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FIRST MOLECULAR IDENTIFICATION OF *GERBILLUS AMOENUS* (RODENTIA, MURIDAE) IN MOROCCO

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Abstract. The taxonomic status of the gerbil *Gerbillus amoenus* in relation to *Gerbillus nanus* and the distribution range of these two species in Africa and/or Asia have long been debated and are not yet fully clarified. In our study, we identify two specimens of small gerbils that we captured in two localities of the south of Morocco, using morphometric and/or molecular tools. The body and skull measurements were not able to unambiguously discriminate between three closely related small gerbils (*Gerbillus amoenus*, *Gerbillus nanus* and *Gerbillus henleyi*). However, the cytochrome b gene analysis showed that our two specimens cluster unambiguously with haplotypes of *G. amoenus*. This represents the first genetic characterization of *G. amoenus* in Morocco. It confirms, based on mitochondrial DNA, that the previously described species living in Africa is indeed *G. amoenus* and not *G. nanus,* the latter species being present strictly in Asia.

INTRODUCTION

The pleasant gerbil, *Gerbillus amoenus* de Winton, 1902, was first described in Egypt and its type locality is Giza Province (ca. 29°59′ N, 31°08′ E). It is a small gerbil that possesses hairless hind feet and a long discoloured tail with inconspicuous brush. It occupies a wide range of habitats including sandy and rocky desert, salt marshes, wasteland and dense vegetation in cultivated areas, oases and wadis (Osborn and Helmy 1980; Abu Baker and Amr 2003; Aulagnier et al. 2008; Hoath 2009; Denys et al. 2017). This gerbil was placed in synonymy under *G. dasyurus* by Ellerman and Morrison-Scott (1951), *G. campestris* by Petter (1975) or *G. nanus* by Corbet (1978). In opposition, it was considered a distinct species by Wassif et al. (1969), Osborn and Helmy (1980), Lay (1983), and Musser and Carleton (2005). Based on the external appearance, *G. amoenus* cannot be distinguished from the dwarf gerbil, *Gerbillus nanus* Blanford, 1875. It also resembles another long-tailed small gerbil with naked hind feet, the pigmy gerbil, *Gerbillus henleyi* de Winton, 1903. These three species have a similar coloration pattern and a few identical cranial features (Osborn and Helmy 1980). They also have the same diploid chromosome number (2n = 52), with aFN (autosomal fundamental number) = 58–60 for *G. amoenus*, aFN = 58–62 for *G. nanus* and aFN = 58–62 for *G. henleyi*. Banding analysis can unambiguously distinguish *G. amoenus* from *G. henleyi*, but *G. amoenus* and *G. nanus* share very similar chromosome morphology (Lay 1983; Volobouev et al. 1995; Dobigny et al. 2002; Hoath 2009; Ndiaye et al. 2013). Using complete cytochrome b gene (*cytb*) sequencing, Ndiaye et al. (2013) showed an important genetic distance (pairwise Kimura two-parameter, or K2P of 6.5%) between African (Libya, Mauritania, Mali) and Asian (Israel, Pakistan) specimens, and proposed to name the African specimens *G. amoenus* and the Asian specimens *G. nanus*, as previously suggested by Aulagnier et al. (2008). Ndiaye et al. (2016a) confirmed this distinction using short *cytb* sequences from Mauritania, Niger, Egypt, Pakistan and Afghanistan. The distribution of *G. amoenus* would extend in North Africa, discontinuously from Morocco to Egypt, and in the Sahel from Mauritania to Chad. For *G. nanus*, the distribution would cover Israel, the Arabian Peninsula, Iraq, Iran, Afghanistan, Pakistan and north-west India. The aim of our study is to identify morphologically and/or genetically two specimens of small naked-footed gerbils captured in Morocco and to provide the first molecular proof of the presence of *G. amoenus* in Morocco.

