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Genetic diversity within two Tunisian wild jirds: *Meriones shawi* and *Meriones libycus* (Rodentia, Gerbillinae)

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Three *Meriones* species inhabit Tunisia, namely *M. shawi*, *M. libycus* and *M. crassus*, but little genetic data exist on these gerbils. We collected *Meriones* from eight localities in Tunisia, and obtained mitochondrial (cytochrome *b*) and nuclear (IRBP) gene sequence data for 37 and 13 specimens, respectively, belonging to two species: *M. shawi* and *M. libycus*. We also optimised three microsatellite markers previously described in *M. unguiculatus* to obtain a finer analysis of their genetic diversity and geographic structure, given their wide distribution. Phylogenetic inferences of cyt *b* and IRBP data for these species, in the context of other gerbillin data, corroborate their taxonomic affinities reported by previous studies. High cyt *b* haplotype diversity was observed in both species (25 haplotypes in 29 and 27 sequences for *M. shawi* and *M. libycus*, respectively) with little geographical structure for *M. shawi* but three divergent groups in *M. libycus*. The average microsatellite diversity within each population was high ($H_O \geq 0.6$, $H_E \geq 0.8$) with *M. libycus* populations attaining the highest values. Population differentiation was moderate for several population pairs ($F_{st} \geq 0.1$), the highest being between *M. shawi* populations. However, genetic distance among populations was not significantly correlated with geographic distance in either *M. shawi* or *M. libycus*. Our results contribute to a better characterisation of Tunisian *Meriones* species, suggesting high geographic structure in mtDNA of *M. libycus* populations within North Africa.

Keywords: Libyan jird heterogeneity, microsatellites, mtDNA, nuclear DNA, phylogeny

Introduction

Jirds of the genus *Meriones* are rodents of the subfamily Gerbillinae, distributed throughout the Palaearctic region: North Africa, the Middle East and Central Asia to eastern Mongolia (Carleton and Musser 1984; Wilson and Reeder 2005). Most jird species inhabit the arid belt of these regions, with numerous authors having contributed to the taxonomy of the group (Blanford 1869; Vinogradov and Argyropolo 1941; Petter 1953, 1955, 1957, 1961; Lay 1967; Hassinger 1968; Petter et al. 1984; Harrison and Bates 1991). Four *Meriones* species have been described in Tunisia (Cabrera 1907; Pavlinov 2000; Wilson and Reader 2005; Aulagnier et al. 2010): *M. libycus* Lichtenstein, 1823, *M. crassus* Sundevall, 1842, *M. shawi* Duvernoy, 1842 and *M. grandis* Cabrera, 1907. Although the latter two species were recognised by Cabrera (1907) and Pavlinov (2000) as valid species of the *M. shawi* complex, earlier studies considered *M. shawi* and *M. grandis* as subspecies of *M. shawi* (Petter 1961; Aulagnier and Thevenot 1986). According to Aulagnier et al. (2010), *M. grandis* is distributed in North and Central Tunisia and *M. shawi* in the South. However, studies of rich fossil records of this genus in the region of Temara on the North Atlantic coast of Morocco suggest that all fossil *Meriones* remains of 'modern' morphology in North Africa are assigned to *M. shawi* without any mention of *M. grandis* (Stoetzel et al. 2010, 2011, 2014; reviewed in Lalis et al. 2016). Thus we conclude that only *M. shawi*, *M. libycus* and *M. crassus* occur in Tunisia with different distribution ranges, and they are recognised by morphological differences (Supplementary Table S1).

Meriones shawi is a ubiquitous species distributed throughout most of Tunisia, including the northern, central and coastal regions of the country but also many southern regions; thus it is found in a wide range of bioclimates from subhumid to arid and in a variety of environments (Aulagnier et al. 2010; HK pers. obs.). In the north this species occupies mainly slopes of wadis, fields near agricultural activities and erosion-control weirs, which it may damage, but is rare in forests and grasslands. In the central and southern regions its burrows occur either at the bottom of slight natural land depressions where the soil stays moist and vegetation is relatively abundant, or on mounds of sand fixed by *Zizyphus lotus* (jujube), *Retama retum* or *Rhus tripartitum*. *Meriones libycus* is distributed throughout southern Tunisia where it is associated with sandy soil but also occurs under some halophytic plants (Aulagnier et al. 2010; HK pers. obs.). *Meriones crassus* is present also

in the south in warm and sandy regions (Aulagnier et al. 2010); this species seems to be rare because previously only two authors have captured it in Tunisia (Choumowitch 1954; Bernard 1969). Many studies have, however, reported some level of sympatry between these species (Osborn and Helmy 1980; Kowalski and Rzebik-Kowalska 1991). For example, in some regions in the south of Tunisia *M. shawi* and *M. libycus* occur in sympatry, although the latter species is dominant (HK pers. obs.). Due to this sympatric occurrence, species identification relies heavily on morphological determination, which may be particularly difficult given that interspecific hybrids may be present, because interfertility between these species was previously confirmed in captivity in Moroccan, Algerian and Tunisian populations (Petter 1957).

To clarify the taxonomic status of *M. shawi* and *M. libycus* in Tunisia considering their important ecological plasticity, especially *M. shawi*, and to provide genetic data for these two taxa and allow a better study of this genus, we performed a genetic analysis of specimens belonging to *Meriones* populations distributed in different regions and habitats, and placed this in the context of other *Meriones* and Gerbillini.

To complement morphological and ecological studies with genetic data, in our study we used cytochrome *b* (cyt *b*) and Interphotoreceptor Retinoid Binding Protein (IRBP) sequences of individuals sampled in this study belonging to *M. shawi* and *M. libycus* and those of other Gerbillini to determine the phylogenetic relationships between these taxa. These two genes have been largely used to infer phylogenetic relationships in muroid rodents (Jansa and Weksler 2004; Veyrunes et al. 2005; Pagès et al. 2010; Ndiaye et al. 2013). We also used microsatellites to assess the genetic diversity and geographic structure of populations distributed throughout Tunisia. Many genomic studies have shown that primer sequences of simple sequence repeats (SSR) are often conserved across related species, which make them especially useful in co-amplification of cross-species genetic markers (de Gortari et al. 1997; Navani et al. 2002; Du et al. 2010).

Material and methods

Study area and sample collection

We studied natural populations of *Meriones* gerbils in Tunisia. A total of 90 adult specimens were collected for this study to evaluate the genetic variation within and between *Meriones shawi* and *Meriones libycus* in Tunisia (Supplementary Table S2).

