1 **What should we call the Levant mole? Unravelling the systematics and demography of**
2 ***Talpa levantis* Thomas, 1906 sensu lato (Mammalia: Talpidae).**

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16

17 **Abstract**

18

19 Turkey hosts five of the eleven species of *Talpa* described to date, Anatolia in particular
20 appearing to be an important centre of diversity for this genus. Of these taxa, the Levant mole,
21 *Talpa levantis* Thomas, 1906 has been suggested to consist of two genetically divergent
22 sublineages, which may represent separate species. Here we use a combination of
23 mitochondrial and nuclear DNA sequences from specimens of *T. levantis* s.lat. collected
24 across the species geographical range to explore the systematics and demographic history of
25 Levant moles. Both mitochondrial and nuclear markers confirm the existence of distinct
26 eastern and western sublineages, which apparently diverged from each other in the early
27 Pleistocene. Given the degree of cytochrome-*b* divergence between these (7.28%), we
28 consider them to represent independent, cryptic species. By including topotypic specimens of
29 *T. levantis* s. str. in our study we are able to show that this name applies to the western
30 sublineage, distributed across most of the Anatolian Black Sea coastal region, from the
31 vicinity of Trabzon in the east, westwards to Marmara. The earliest name available for the
32 eastern taxon, found in Transcaucasia and adjacent parts of northeastern Anatolia, is *T.*
33 *transcaucasica* Dahl, 1944. Cytochrome-*b* haplotype diversity in *T. levantis* is relatively high,
34 demographic analyses suggesting that the species may have survived in multiple, separate,
35 refugial areas during the Pleistocene. Our work brings the total number of named mole
36 species recognized in Turkey to six, emphasising the importance of this region as a global
37 centre of mole diversification.

38 **Key words:** *Talpa*; Cytochrome *b*; *BRCA2*; Phylogeny; Anatolia

39

40 **Introduction**

41 *Talpa* Linnaeus, 1758 is a strictly subterranean genus of moles, distributed throughout the
42 western Palearctic region, from the Iberian Peninsula to China and Siberia (Hutterer, 2005),
43 which has been recovered as monophyletic in a number of phylogenetic studies (e.g.,
44 Colangelo et al., 2010; Bannikova et al., 2015). Bannikova et al. (2015) dated the most recent
45 common ancestor of *Talpa* to 5.49–7.64 Myr, based on four concatenated nuclear genes, a
46 time window in keeping with that suggested by Colangelo et al. (2010) from studies of
47 mitochondrial cytochrome *b* (cyt-*b*) sequences, and corresponding to the Late Miocene (latest
48 Tortonian and Messinian stages). During this time several dramatic changes to terrestrial
49 environments and ecosystems occurred, large areas of continents experiencing drying,
50 enhanced seasonality, and a consequent restructuring of terrestrial plant and animal
51 communities (Herbert et al., 2016). The most recent version of *Mammal Species of the World*
52 (Hutterer, 2005) recognized nine valid species in the genus, divided into a western group
53 including the common mole *T. europaea* Linnaeus, 1758, the blind mole *T. caeca* Savi, 1822,
54 the Roman mole *T. romana* Thomas, 1902, the Levant mole *T. levantis* Thomas, 1906, the
55 Iberian blind mole *T. occidentalis* Cabrera, 1907 and the Balkan mole *T. stankovici* Martino
56 and Martino, 1931 and an eastern group comprised of the Siberian mole *T. altaica* Nikolasky,
57 1883, the Père David's mole *T. davidiana* Milne-Edwards, 1884 and the Caucasian mole *T.*
58 *caucasica* Satunin, 1908. Based on genetic data, Bannikova et al. (2015) recognized three
59 additional species: *T. talyschensis* Vereschagin, 1945; *T. ognevi* Stroganov, 1948 and *Talpa*
60 ex gr. *levantis*. More recently two new mole species, *T. aquitania* Nicolas, Martínez-Vargas
61 and Hugot, 2017 (Nicolas et al., 2017a) from southern France and northern Spain and *T.*
62 *martinorum* Kryštufek, Nedyalkov, Astrin and Hutterer, 2018 from the south-western Black
63 Sea coast (Thrace), were described, initially on the basis of genetic data(Nicolas et al. (2017b;
64 Kryštufek et al. 2018). While most species of *Talpa* are narrowly endemic with predominantly
65 non-overlapping ranges, one species, *T. europaea,* is widespread and relatively eurytopic
66 across much of Europe, with a range extending from the Ebro River in Spain to the Ob and
67 Irtish Rivers in Russia (Mitchell-Jones et al., 1999; Hutterer, 2005; Loy et al., 2005; Wilson
68 and Reeder, 2005; Nicolas et al., 2017b).
69

70 The descriptions of the majority of the above *Talpa* species are based primarily on
71 morphometry (Corti et al., 1985; Corti and Loy, 1987; Loy et al., 1993; Kryštufek, 1994;
72 Rohlf et al., 1996; Loy and Capanna, 1998; Kryštufek and Benda, 2002; Kryštufek et al.,