MATERIALS AND METHODS

During field work in October 2014 and December 2014 in Morocco, we captured two small naked-footed gerbils alive using Sherman traps: one in the arid region of

Aousserd (extreme south of Morocco) and one in the semi-arid region of Akhfennir (south-west of Morocco) (see Table 1). One of the two specimens (AKH2) was considered for morphometric identification. The body and skull of the other specimen (AOS1) were in too poor a condition to be included in the morphometric analysis. We used the standard external measurements (in mm), head-body length (HB), tail length (T), hind feet length (HF) and ear length (E). We also took the skull measurements (in mm) on both dorsal and ventral views of the skull: greatest length of skull (GLS), breadth of braincase (BB), least interorbital constriction (IO), length of nasals (LN), width of zygomatic arch (WZYG), length of anterior palatine foramina (LAF), length of upper molar series (M1M3) and diagonal length of tympanic bulla (LTB). For the sake of comparison, we measured 10 skulls of *G. nanus* from karyotyped adult individuals from the collections of the 'Museum National d'Histoire Naturelle' of Paris, France (MNHN). Among these 10 skulls, we labelled those from Africa (Mauritania, Mali, Niger) as *G. amoenus* and those from Asia (Saudi Arabia) as *G. nanus*, based on Ndiaye et al. (2013, 2016a) (see Table 2). We also measured four skulls of *G. henleyi* from sequenced adult individuals of the collections of the Laboratory 'Biodiversity, Ecology and Genome' in the Faculty of Sciences of Rabat, Morocco (FSR) (see Table 2). The voucher of the measured specimen (AKH2) is kept in the FSR collections (FSR-MAR14-AKH2).

For the genetic identification, we extracted the DNA from a piece of liver using the QIAGEN Kit (DNeasy Blood & Tissue Kit) following the manufacturer recommendations. Then we amplified the cytochrome b gene (1040 base pairs, or bp) via polymerase chain reaction using the primers L7 (ACC AAT GAC ATG AAA AAT CAT CGT T) and H15915 (TCT CCA TTT CTG GTT

Table 1. List of sequenced specimens used in the molecular analysis.

Species	Specimen code	Country (locality name)	Latitude and longitude	GenBank number	Reference
Gerbillus amoenus	AOS1	Morocco (Aousserd)	22°36′N, 14°17′W	MN395833	This work
Gerbillus amoenus	AKH2	Morocco (Akhfennir)	28°05′N, 12°04′W	MN395832	This work
Gerbillus amoenus	1997016	Mauritania (15 km N of Nouakchott)	18°12′N, 16°02′W	AJ851270	Chevret and Dobigny (2005)
Gerbillus amoenus	1553	Libya (23 km N of Sabha)	27°14′N, 14°24′E	JQ753052	Ndiaye et al. (2013)
Gerbillus amoenus	JMD930	Mauritania (80 km N of Nouakchott)	18°44′N, 15°37′W	JQ753059	Ndiaye et al. (2013)
Gerbillus amoenus	TES2	Mali (6 km SSE of Tessalit)	20°12′N, 01°01′E	JQ753060	Ndiaye et al. (2013)
Gerbillus amoenus	TES23	Mali (1 km N of Tessalit)	20°15′N, 00°59′E	JQ753061	Ndiaye et al. (2013)
Gerbillus amoenus	N3009	Niger (Ourou, Air)	19°10′N, 07°58′E	KM236112	Ndiaye et al. (2016b)
Gerbillus amoenus	1999032	Mali (Edjerir)	18°12′N, 01°24′E	LN606685	Ndiaye et al. (2016b)
Gerbillus amoenus	M005255	Egypt (Faiyum)	29°34′N, 30°54′E	KX786153	Khalifa et al. (2018)
Gerbillus amoenus	M005256	Egypt (Faiyum)	29°34′N, 30°54′E	KX792471	Khalifa et al. (2018)
Gerbillus amoenus	M005257	Egypt (Faiyum)	29°34′N, 30°54′E	KX792472	Khalifa et al. (2018)
Gerbillus amoenus	M005258	Egypt (Faiyum)	29°34′N, 30°54′E	KX792473	Khalifa et al. (2018)
Gerbillus amoenus	M005259	Egypt (Faiyum)	29°34′N, 30°54′E	KX792474	Khalifa et al. (2018)
Gerbillus amoenus	M005262	Egypt (Wadi El Natroun)	30°23′N, 30°22′E	KX786154	Khalifa et al. (2018)
Gerbillus amoenus	M005263	Egypt (Wadi El Natroun)	30°23′N, 30°22′E	KX792475	Khalifa et al. (2018)
Gerbillus nanus	7045	Israel (20 km ESE of Sde Boker)	30°48′N, 34°59′E	JQ753051	Ndiaye et al. (2013)
Gerbillus nanus	Gn1	Israel (Aïr bar, Arava)	29°51′N, 35°03′E	JQ753053	Ndiaye et al. (2013)
Gerbillus nanus	Gn2	Israel (Arava)	29°51′N, 35°03′E	JQ753054	Ndiaye et al. (2013)
Gerbillus nanus	Gn3	Israel (Aïr bar, Arava)	29°51′N, 35°03′E	JQ753055	Ndiaye et al. (2013)
Gerbillus nanus	Gn4	Israel (Aïr bar, Arava)	29°51′N, 35°03′E	JQ753056	Ndiaye et al. (2013)
Gerbillus nanus	Gn5	Israel (Aïr bar, Arava)	29°51′N, 35°03′E	JQ753057	Ndiaye et al. (2013)
Gerbillus nanus	Gn6	Israel (Aïr bar, Arava)	29°51′N, 35°03′E	JQ753058	Ndiaye et al. (2013)
Gerbillus nanus	1988007	Pakistan (Sind Desert)	–	JQ753063	Ndiaye et al. (2013)
Gerbillus nanus	N1	Israel (Hatzeva N. Reserve)	30°43′N, 35°16′E	KM236130	Ndiaye et al. (2016b)
Gerbillus nanus	N2	Israel (Hatzeva N. Reserve)	30°43′N, 35°16′E	KM236131	Ndiaye et al. (2016b)
Gerbillus nanus	N3	Israel (Hatzeva N. Reserve)	30°43′N, 35°16′E	KM236132	Ndiaye et al. (2016b)
Gerbillus nanus	N4	Israel (Hatzeva N. Reserve)	30°43′N, 35°16′E	KM236133	Ndiaye et al. (2016b)
Other Gerbillinae					
Gerbillus henleyi				MH660911	Bouarakia et al. (2018)
Gerbillus hoogstrali				JN021414	Ndiaye et al. (2012)
Gerbillus poecilops				JQ753064	Ndiaye et al. (2013)
Gerbillus simoni				MH660910	Bouarakia et al. (2018)