Tunisia is divided into different bioclimatic regions ranging from humid to Saharan, and is strongly influenced by the Tunisian Dorsal mountain, which separates Mediterranean areas from the arid climates in the south (Bellil 1979) (Figure 1). It is characterised by a mosaic of juxtaposed biotopes with distinct local characteristics (Karray 1979); these mainly include Kroumeria and Mogod with a humid bioclimate, the Tell region with a semi-arid continental bioclimate bordered to the south by the Tunisian Dorsal mountain, the Cape Bon peninsula with semi-arid maritime bioclimate, the Steppe region with an arid continental bioclimate, the Sahel Plain with an arid maritime bioclimate, the Jeffara region (a vast plain straddling the south-east of Tunisia and north-western Libya) with an arid (continental and maritime) bioclimate, Chott El Jerid (the largest plain in Tunisia bleached by salt efflorescence showing high salinity), and the Big Oriental Erg and the Dahar region are characterised by a Saharan bioclimate (Figure 1).

Animals were captured alive using live rat traps and following two geographical gradients: north–south and southwest–southeast, between 2007 and 2013 from eight locations with distinct climates (Figure 1, Supplementary Table S2). Capture permits (no. 264 and 1551) were provided by the Tunisian Forestry Management Department. Dghoumes (north of Chott el Jerid) is the only locality sampled where the *M. shawi* population (Msh-D) occurs in sympatry with *M. libycus* (Mli-D). We trapped *M. shawi* from four other localities: the populations in the north from Haouaria (Msh-H) in Cape Bon peninsula and in Siliana (Msh-S), separated by the Tunisian Dorsal from the Bouhedma (Msh-B) and Skhira (Msh-Sk) populations. The Msh-B and Msh-Sk populations are the closest to each other but are separated by Sebkha en -Noual (a plain with high salinity). *Meriones libycus* was captured from three

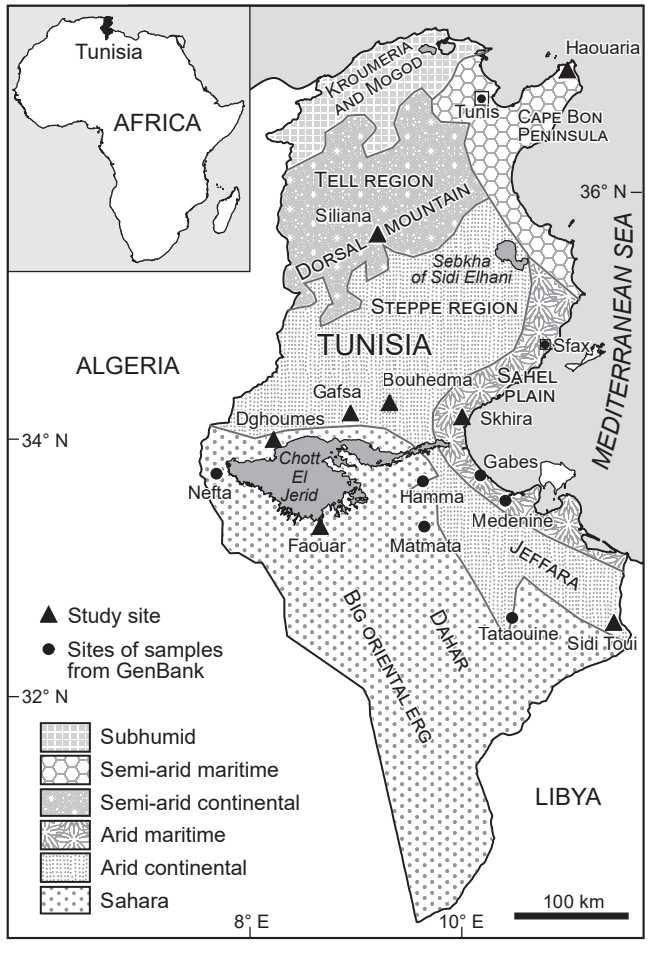

Figure 1: Map of Tunisia showing geographic locations and sampling areas according to climatic zones. Geographic coordinates are provided in Supplementary Table S2

other localities: Gafsa (Mli-G) separated from Mli-D by Mount Bouhlel (approximately 300 m above sea level [asl]) that defines the limits of the Tozeur and Gafsa regions, and Faouar (Mli-F) separated from Msh-D and Mli-D by Chott el Jerid. We trapped *M. libycus* (Mli-T) and specimens of *Meriones* (Msp-T) of uncertain specific identity in Sidi Toui, in south-eastern Tunisia in the Jeffara; these were the most isolated populations we studied. The individuals of undetermined classification have some external characters in common with both *M. shawi* and *M. libycus* as shown in Supplementary Table S1 and we used cyt *b* and IRBP sequences to determine their species status (*M. shawi*, *M. libycus* or hybrids *M. shawi* × *M. libycus*). Animals were euthanised by isoflurane inhalation (Gaertner et al. 2008), then tissues were collected and preserved at −20 °C or in 95% ethanol and stored in the collections of the Laboratory of Animal Ecology – Sciences Faculty of Tunis, Tunisia. Maps were developed using a geographic information system with Quantum GIS 1.8.0 (Sillero and Tarroso 2010; Tlili et al. 2014) and processed with Inkscape 0.48.5. Coordinates of sampling localities are given in Supplementary Table S2.

DNA extraction

DNA was extracted from tissues preserved by freezing or in 95% ethanol (earlobe, toes, kidneys, muscles or skin) of 90 specimens (males and females) using the QIAamp® DNA extraction kit (Qiagen) following the manufacturer's instructions. The resulting DNA was quantified with a NanoDrop spectrophotometer (Thermo Scientific).

Cytochrome b and IRBP sequencing and phylogenetic analyses

The complete mitochondrial cyt *b* gene (1 140 bp) was amplified for 37 specimens, chosen to represent different sampling populations, using the primers L7 (5′–ACCAATGACATGAAAAATCATCGTT–3′) and H6 (5′–TCTCCATTTCTGGTTTACAAGAC–3′) (Montgelard et al. 2002). When necessary we amplified shorter fragments with the internal primers H8 (5′–CCTCAGAATGATATTTGTCCTC–3′) and L2 (5′–TACCATGAGGACAAATATC–3′) or L24 (5′–CCATGGGGACARATATCATTYTGAGG–3′) (Veyrunes et al. 2005). The amplification reactions were performed in volumes of 25 μl including 1.25 μl of each primer at 10 μM, 2 μl of 2.5 mM dNTPs, 2.5μl of 10× reaction buffer, 2 μl of 25 mM $MgCl_2$, 13.25 μl purified water and 0.25 μl Taq DNA polymerase (5 U μl^{-1}; Promega). A volume of 2.5 μl DNA was used for each amplification reaction, carried out with a Labover PTC100 thermocycler using a program of 40 cycles (30 s at 94 °C, 60 s at 50 °C and 1 min at 72 °C) with an initial denaturation step of 5 min at 94 °C and a final extension cycle of 10 min at 72 °C. The PCR products were purified and sequenced in both directions (forward and reverse) by Cogenics (Grenoble, France) with the primers L7 and H6, and if necessary the internal primers.

