a
Ldh-3	a	a
Ldh-4	a	a
Ldh-5	a	a
Mdh-1	a	a
Mod-1	a	a
Idh-1	a (0.556); b (0.444)	b
Got-1	a (0.222); b (0.778)	a
Gdh-1	a	a
Est-1	a	a
Est-2	a (0.556); b (0.444)	a (0.500); b (0.500)
Est-3	a (0.667); b (0.333)	a
Est-4	a	a
Pal-1	a (0.389); b (0.611)	a (0.500); b (0.500)
Alb-1	a (0.111); b (0.889)	b
Trf-1	a (0.222); b (0.778)	b

Average of Nei's (1972) genetic identity (I) between the two karyotype forms I and II or populations at the 17 structural genetic loci was 0.932 and Nei's (1972) genetic distance (D) was 0.070.

Phylogenetic analysis

From the analysis of the 7 polymorphic loci, 7 it was found that all of these loci were represented by 7 different electromorphs. These 7 protein variants were identified and shared (symlesiomorphic) by the two forms. Analysis of the mean of F-statistics of genotype frequency across the 7 loci indicated that ca 19 % (F_{ST} = 0.192, range = 0.003- 0.636) of variation was due to 21 % (F_{ST} = 0.206) of total population (F_{IT}) and 2 % (F_{ST} = 0.017) of subpopulation (F_{IS}) variation. In addition, Wright's (1978) non-hierarchical analysis of average of F-statistics demonstrated that an average of ca 17 % (F_{DT} = 0.169, range = 0.000- 0.616) of variation was due to ca 0.380 of total limiting and 0.065 of sampling variation.

DISCUSSION

Results of electrophoretic analysis at 17 genetic loci encoding 7 enzymatic and 3 non-enzymatic proteins showed relatively low genetic variation between individuals of the two karyotype forms I (2n = 60) and II (2n = 62) of *Nannospalax ehrenbergi* collected from El-Hammam, Matruh, Egypt. Only 7 (41.2 %) loci (Idh-1, Got-1, Est-2, Est-3, Pal-1, Alb-1 and Trf-1) were polymorphic and 10 (58.8 %) loci (Ldh-1, Ldh-2, Ldh-3, Ldh-4, Ldh-5, Mdh-1, Me-1, Gdh-1, Est-1 and Est-4) were monomorphic with the same allele fixed in all individuals from both forms or populations. As a rule, none of the populations exhibited a unique allele at any of the 7 polymorphic loci and no sex or color-linked alleles were observed in all of the examined populations. Within each population or form, no individuals presented unique alleles or genetic frequencies that allow characterization of subpopulation or subforms. On the average, the means of genetic variability indices were A = 1.30 ± 0.11, P = 29.42 %, H_e = 0.175 ± 0.503 and H_o = 0.124 ± 0.496. However, the mean of Nei's (1972) genetic distance (D) and identity (I) between the two karyotype forms were 0.070 and 0.932, respectively. This result of close similarity in genetic content between individuals of the two forms seemingly characterizes the majority of organisms of which data are available (see review by Selander and Johnson, 1973; Avise, 1974; Ayala, 1975; Nevo, 1999; Nevo *et al*., 1990, 1994a, 2000; Shahin, 2003; Kankılıç *et al*., 2005). Estimates of levels of genetic variability as well as levels of genetic similarity

Table II.- Genetic variability between the two karyotype forms of the blind mole rat *Nannospalax ehrenbergi*. A locus was considered polymorphic if the frequency of the most common allele does not exceed 0.95. n = population size; ± = standard error.

Karyotype form or population (n)	Mean no. of alleles per locus (A)	Percentage of polymorphic loci (P)	Mean heterozygosity	
			Hardy-Weinberg expected (H_e)	Direct-count observed (H_o)
Form I (9)	1.41 ± 0.12	41.18	0.174 ± 0.055	0.072 ± 0.041
Form II (1)	1.18 ± 0.10	17.65	0.176 ± 0.950	0.176 ± 0.950
Mean	1.30 ± 0.11	29.42	0.175 ± 0.503	0.124 ± 0.496

suggest that the two karyotype forms were closely related to each other and are comparatively either within the range or quite different from other taxa (for details, see Nevo *et al*., 1974, 1990, 1994a; Avise and Smith, 1977; De Sousa *et al*., 1996; Gardenal *et al*., 1990; Nevo, 1999; Shahin, 2003; Kankılıç *et al*., 2005).

Mole rats of *N. ehrenbergi* studied here are subterranean generalists. They inhabit an area of a semi-arid desert, El-Hammam locality, which has a Mediterranean climate characterized by a brief, mild, rainy winter and long warm summer months (May to September) of clear sky, high radiation, and no rain. The average annual rainfall is approximately 140 mm/yr and 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. Thus, they are particularly adapted to dry regions and nearly occupy a similar ecological niche. The effective population size and reproductive characteristics of *S. ehrenbergi* were previously studied (Nevo, 1969; Heth *et al*., 1987; Zuri and Terkel, 1998; Gazit and Terkel, 2000; Dewey, 2003). Therefore, one main source of environmental variability should be considered in this area; the weather conditions which vary over the year and temperature ranges from 7°C to around 30.5°C. In such case, external temperature could be a selective factor.