Table 2. Skull measurements of one newly collected specimen and comparison with the measurements of karyotyped adult individuals of *Gerbillus amoenus* and *Gerbillus nanus* (MNHN collections) and sequenced adult individuals of *Gerbillus henleyi* (FSR collections).

Species	Country (locality)	Specimen code	GLS	BB	IO	LN	WZYG	LAF	M1M3	LTB
Gerbillus amoenus	Morocco (Akhfennir)	FSR-MAR14-AKH2	25.63	13.5	4.43	10.75	10.58	4.41	3.58	9.65
	Mauritania (N. Nouakchott)	MNHN-ZM-MO-1997-1452	25.38	13.36	4.57	10.78	10.75	4.38	3.72	9.21
	Mali (In Tebezas)	MNHN-ZM-MO-2004-905	24.36	13.77	4.67	9.73	10.38	4.19	3.26	–
	Mali (Tidermène)	MNHN-ZM-MO-2004-907	23.89	13.14	4.47	9.96	9.93	3.86	3.18	9.19
	Mali (Tillemsi)	MNHN-ZM-MO-2004-904	25.12	13.91	4.23	10.62	10.46	4.23	3.59	10.01
	Mali (Tessalit)	MNHN-ZM-MO-2004-903	25.17	13.21	4.3	10.56	10.09	3.88	3.38	9.36
	Niger (Agadez)	MNHN-ZM-MO-2003-564	24.83	–	4.32	10.24	10.16	3.88	3.58	9.31
	Niger (Bosso)	MNHN-ZM-MO-2003-599	25.58	13.98	5.32	11.4	10.81	3.85	3.46	9.78
	Niger (Ourou-Aïr)	MNHN-ZM-MO-2003-594	25.12	13.71	4.49	10.51	10.39	3.79	3.4	9.77
Gerbillus nanus	Saudi Arabia (Taif)	MNHN-ZM-MO-1992-771	28.29	14.82	4.69	12.16	11.29	4.65	3.86	10.92
	Saudi Arabia (Al Hofuf)	MNHN-ZM-MO-1990-4	27.7	14.94	4.55	12.14	11.19	4.61	3.58	10.62
Gerbillus henleyi	Morocco (Ain Beni Mathar)	FSR-MAR14-BMT1	21.52	11.98	3.93	8.83	8.99	3.55	2.8	6.74
	Morocco (Ain Beni Mathar)	FSR-MAR14-BMT2	22.38	12.45	4.02	9.1	9.38	3.82	3.03	7.22
	Morocco (Ain Beni Mathar)	FSR-MAR14-BMT4	22.35	12.5	4.15	8.81	8.98	3.78	3.02	7.14
	Morocco (Ain Beni Mathar)	FSR-MAR15-BMT18	22.49	12.39	4.07	8.84	9.46	3.55	3.09	6.9