Two pairs of primers were used to amplify two overlapping fragments of the first exon of the IRBP gene: I1 (5′–ATGGCCAAGGTCCTCTTGGATAACTACTGCTT–3′) and J2 (5′–CGCAGGTCCATGATGAGGTGCTCCGTGTCC TG–3′); and I2 (5′–ATCCCCTATGTCATCTCCTACYTG –3′) and J1 (5′–CCACTGCCCTCCCATGTCTG–3′) as described by Poux and Douzery (2004). PCR products were purified and sequenced by Biofidal (Vaulx en Velin, France). The resulting cyt *b* and IRBP sequences were aligned with Muscle (http://www.ebi.ac.uk/Tools/msa/muscle/), implemented in Seaview (Gouy et al. 2010). The new sequences have been deposited in GenBank under the accession numbers KP899521 to KP899542 and LN868360 to LN868387.

The cyt *b* data set included 19 sequences of *M. libycus* and six additional *Meriones* species retrieved from GenBank (*M. unguiculatus*, *M. crassus*, *M. rex*, *M. tamariscinus*, *M. chengi* and *M. meridianus*). For the IRBP data, sequences from three species of *Meriones* (*M. unguiculatus*, *M. crassus* and *M. meridianus*) were retrieved from GenBank. For the two data sets sequences from four closely related genera – *Psammomys*, *Rhombomys*, *Taterillus* and *Gerbillus* – were included as outgroups (Supplementary Table S2). The best models fitting our data (GTR+I+G and TVM+I+G for cyt *b* and the IRBP gene, respectively) were determined with jModeltest 2.1.4 (Darriba et al. 2012) using the Akaike information criterion (AIC; Akaike 1973). Phylogenetic reconstructions were performed using maximum likelihood (ML) with PhyML 3.1 (Guindon et al. 2010) and Bayesian inference (BI) with MrBayes 3.2 (Ronquist et al. 2012). Nodal support was estimated using 1 000 bootstrap (BP) replicates in ML analyses, whereas posterior probabilities (PP) were obtained from the BI analyses. The BI Monte Carlo Markov Chain analyses were run for 10 000 000 generations with trees sampled every 500 generations. The burn-in was determined with Tracer 1.6 (Rambaut et al. 2014) and we checked that the average standard deviation of split frequencies remained <0.01 after the burn-in threshold. We discarded the first 10% of the trees as burn-in and visualised the tree with Figtree 1.4 (Rambaut 2012).

Pairwise Kimura two-parameter (K2P) genetic distances were obtained with the cyt *b* data set with Mega 6 (Tamura et al. 2013), and the standard errors were estimated through a bootstrap procedure (1 000 replicates).

Haplotype networks were constructed in Network 4.612, applying the reduced median-joining method (Bandelt et al. 1999) with 29 sequences for *M. shawi* and 27 sequences for *M. libycus*.

Microsatellite genotyping

When we started this study no microsatellite data had been published for any African wild jird. Thus we tested nine microsatellite markers previously described in *Meriones unguiculatus* (Table 1; Neumann et al. 2001) in a representative subsample of each species. Four of the markers showed variation and were therefore further analysed in this work (Table 1). *Mung*μ3, described as a simple AC repeat in *M. unguiculatus*, showed a different structure in the wild African *Meriones* individuals sequenced (*M. shawi* and *M. libycus*) with some individuals showing an interrupted repeat with a smaller CA repetition 5′ to the larger AC tract. *Mung*μ5 showed two $(GT)_n$ repeat tracts interrupted by a variable sequence. In the individuals sequenced (two *M. shawi* and three *M. libycus*),

Table 1: Microsatellites analysed in this study. Marker designations and primers are from Neumann et al. (2001). Microsatellites shown to be polymorphic in wild African *Meriones* species are highlighted in bold

Primer	Sequence (5′–3′)	Label	Repeat type[a]
Mungμ1	F: TGTGGCTGGCATCCTA		MU:
	R : AAGCAATTCTGTCTCTGTCTG		Msh, Mli: ND
Mungμ2	F: AGCCTTTATAGATGAGCAAGT		MU:
	R: GCCTACTAATGGTGAACTGA		Msh, Mli: ND
Mungμ3	**F: CAGGCACCCCCAGTTT**	**TET**	**MU, Msh: (AC)n**
	R: GTCTACACAGGCTGAGGATGT		**Msh and Mli: (CA)3-5 T $(AC)_n$**
Mungμ4	F: GGCTCCTGATTCTACATTTCT		MU:
	R: CAACCATTGGCAACTCTC		Msh, Mli: ND
Mungμ5	**F: GCTGGGCTTTAATGTTTATTT**	**FAM**	**MU: $(GT)_n$**
	R: GGTGGCTCACACTTTCTGT		**Msh:$(GT)_n$ (GATAGACAGACAGA) $(GT)_n$**
			Mli: $(GT)_n$ (CT) $(GT)_n$
Mungμ6	F: TTTCTGGGGTCTCTTTCTCTC		MU:
	R: CCATTCTGCAAGACTCCTCT		Msh, Mli: ND
Mungμ7	**F: AGTCCCTATTACATCCACAAG**	**TET**	**MU, Msh, Mli: $(GA)_n$**
	R: TTATCCTGCAAAGCCTAAG		
Mungμ8	F: TGGGTCCTTTGGAAGA		MU:
	R: TGGGTCCTTTGGAAGA		Msh, Mli: ND
Mungμ9	**F: GACAGAGTGGGAGGGGTATGT**	**FAM**	**MU, Msh, Mli: $(CA)_n$**
	R: TGGCAAGTTTGGTTTGTTTGA		

[a] In *M. unguiculatus* (MU), *M. shawi* (Msh), *M. libycus* (Mli); ND = not determined

Mungμ7 displayed a simple GA repeat, as described in *M. unguiculatus*. *Mungμ9* also showed a simple CA repeat in the two species (three *M. shawi* and two *M. libycus*). For *Mungμ3* and *Mungμ7* variation in the flanking regions of the repeats was also detected in some individuals, impeding the establishment of a direct correlation between allele size and repeat length, and therefore only allele size was taken into account in the analyses.

Microsatellites were amplified in duplex: *Mungμ3* with *Mungμ9* and *Mungμ5* with *Mungμ7*. PCRs were performed according to the following steps: 0.5 μl of each primer and 1 μl of genomic DNA (10–17 ng μl^{-1}) were added to a total volume of 10 μl using Bioline 2× MyTaq™ HS Mix DNA polymerase. After an initial denaturation step of 15 min at 95 °C, amplification proceeded for 32 cycles of 30 s at 94 °C, 30 s of annealing at a gradient temperature of 64 °C (for *Mungμ3* and *Mungμ9*) and 60 °C (for *Mungμ5* and *Mungμ7*) and extension at 72 °C for 30 s. A final elongation step of 10 min was carried out at 72 °C. Next, 1 μl of the PCR product was loaded on a polyacrylamide gel, electrophoresed and bands were visualised by colouration. Forward primers marked with fluorescence were obtained from Thermo Scientific. PCR products were then run on an Applied Biosystems 3130 DNA Analyser with the GeneScan™ 500 ROX™ size standard (Applied Biosystems). Given that these markers had been described in a different species (Neumann et al. 2001) we performed a preliminary characterisation by direct sequencing of the amplification products of individuals apparently homozygous for the amplicon size. *Mungμ5* showed many alleles with intermediate sizes suggesting a highly irregular structure and hindering precise allele assignments without extensive cloning. This marker was therefore not included in the analyses that followed.