Many accounts have been cited about the main causes of genetic variability and relatively low heterozygosity exhibited by all subterranean and fossorial mammals and many of vertebrate species. For example, it has been reported that both random and deterministic factors, including genetic drift, selection, migration, mutation and historic events, may affect the population size and breeding and thereby causing homozygosity and reducing heterozygosity (Nevo *et al*., 1974). In addition, an increase in genetic variability could be adaptive strategy in an unexpected environment (Nevo, 1978); and variability can also remain weak in an ecologically diversified environment (Pasteur *et al*., 1978). Moreover, under stable conditions in a uniform trophic environment, genetic variability could accumulate (Ayala and Valentine, 1974). Furthermore, it has been pointed out that the genetic variation observed among populations living in nearly stable environmental conditions could be suggestive of differences in vagility and inbreeding (Gorman *et al*., 1977). Normally, high vagility and consequent low inbreeding results in relatively high levels of genetic variation. Thus, like in many other rodent species, the considerably low levels of genetic variability observed herein could be explained as 1) an adaptive strategy for homozygosity in the relatively uniform environment, 2) historic events that limited their geographic distribution in certain habitats, random genetic drift which resulted in nearly similar averages of heterozygosity between the two populations (although general uniformity of fixed or prevalent alleles among them negates drift and suggests strong uniform selection), and 3) ecological variations that lead to a relatively irregular environment. Regarding the effect of gene flow, the striking similarity of allelic patterns across loci among populations would suggest that they have a common gene pool shared by all individuals. Alongside these interpretations, Nevo *et al*. (1984) concluded that the levels of genetic diversity are significantly correlated with ecological parameters (life zone, geographical range, habitat type and range, and climate region), demographic parameters (species size and population structure, gene flow and sociability), and a series of life history characteristics (longevity, generation length, fecundity, origin and parameters related to the mating system and mode of reproduction). Also, Nevo *et al*. (1990) reanalyzed the environmental theory of genetic diversity, particularly the hypothesis of niche-width variation (Van Valen, 1965) that predicts positive correlation between ecological and phenotypic heterogeneities in subterranean, fossorial, and aboveground small mammals. Indeed, they found that the narrow-niche fossorial and subterranean species (Nevo, 1979) displayed significantly lower levels of observed heterozygosity than did the small mammalian species living aboveground (for details, see Nevo *et al*., 1994a) and references therein). In *N. ehrenbergi* (Nevo, 1991) and *N. leucodon* (Nevo *et al*., 1989), it has been suggested that adaptive radiation and heterozygosity are positively correlated with aridity stress and climatic unpredictability (Nevo and Cleve, 1978). Specifically, in Egyptian, Turkish and Israeli *S. ehrenbergi*, Nevo *et al*. (1994a) and (1995) found that heterozygosity increases toward the ecologically harsh, arid, and climatically unpredictable. More broadly, Nevo *et al*. (1994a) added that allozyme diversity is significantly correlated with the external physical (both climatic and edaphic) and biotic (parasite infection and plant cover) environment and migration is not influential based upon spatial autocorrelation of allozyme frequencies.

On the other hand, the significant close similarity of observed and expected heterozygosity between the two populations may e due to 1) selection, either for the homozygotes or against the heterozygotes, 2) positive assortative mating, or 3) any other special explanation such as that the homozygotes are more active than the heterozygotes and then they were frequently more trapped.

As reported by Shahin *et al*. (2018), the two karyotype forms I and II (species) possess a diploid number (2n) of 60 and 62 chromosomes and a fundamental number (NF) of 73 and 77, and autosomal number (NFa) of 70 and 74, respectively. Analysis of genetic data showed

that the two forms were differentiated by heterogeneity in the occurrence alleles of only 5 (29.4 %) loci of the 17 genetic loci. This reflects their strong phenotypic and genotypic affinities (I = 0.932). In the remaining 12 loci, the same allele was either fixed or predominant in both of them, or completely absent at least in one of them (Table I). Karyotypically, the two species are different from each other in the 2n, NF and NFa due to the possession of form II one metacenteric pair of chromosomes more than form I. This increase in 2n, which is presumably occurred as a result of Robertsonian fission, *i.e.,* metacentric fission, could be explained in terms of speciation and adaptation to environmental conditions, particularity the aridity and relatively high temperature characterizing El-Hammam region (Shahin *et al*., 2018). In addition, the latter authors found that there are differences in the morphology of chromosomes between the two species which have been attributed to centromeric translocation. This chromosomal variation is also associated by obvious differences in the morphological (external and craniodental) and penial characters (for details, see Shahin *et al*., 2018).

On the basis of divergence time, divergence between the two species would have occurred during Pleistocene (ca 1.26 million years ago).

CONCLUSION

Results of the present study revealed that the levels of genetic variability between the two forms were relatively low and are comparatively either within the range or quite different from those of the same species occurring elsewhere. The means of expected (H_e) and observed (H_o) heterozygosity were 0.175 ± 0.503 and 0.124 ± 0.496, respectively, the mean percentage of polymorphic loci (P) was 29.42 %, while the mean number of alleles per locus (A) was 1.30 ± 0.11. In addition, the means of genetic identity (I) and genetic distance (D) were 0.932 and 0.070, respectively, indicating that they were genetically highly similar. Therefore, the biochemical data are comparatively concordant with the chromosomal and morphological observations carried out on the same individuals from El-Hammam locality and strongly supports the recent hypothesis of occurrence of new speciation in *N. ehrenbergi* that led to the formation of a new putative biological species with different chromosomal, morphological, and penial characters. The two species were diverged by the late Pleistocene (ca 1.26 million years ago).

ACKNOWLEDGEMENTS

We are grateful to the Bedouins who guided us to the sites of collection of samples from El-Hammam, Matruh, Egypt. Also, we are grateful to the peoples who presented a great help to trap the animals alive. In addition, we thank the two anonymous reviewers for their valuable comments on the manuscript.

Statement of conflict of interest

The authors declare that there is no any conflict of interests regarding the publication of this article.

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