TAC AAG AC) (Ducroz et al. 2001). The PCR mix contained a total volume of 20 µl, composed of 3.44 µl of milliQ water, 2 µl of Taq polymerase buffer, 1 µl of Dimethyl Sulfoxide (DMSO), 0.8 µl of Nucleotides Mix (6.6 mM), 0.32 µl of each primer (10 pM/µl), 0.12 µl of Taq Polymerase and 2 µl of DNA. PCR started with the initial denaturation step of 3 min at 94°C, followed by 38 cycles of 30 sec at 94°C, 40 sec at 52°C, and 90 sec at 72°C, with the final extension step of 5 min at 72°C. Double-stranded PCR products were purified and sequenced in both directions by Eurofins (France). The two obtained sequences (1133 bp for AOS1 and 1140 bp for AKH2) were submitted to GenBank (see Table 1). We included in the molecular analysis 28 *cytb* sequences for *G. amoenus*/*nanus* present in the GenBank database. Based on Ndiaye et al. (2013, 2016a), we labelled the 16 sequences from Africa as *G. amoenus* and the 12 sequences from Asia as *G. nanus* (see Table 1). The five Egyptian sequences of *G. amoenus* (KT721321 to KT721325) from Ndiaye et al. (2016a) were not included in the analysis because they are shorter than 239 bp. Also, 12 sequences of *G. nanus* in GenBank were not included due to their small length (between 228 and 416 bp).

We aligned the sequences in BioEdit (Hall 1999) and removed the beginning and the end of all the sequences used in the analysis because these parts of the sequences were missing in all the seven individuals of *G. amoenus* from Egypt available in GenBank (Khalifa et al. 2018), and we maintained a fragment of 942 bp. We constructed a phylogenetic tree using the maximum likelihood (ML) method in the software MEGA 7.0.26 (Tamura et al. 2013) to estimate the evolutionary relationships among the sequences. We used jModeltest 2.1.10 (Darriba et al. 2012) to define the General Time Reversible (GTR) + I + G model (Gu et al. 1995) as the best-fit model of nucleotide substitution according to the Akaike information criterion (Akaike 1973). We tested the robustness of the obtained topologies in all the treatments using 1000 bootstrap replicates. We rooted the phylogenetic tree with one member of the subgenus *Gerbillus* (*Gerbillus hoogstrali*), one member of the subgenus *Dipodillus* (*Gerbillus simoni* referred to as *Dipodillus simoni* in GenBank) and the two other members of the subgenus *Hendecapleura* (*Gerbillus henleyi* and *Gerbillus poecilops*), to which *Gerbillus amoenus* and *Gerbillus nanus* belong (see Table 1). We also calculated for *G. amoenus* and *G. nanus* the number of polymorphic sites (*Np*), the number of haplotypes (*h*), haplotype diversity (*Hd*), nucleotide diversity (*Pi*) and the average number of nucleotide differences (*k*) (Nei 1987) using DnaSP 5.10 (Librado and Rozas 2009).

RESULTS

The body measurements of the individual AKH2 are: HB = 78, T = 105, HF = 21, E = 11; while its skull measurements are presented in Table 2. The individual AKH2 seems to be slightly similar in skull size to the African individuals (*G. amoenus*) of the MNHN collections.

Our genetic analysis allowed us to unmistakably identify the two specimens (AOS1 and AKH2) as *G. amoenus* (see Fig. 1). Also, no phylogeographic structure is recorded within Africa. Although Egypt is located next to Israel, all the Egyptian specimens fall within *G. amoenus* and those from Israel fall within *G. nanus*. For the 16 specimens of *G. amoenus*, the number of polymorphic sites (*Np*) is 53, the number of haplotypes (*h*) is 16, haplotype diversity (*Hd*) is 0.956 ± 0.016, nucleotide diversity (*Pi*) is 0.011 ± 0.001 and the average number of