Microsatellite sequences obtained were deposited in GenBank under accession numbers KX775964 to KX775982.

Genetic analyses

Mungμ3, *Mungμ7* and *Mungμ9* were used in the analyses. Genetic diversity was estimated by taking each sampling location independently. Data were checked for null alleles with the program Micro-Checker (Oosterhout et al. 2004). Genetic diversity was measured using both allelic richness (AR) and standard heterozygosity; we excluded the two populations with fewer samples (<5) and for which the sampling did not reflect the effective population size (Msh-H and Mli-F) and considered two different *g* values, one for each species, given that the minimum number of specimens sampled per population was different (seven for *M. shawi* and six for *M. libycus*).The parameter *g* can be considered a standardised sample size and when comparing AR between several populations, *g* must be less than the smallest sample size (Kalinowski 2004). Linkage disequilibrium was also tested for the three loci genotyped. Heterozygosity (H_E) from Hardy–Weinberg, and Mantel tests were calculated with Arlequin 3.5.1.2. Allelic richness was standardised for sample size using the repeated sampling procedure implemented in HP-Rare (Kalinowski 2005).

Analysis of genetic structure

We have assessed population differentiation using the number of different alleles (F_{st}) and the squared number of repeat differences (R_{st}), to accommodate the two alternative mutation models generally assumed for microsatellite evolution: the infinite alleles model (IAM; Kimura and Ohta 1978) and the stepwise mutation model (SMM; Kimura and Ohta 1978; Shriver et al. 1993; Valdes et al. 1993), respectively.

Results and discussion

Cytochrome b and IRBP phylogenetic relationships

The present study is the first to address the genetic

diversity of two wild *Meriones* species in Tunisia. We generated 1 140 bp of cyt *b* sequence data for 37 of the Tunisian wild *Meriones* specimens sampled in this study (23 *M. shawi*, nine *M. libycus* and five Msp-T), chosen to represent the sampling populations (Supplementary Table S2). We combined these with 37 sequences retrieved from GenBank in the phylogenetic analyses.

The cyt *b* tree (Figure 2a) showed that our Tunisian samples fell within two robustly supported monophyletic groups, one within *M. libycus* (BP = 100/PP = 1) for the nine samples and the second one comprising *M. shawi*, Msp-T and Msp_Algeria (AJ851264) (BP = 100/PP = 1). These results lead us to conclude that the Msp_Algeria and four specimens of the Msp-Ts (T1–T4) were in fact *M. shawi*, the identity of the remaining specimen (T) still uncertain (*M. shawi* or hybrid). The phylogenies supported our morphological identification of the two species. Moreover, the cyt *b* data obtained were congruent with the previous study of Bray et al. (2014) confirming the stratification within *M. libycus* according to geographic origin. Sequences of *M. libycus* were divided into three robustly supported groups (BP = 100/PP = 1) (Figure 2a); the first group (Mli1) contained sequences from Tunisia and one from China, the second group (Mli2) comprised sequences from Saudi Arabia, and our sequences from Tunisia generated in the current study constituted the third group (Mli3). Despite a closer proximity between Tunisia and Arabia, our sequences were highly divergent from the Arabian ones and, surprisingly, also from the group of Tunisian samples previously analysed by Bray et al. (2014), which are closer to the single Chinese sample. Our samples were more closely related to those from Saudi Arabia (BP < 50/PP = 0.69) than to other Tunisian sequences previously reported. The divergence between these three groups is rather high (K2P = 8%; Table 2) which often corresponds to the genetic distance observed between sister species in rodents (Baker and Bradley 2006; Boratynski et al. 2012; Ndiaye et al. 2012), and were similar to the genetic difference of 7.7–7.8% estimated by Lalis et al. (2016) in *M. shawi* between two groups, one group of Moroccan specimens and the second of Tunisian and Algerian specimens with some specimens from Guenfouda the most eastern Moroccan locality. By comparison the mean genetic divergence was very low within our samples of *M. shawi* from Tunisia (K2P = 0. 5%). Despite the wide distribution and ecological plasticity of the Tunisian *M. shawi*, these results reflect a high degree of genetic homogeneity for this species and support the existence of one clade in Tunisia, which probably corresponds to the clade C of Lalis et al. (2016).

Meriones libycus was grouped with *M. rex* and *M. crassus* with moderate support (BP = 69/PP = 0.96); their next closest relative was *M. shawi*, followed by *M. meridianus* and *M. unguiculatus*, and *Rhombomys.* We also noted that the cyt *b* sequence AB381900 classified as *M. chengi* is probably *M. meridianus*. Based on the cyt *b* phylogeny the genus *Meriones* might not be monophyletic as *M. tamariscinus* is more distantly related to the other *Meriones* than to *Rhombomys*. This result is congruent with Ito et al. (2010) and Alhajeri et al. (2015), who observed that *M. tamariscinus* is more distantly related to other *Meriones* species than *Rhombomys*, *Psammomys* and *Brachyones*.

For the IRBP gene we generated 1 262 bp of sequence for 13 specimens (four *M. shawi*, five *M. libycus* and four Msp-T). Phylogenetic analysis results (Figure 2b) were congruent with the cyt *b* results and showed that our samples belonged to two monophyletic groups, one for *M. libycus* (BP = 88/PP = 0.99) regrouped with *M. crassus* (BP < 50/PP = 0.55), and one for *M. shawi* and the four Msp-T samples (BP = 95/PP = 1) confirming their classification as *M. shawi*. We also suggest that the only IRBP sequence of *M. shawi* (KC953400) retrieved from GenBank is probably *M. unguiculatus* (Figure 2b). As we do not have IRBP sequences of *M. tamariscinus* we cannot confirm the possible paraphyly of *Meriones*, but in the IRBP phylogeny, *Psammomys* was more closely related to *Meriones* than *Rhombomys* (BP = 86/PP = 1).