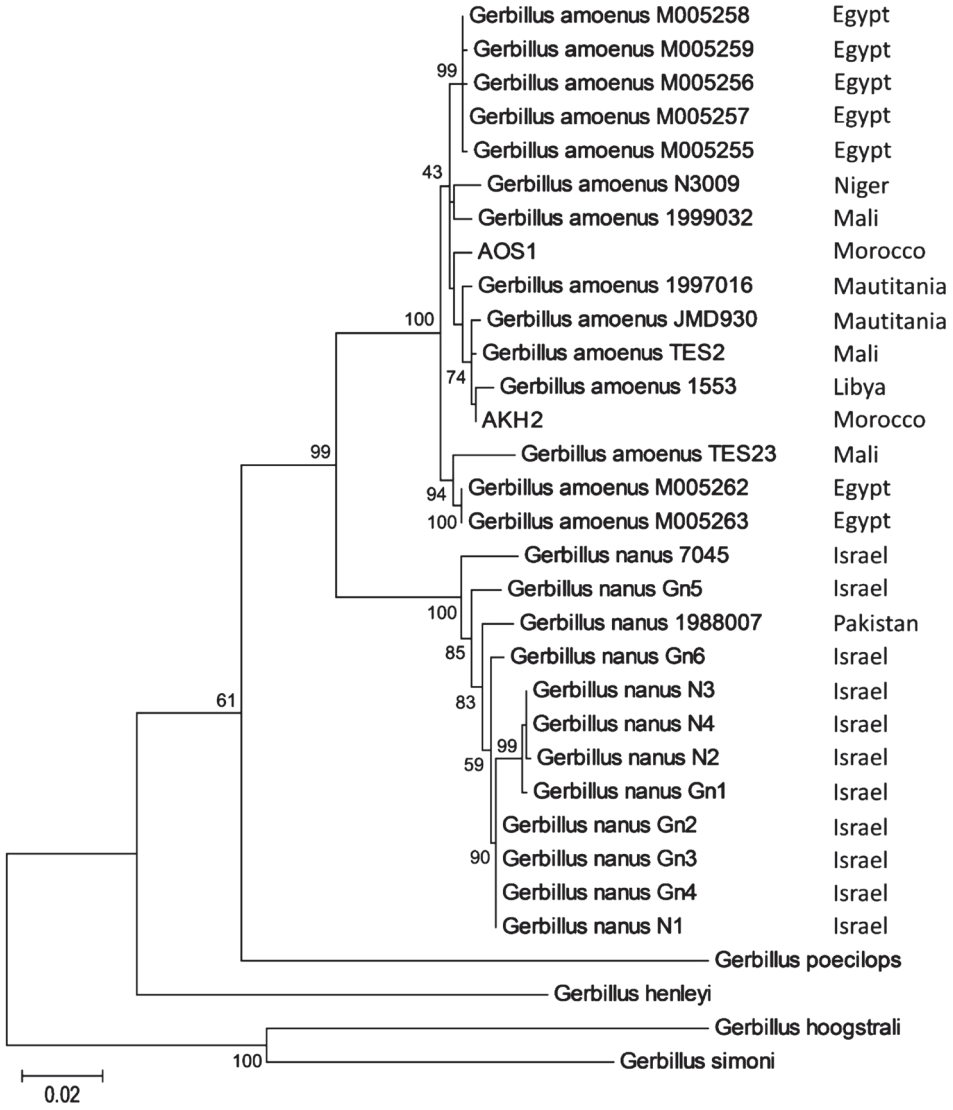

Figure 1. Phylogenetic tree of *cytb* sequences of *Gerbillus amoenus* and *Gerbillus nanus* recovered by maximum likelihood analysis (GTR + I + G substitution model). Numbers at nodes represent ML bootstrap support. To improve clarity, values of the most apical nodes are not included. The scale bar represents the branch length measured in the number of substitutions per site.

nucleotide differences (*k*) is 10.712. A comparison with the 12 specimens of *G. nanus* indicates 43 polymorphic sites, eight haplotypes, haplotype diversity of 0.894 ± 0.078, nucleotide diversity of 0.011 ± 0.003 and an average number of nucleotide differences of 10.591.

DISCUSSION

In our morphometric study, the specimen AKH2 seems to be slightly similar in skull size to *G. amoenus* when compared to the MNHN specimens. Comparison with published values shows that the body and skull measurements of AKH2 can be attributed to either *G. amoenus* or *G. nanus* (Ranck 1968; Osborn and Helmy 1980; Kowalski and Rzebik-Kowalska 1991; Abu Baker and Amr 2003; Aulagnier et al. 2008; Hoath 2009; Granjon and Duplantier 2009; Happold 2013; Aulagnier et al. 2017; Hadjoudj 2017). Thus, the morphological identification cannot be trusted to rigorously differentiate between these two sister species. Additionally, morphological variability in the mean body measurements, tail length and inflation of auditory bulla of *G. amoenus* was previously reported for populations from Egypt and Libya (Ranck 1968). These measurements can also be mistaken for those of another small naked-footed gerbil present in the south of Morocco, *Gerbillus henleyi*, but they are smaller on average in this latter species (Granjon 2013; Happold 2013; Bouarakia et al. 2018). We also note that our specimen differs to a small degree from *G. henleyi* in the morphology of the upper M1 molar and in the length of the auditory bullae on the occipital condyle level (Petter 1961, 1975).