Cytochrome b genetic variation

In order to assess intraspecific structure, one haplotype network was constructed for each species (Figure 3). Within *M. shawi* 25 haplotypes were resolved and only four were shared by more than one individual, indicating a high frequency of unique mutations. Moreover, some haplotypes have accumulated a large number of mutations and the samples of undetermined classification (Msp-T: T, T1–T4) were as divergent from the ancestral *M. shawi* haplotype as are other *M. shawi* samples such as S8 (by nine and 11 mutational steps for T and S8, respectively), the latter clearly identified as belonging to this species from the population of Siliana locality where only *M. shawi* lives. Haplotype diversity for mtDNA was high in *M. shawi* and, even though some reticulation was observed, the star-like shape of the network obtained for this species and the paucity of clear population-specific clusters reflected little geographical structure, suggesting a rapid population expansion in the colonisation of Tunisia by extant *M. shawi*. We obtained sequence data for nine *M. libycus* samples, five from Dghoumes (Mli-D8, Mli-D11, Mli-D12, Mli-D14 and Mli-D15), three from Sidi Toui (Mli-T3, Mli-T6 and Mli-T9) and one from Gafsa (Mli-G1). We took advantage of previously reported cyt *b* sequences (Supplementary Table S2) from Tunisia, Saudi Arabia and China (Ito et al. 2010; Bray et al. 2014). All of these sequences were combined and trimmed to the same length (932 bp). Similar to its congener *M. shawi*, *M. libycus* showed a high diversity of cyt *b* haplotype (25 haplotypes in 27 sequences). Among our new sequences were resolved seven haplotypes of which only two were shared by more than one individual. In accordance with the phylogenetic tree (Figure 2a), the seven haplotypes detected in our Tunisian samples were clustered even though two of the haplotypes were more divergent (13 mutations to closest median vector), and highly and almost equally divergent from the Arabian and the remaining Tunisian sequences (66 and 69 mutations between the closest median vectors, respectively). The haplotype network showed considerable reticulation within the cluster, which may reflect homoplasy, suggesting a relatively old origin for this cluster in Tunisia. The sequences from Saudi Arabia (Bray et al. 2014) and the group of Tunisian sequences

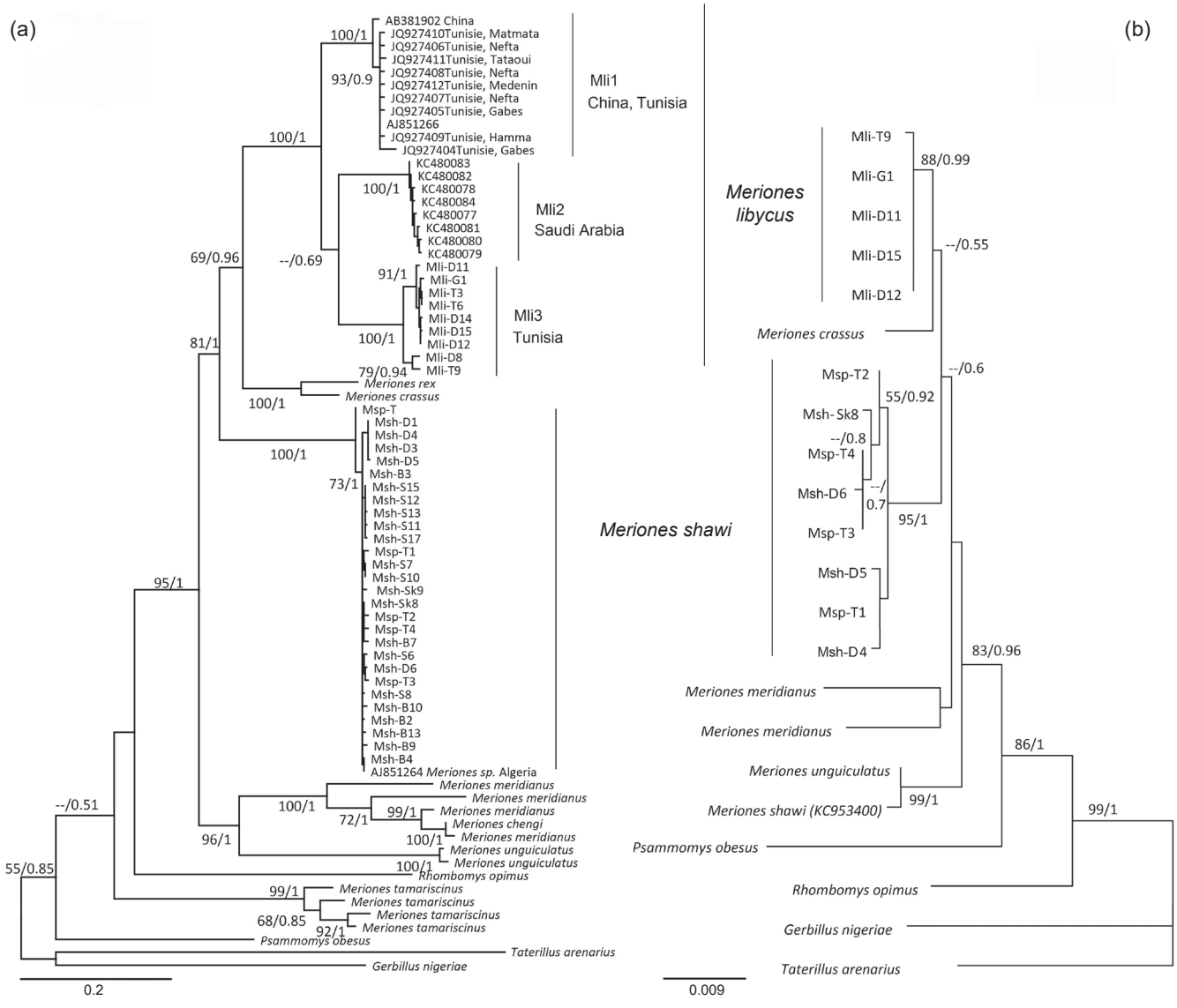

Figure 2: Maximum likelihood phylogenies obtained with cytochrome *b* (a) and IRBP (b) sequence data. For each node the support is indicated as follows: bootstrap percentage/posterior probability except when the node was not supported; -- indicates that the corresponding value is below 50%

Table 2: K2P genetic distances (percentage of mean and standard deviation) between and within (in bold) genetic clades

Species	Mli1	Mli2	Mli3	*M. rex* and *M. crassus*	*M. shawi*	*M. meridianus*	*M. unguiculatus*
Mli1	**0.7 ± 0.1**						
Mli2	8.9 ± 0.9	**0.5 ± 0.1**					
Mli3	8.2 ± 0.8	8.3 ± 0.9	**1.0 ± 0.2**				
M. rex and *M. crassus*	11.8 ± 1.0	12.2 ± 1.1	12.6 ± 1.0	**5.5 ± 0.7**			
M. shawi	13.1 ± 1.0	14.6 ± 1.3	13.4 ± 1.1	12.6 ± 1.1	**0.5 ± 0.1**		
M. meridianus	16.2 ± 1.1	14.4 ± 1.1	16.5 ± 1.1	15.3 ± 1.0	16.6 ± 1.1	**7.4 ± 0.5**	
M. unguiculatus	16.8 ± 1.2	16.5 ± 1.3	16.2 ± 1.2	16.4 ± 1.2	15.9 ± 1.2	16.0 ± 0.1	**0.7 ± 0.2**

obtained from GenBank formed two clear population-specific clusters; one more divergent sequence stands out in the Tunisian cluster, separated by nine mutations from the common stem, the same distance shown by the single Chinese sample available.

These results suggest high mtDNA heterogeneity for *M. libycus* in Tunisia, especially as these populations are not very far from those that we sampled (Figure 1) and are all located in southern Tunisia. Despite the high level of divergence between the two groups (K2P = 8%; Table 2)

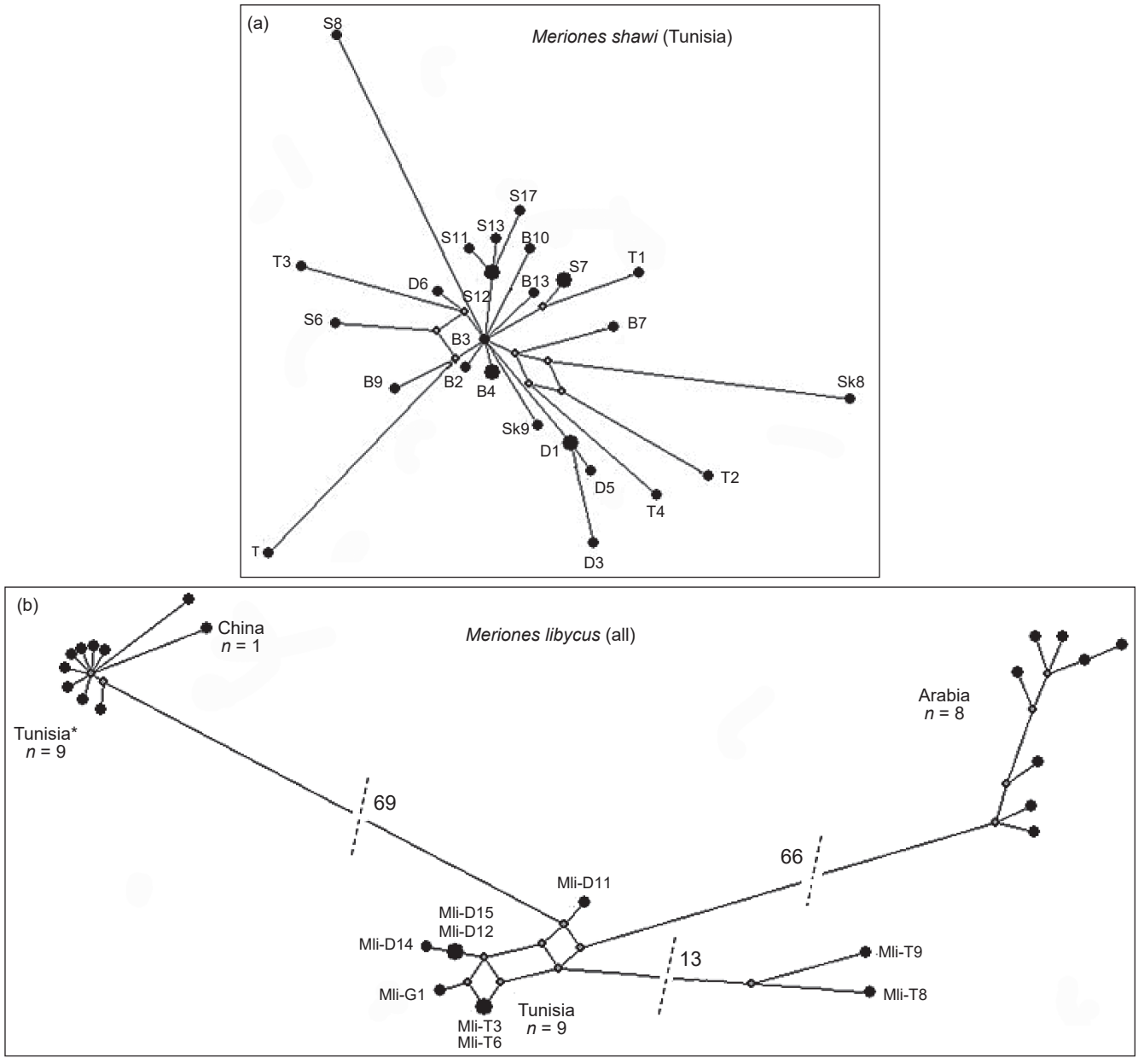

Figure 3: Median-joining networks of cyt *b* haplotypes. Circle area is proportional to haplotype frequency and branch length is proportional to the number of mutations. (a) *Meriones shawi* and Msp-T haplotypes found in this study (T, T1–T4); (b) *M. libycus* haplotypes from this study together with those from previous studies (Supplementary Table S2). The longest branches have been trimmed and the corresponding number of mutations is indicated. Symbols: T = Msp-T, SK = Msh-Sk, D = Msh-D, S = Msh-S, B = Msh-B, * = Tunisian *M. libycus* samples retrieved from GenBank

we cannot attribute the two groups to different subspecies. Nevertheless, three subspecies have been previously reported in Tunisia by many authors: *M. l. erythrourus*, *M. l. schouesboei* and *M. l. syrius* (Lataste 1887; Blanc 1935; Harrison and Bates 1991; IUCN 2009). *Meriones libycus erythrourus* and *M. l. syrius* have also been reported for Saudi Arabia (Harrison and Bates 1991), whereas Bray et al. (2014) suggested that Arabian samples analysed were clearly genetically distinct from the Tunisian variant. These results show that the status of *M. libycus* and its possible subspecies is still not clear, and that more samples and genetic data are needed to confirm and clarify the subdivision within this species.

Microsatellite diversity of Tunisian wild Meriones populations

Microsatellite distributions (Figure 4) were in accordance with our phylogenies, classifying the Msp-Ts within *M. shawi* given the presence of *M. shawi* private alleles in Msp-T. When considering all populations of a species together, *M. shawi* ($n = 82$) and *M. libycus* ($n = 70$) present similar average numbers of alleles per locus (14.3 and 13,

respectively). Considering the differences in sample size we calculated the AR for each population sampled. The AR values obtained were rather homogeneous within each species, suggesting that at the level of sampling performed here, there were no clear differences in the diversity levels across populations within each species.

The average genetic diversity within each population of Tunisian *Meriones* was high ($H_O \geq 0.6$, $H_E \geq 0.8$) (Table 3), as previously observed for these markers in their congener *M. unguiculatus* (Neumann et al. 2001) and for other microsatellites in Moroccan *M. shawi* (Lalis and Lambourdière 2014), with *M. libycus* populations attaining the highest values of H_O. The heterozygosity per locus varied considerably among populations as well as across loci within populations. Across *M. shawi* populations, microsatellite locus 1 (*Mungμ3*) showed the highest diversity (mean $H_O = 1$, mean $H_E = 0.911$), whereas across *M. libycus* populations *Mungμ7* attained the highest diversity values (mean $H_O = 0.896$, mean $H_E = 0.928$).