In our genetic study, by adding new sequences of *Ger-*

billus amoenus/*nanus* from Morocco, Egypt and Niger to the molecular analysis, we obtained two strongly-supported clades distinguishing between African (Libya, Mali, Mauritania, Niger, Egypt, Morocco) and Asian localities (Israel, Pakistan). This result is congruent with the genetic structuration first uncovered by Ndiaye et al. (2013) in the form of two well-supported reciprocally monophyletic *cytb* clades within the *Gerbillus nanus* species complex: one comprising African individuals (Libya, Mali, Mauritania) and the other Asian individuals (Israel, Pakistan), with a K2P genetic distance between these two clades reaching 6.5%.

This genetic divergence being greater than the one found between unambiguously identified species of the genus *Gerbillus*, like *G. occiduus*/*G. tarabuli* (K2P distance 1.8%) or *G. pyramidum*/*G. perpallidus* (K2P distance 3.1%), Ndiaye et al. (2013) concluded that these two clades correspond to two sister species with exclusive distribution areas, the African species called *Gerbillus amoenus* and the Asian one *Gerbillus nanus*. Later, Ndiaye et al. (2016a) demonstrated that this differentiation between *G. amoenus* from Africa and *G. nanus* from Asia can also be found when using short *cytb* sequences (239 bp) from museum specimens (from Egypt, Niger, Mauritania, Pakistan and Afghanistan).

One could argue that the distinction between the two species is only based on mitochondrial DNA and that it should also be proved by nuclear data. Ndiaye et al. (2016b) showed some differentiation between these two species based on one nuclear gene (first exon of the gene for the interphotoreceptor retinoid-binding protein, or IRBP), even if they do not form two reciprocally highly supported monophyletic groups. A similar low level of support was also identified in many other closely related *Gerbillus* species and can be explained by a low degree of variability in this gene. Because of incomplete lineage sorting, reciprocal monophyly is not necessarily a property of species, especially recently diverged species (Knowles and Carstens 2007). For diploid organisms, the effective population size of nuclear DNA is four times higher than that of the haploid and maternally inherited mitochondrial DNA. The faster coalescence of mitochondrial loci explains why a species can appear as monophyletic in a mitochondrial tree but not in a nuclear tree, in particular in the case of recent divergence events (Leliaert et al. 2014). Pending additional in-depth analyses investing the genetic divergence between *G. nanus* and *G. amoenus* based on numerous nuclear genes and/or microsatellite data, we consider in this paper that *G. nanus* and *G. amoenus* are two distinct species that can be discriminated based on mitochondrial DNA data. We are fully aware that the mitochondrial DNA represent only the maternal line and that it is possible that individuals from *G. nanus* species could have *G. amoenus* haplotypes if natural hybridization exists between these species, but up to now this has been never reported.

In our study, the clear mitochondrial distinction between the Egyptian specimens (*G. amoenus* haplotypes) and those from Israel (*G. nanus* haplotypes) shows that a geographic barrier present in this region, probably represented by the Sinai Desert or the Nile River, may have caused differentiation in allopatry on both sides of the Red Sea (Ndiaye et al. 2016b). Further studies should be undertaken to determine the factors that separated these two species. Moreover, important intraspecific variability is displayed within the range of *G. amoenus* in Africa. In comparison, and although the specimens of *G. nanus* come from only four localities from two countries, *G. nanus* seems as diverse as *G. amoenus*. Similar intraspecific variability was found in another widely distributed North African gerbil, *G. campestris* (Nicolas et al. 2014; Bouarakia et al. 2019).

The strong clustering of the Moroccan specimens within the African clade represents the first genetic characterization of the species *G. amoenus* in Morocco. The clear molecular distinction based on mitochondrial DNA between the two sister species and their exclusive distribution areas between the two sides of the Red Sea make it very unlikely that *G. nanus* can be found in North-West Africa and Morocco. Therefore, we conclude that the species present in Morocco is improperly labelled *G. nanus*, and it should be named *G. amoenus* instead.

Additional sampling from North Africa, especially from Algeria and Egypt, and more advanced molecular and cytogenetic studies are needed to elucidate the intraspecific diversity of *G. amoenus*, its separation from *G. nanus* and to detect any possible natural hybridization between them.

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