When populations were tested for Hardy–Weinberg equilibrium (HWE), five out of 33 tests across 10 populations resulted in a significant deviation, but after Bonferroni correction for multiple testing only two remained significant (Mli-G for *Mungμ9* and Msh-B for *Mungμ7*). For *M. unguiculatus* Neumann et al. (2001) reported significant deviation for *Mungμ9* ($p < 0.05$). The deviations observed are heterozygote deficits, which may result from inbreeding or the presence of null alleles. We found evidence for null alleles, for two loci in *M. shawi* and *M. libycus*. There was a significant excess of homozygotes for *Mungμ9* in *M. shawi* from Siliana (Msh-S; $p < 0.01$, Micro-checker) and *M. libycus* from Gafsa (Mli-G; $p < 0.01$, Micro-checker), and for *Mungμ7* in two *M. shawi* populations from Bouhedma (Msh-B; $p < 0.001$, Micro-checker) and Dghoumes (Msh-D; $p < 0.025$, Micro-checker). For *Mungμ7* genotyping failed in some individuals of *M. shawi* (5%, all from Skhira, Msh-Sk) and *M. libycus* (17%, all from Gafsa and Sidi Toui), which may suggest the existence of null homozygotes at this locus.

The test of linkage disequilibrium showed significant values for three pairs of markers but restricted to a few populations: *Mungμ*3-*Mungμ7* in Msh-Sk, *Mungμ*9–*Mungμ7* in Msh-Sk and Mli-G, and *Mungμ*3–*Mungμ*9 in Mli-G, which was not consistent with their congener *M. unguiculatus*, for which no evidence for linkage was reported and all markers were considered genetically independent (Neumann et al. 2001).

At the level of sampling performed here, our microsatellite analysis suggests that there are no clear differences in the diversity levels of the populations within each species. Thus the populations studied here are close to HWE with few significant deviations resulting from heterozygote deficits, which were not consistent across loci. Although linkage disequilibrium was detected for three pairs of markers, the signal was restricted to a few populations and likely reflects spurious effects.

Analysis of genetic structure

Within each species, population differentiation was moderate for several population pairs ($F_{st} \geq 0.1$), the highest being between *M. shawi* populations, with three tests significant after Bonferroni correction (Table 4), probably reflecting the presence of private alleles. Nevertheless these results should be interpreted with caution given the likely presence of null alleles in some of these populations. *Meriones shawi* from Dghoumes (Msh-D), the southwestern-most population sampled, which is separated from the northern populations by Mount Bouhlel, showed the highest differentiation values (both F_{st} and R_{st}) from all other populations; the populations from

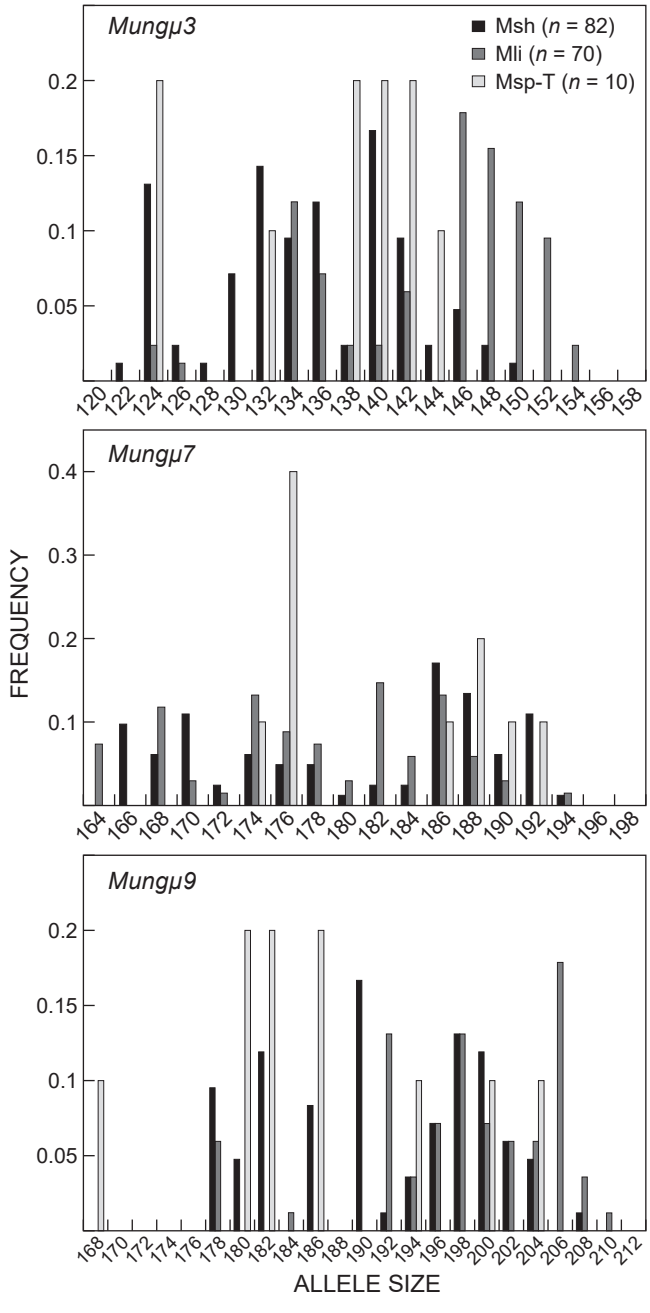

Figure 4: Microsatellite allele distributions for Tunisian wild *Meriones* species. Microsatellite allele frequency distributions for the two *Meriones* species sampled (*M. shawi* and *M. libycus*) and the Msp-T sample. Msh = *M. shawi*, Mli = *M. libycus*, *n* = number of alleles

Bouhedma (Msh-B) and Skhira (Msh-Sk) showed high differentiation by R_{st} but not by F_{st}.

Meriones libycus populations showed higher genetic homogeneity, with most population comparisons resulting in F_{st} values below 0.1, except in the case of population Mli-F with Mli-G, when the number of repeat differences is considered. Although habitat fragmentation due to natural barriers can result in genetic drift by limiting gene flow between nearby populations (Wright 1931; Taylor and Hoffman 2012), our results did not confirm such geographic impact in these species. In fact, high genetic differentiation is not detected between populations north of the Tunisian Dorsal (Msh-S and Msh-H) and those south of it (Msh-B and Msh-Sk), though this mountain chain also represents a climate barrier, harbouring different plant ecosystems in the semi-arid region to the north compared with those found in the more arid regions in the centre and south. Moreover, Mantel tests did not show a significant correlation between geographic distance, measured in latitude and longitude, and microsatellite genetic distances in either of the

Table 3: Molecular diversity indexes for populations of wild Tunisian *Meriones* species and heterozygosity means and HWE deviation significance of *M. unguiculatus* (Neumann et al. 2001). Significant comparisons following Bonferroni correction are highlighted in bold. The highest heterozygosity means are highlighted in bold italics. *N* = sample size (individuals); *A* = average number of alleles per locus; AR = allelic richness corrected by rarefaction, excluding the populations with lower number of samples (Msh-H and Mli-F) and considering two different *g* values; H_O = observed heterozygosity; H_E = expected heterozygosity; ND = not determined; ns = non-significant

Population	*N*	*A*	AR	H_O/H_E			Average H_O/H_E	HWE *p*-value[a]		
				Mungμ3	*Mungμ7*	*Mungμ9*		*Mungμ3*	*Mungμ7*	*Mungμ9*
Meriones shawi										
Haouaria (Msh-H)	4	5.00	ND	1/0.929	0.333/0.867	0.5/0.857	0.611/0.884	1	0.067	0.087
Siliana (Msh-S)	11	7.67	4.08	1/0.887	0.636/0.835	0.454/0.740	0.697/0.821	0.815	0.123	0.163
Bouhedma (Msh-B)	11	9.67	4.76	1/0.922	0.273/0.922	0.818/0.874	0.697/0.906	1	**0**	0.557
Dghoumes (Msh-D)	8	5.67	3.69	1/0.85	0.375/0.808	0.5/0.667	0.625/0.775	0.231	0.066	0.124
Skhira (Msh-Sk)	7	8.33	4.83	1/0.967	0.4/0.844	0.571/0.901	0.657/0.904	0.23	0.33	0.231
Sidi Toui (Msp-T)	5	6.33	ND	1/0.911	0.6/0.844	1/0.933	0.867/0.896	0.136	0.167	1
Mean (H_O/H_E)				***1*/*0.911***	0.436/0.853	0.640/0.828				
Meriones libycus										
Dghoumes (Mli-D)	15	9.67	6.31	1/0.915	0.867/0.885	0.867/0.862	0.911/0.887	0.969	0.396	0.578
Sidi Toui (Mli-T)	12	10.00	6.46	0.786/0.881	0.917/0.917	0.857/0.889	0.853/0.896	0.847	0.606	0.391
Faouar (Mli-F)	2	3.33	ND	0.5/0.5	1/1	1/1	0.833/0.883	1	1	1
Gafsa (Mli-G)	6	8.67	6.55	0.727/0.866	0.8/0.911	0.545/0.9	0.691/0.892	0.33	0.496	**0**
Mean (H_O/H_E)				0.753/0.79	***0.896*/*0.928***	0.817/0.912				
M. unguiculatus				***0.825***/0.804	0.7/0.804	0.675/***0.809***		ns	ns	**<0.05**

[a] HWE *p*-value = Hardy–Weinberg *p*-value corrected for multiple comparisons

Table 4: Genetic distance measures for the populations of wild African *Meriones* species based on microsatellites. Significant comparisons following Bonferroni correction are highlighted in bold. *M. shawi*: Msh-H = Haouaria, Msh-S = Siliana, Msh-B = Bouhedma, Msh-D = Dghoumes, Msh-Sk = Skhira, Msp-T = Sidi Toui; *M. libycus*: Mli-D = Dghoumes, Mli-T = Sidi Toui; Mli-F = Faouar, Mli-G = Gafsa

F_{st}	Msh-S	Msh-B	Msh-D	Msh-Sk	Msh-H	Msp-T	Mli-D	Mli-T	Mli-F
Msh-S									
Msh-B	**0.0635**								
Msh-D	**0.1338**	**0.0966**							
Msh-Sk	0.0465	0.0000	0.0644						
Msh-H	0.0816	0.0000	0.1032	0.0446					
Msp-T	0.0681	0.0165	0.0838	0.0076	0.0098				
Mli-D	–	–	–	–	–	–			
Mli-T	–	–	–	–	–	–	0.0189		
Mli-F	–	–	–	–	–	–	0.0219	0.0459	
Mli-G	–	–	–	–	–	–	0.0000	0.0000	0.0311
R_{st}	Msh-S	Msh-B	Msh-D	Msh-Sk	Msh-H	Msp-T	Mli-D	Mli-T	Mli-F
Msh-S									
Msh-B	0.0000								
Msh-D	0.1947	0.1003							
Msh-Sk	0.0504	0.1353	0.2519						
Msh-H	0.0000	0.0000	0.1352	0.0241					
Msp-T	0.0000	0.0000	0.2754	0.1631	0.0000				
Mli-D	–	–	–	–	–	–			
Mli-T	–	–	–	–	–	–	0.0364		
Mli-F	–	–	–	–	–	–	0.0782	0.0000	
Mli-G	–	–	–	–	–	–	0.0000	0.0000	0.1295

Meriones species analysed ($r = -0.037$, $p = 0.565$; and $r = -0.158$, $p = 0.580$, respectively). However, the fact that the two populations Msh-D and Mli-F showed high differentiation from northern populations of the corresponding species suggests that the local differentiation of these two populations may be due to ecological and climate preferences, especially because the Dghoumes and Faouar regions show the highest average temperature (21 °C) and the lowest average rainfall (<100 mm y^{-1}) (Supplementary Table S2).

The analysis of population differentiation suggested also that *M. shawi* populations are more divergent than those of *M. libycus* on autosomes, although more markers are necessary to confirm this result. The fact that the cyt *b* sequences are more divergent for *M. libycus* than *M. shawi* is not irreconcilable with the microsatellite data, given the different inheritance modes and evolutionary properties of these loci, with mtDNA undergoing stronger genetic drift. The analysis of more polymorphic microsatellite loci, such as those recently identified within *M. shawi* (Lalis and Lambourdière 2014), will be very useful to complement the study of population genetics for this rodent and related species.

Conclusion

The present study is the first to address the genetic diversity of two wild *Meriones* species in Tunisia using mitochondrial (cyt *b*) and nuclear (IRBP) genes combined with microsatellite data. Overall, analysis of mtDNA revealed high divergence between our *M. libycus* specimens and previously reported Tunisian samples, whereas *M. shawi* sequences differed by a relatively low number of mutations from a common ancestor. However, our microsatellite data did not show geographic structure for either *Meriones* species within Tunisia. Our results contribute to a better characterisation of North African *Meriones* species and are suggestive of high geographic structure in *M. libycus* populations in mtDNA even within Tunisia, suggesting an older and possible multiple colonisation events for this species compared with *M. shawi*. Thus, the present study further strengthens the need to refine the genetic characterisation of this species, given the evidence of high mtDNA heterogeneity for *M. libycus* populations. More samples and genetic markers are still needed to confirm and refine the genetic structure of this species in Tunisia as well as at a larger geographic scale.

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