73 2018; Selçuk et al., 2018; Sansalone et al., 2019) and dental traits (Nicolas et al., 2017a;
74 Kryštufek et al., 2018). However, the highly conservative morphology of the genus, resulting
75 from the functional constraints associated with fossoriality, have led to inconsistent
76 morphological recognition of taxa that have confused taxonomic assignments in the past
77 (Kryštufek and Vohralík, 2001; Bannikova et al., 2015; Kryštufek et al., 2018). Cytogenetic
78 studies have also shown that the karyotypes of *Talpa* species are quite stable, with a diploid
79 chromosome number (2n) of 34, and 62–64 autosomal arms (NFa) (Meylan, 1966; Dzuev et
80 al., 1972; Todorovic´ et al., 1972; Capanna, 1981; Jimenez et al., 1984; Gornung et al., 2008;
81 Sözen et al., 2012) throughout the genus, with the exception of *T. caeca* (2n = 36, NFa = 64)
82 and *T. caucasica* (2n = 38, NFa = 62). Additionally it has been shown that *Talpa* species with
83 almost identical karyotypes (*T. europaea* and *T. romana*) differ only slightly in localisation of
84 5S rRNA genes (Gornung et al., 2008), further suggesting taxa are not easily distinguished on
85 cytogenetic features.
86
87 In contrast to this morphological and karyotypic homogeneity, molecular genetic techniques
88 have been highly successful in *Talpa* taxonomy - highlighting the existence of
89 morphologically cryptic, but genetically divergent lineages, which appear to constitute
90 species. Bannikova et al. (2015) recently separated three such well-defined lineages in the
91 Caucasus and Anatolia: *T. talyschensis* Vereschagin, 1945, *T. ognevi* Stroganov, 1948, and
92 *Talpa levantis* sensu lato (s.l.). The first of these taxa was formerly considered a junior
93 synonym of *T. levantis*; the second a junior synonym of *T. caucasica* (Hutterer, 2005). These
94 findings highlight the fact that lineage diversity within the genus *Talpa* likely remains under-
95 estimated, particularly in areas known to habour high biodiversity, and which likely served as
96 refugia during Pleistocene glaciations. Turkey is likely to be one such area, since it not only
97 hosts five out of the eleven currently known *Talpa* species (*T. caucasica, T. davidiana*, *T.*
98 *europaea, T. levantis,* and *T. martinorum* – Kryštufek and Vohralík, 2009; Kryštufek et al.,
99 2018; Selçuk et al., 2018) but it is also the region in which a number of other recent small
100 mammal species/lineages originated (Gündüz et al., 2007). More generally, it is an important
101 biodiversity hotspot in the West Palaearctic due to its complex topography, climatic
102 conditions, and tectonic history (Myers et al., 2000). *Talpa* are strictly subterranean animals,
103 with limited dispersal capability (Steinberg and Patton, 2000) and a high degree of
104 territoriality (Ognev, 1928; Stein, 1950; Godfrey, 1957; Loy et al., 1994), and their
105 diversification is likely to have been strongly impacted by such factors.
106

107 The Levant mole was first described from the vicinity of Trabzon (Maçka, Altındere, north-
108 eastern Turkey) as a subspecies of the European *T. caeca* Savi, 1822 – *Talpa caeca levantis*
109 Thomas, 1906. After being considered as subspecies of *T. caeca* for a period of time
110 (Spitzenberger and Steiner, 1962; Osborn, 1964; Grulich, 1972), it was elevated to species rank
111 by Spitzenberger (in Felten et al., 1973) based on external measurements and craniometrical
112 variables, especially the relationships between condylobasal length and rostral breadth.
113 Subsequent studies showed that the two species are clearly distinguished on diploid karyotypes
114 (2n = 36 in *T. caeca* vs. 2n = 34 in *T. levantis*) and skull morphology (Zima and Král, 1984;
115 Kryštufek, 1994; Selçuk and Kefelioğlu, 2017). Bannikova et al. (2015) showed that these two
116 taxa are not particularly closely related, estimating that they diverged as early as the Late–
117 Middle Pliocene, approximately 2.68 Myr, based on four concatenated nuclear genes. The range
118 of the *T. levantis* s.l. extends west–east across the Anatolian Black Sea region, from South-
119 eastern Bulgaria and Turkish Thrace as far as the southwestern coast of the Caspian Sea
120 (Osborn, 1964; Doğramaci, 1989; Sokolov and Tembotov, 1989; Vohralík, 1991, Kryštufek,
121 2001; Popov and Miltchev, 2001; Bannikova et al., 2015). As such, it is the most common and
122 widespread mole in Turkey, and inhabits various habitats from sea level to ca. 2000 m, in areas
123 with rainfall ranging from 1000 to 2500 mm/year (Doğramaci, 1989; Kryštufek, 2001; Popov
124 and Miltchev, 2001; this study). Bannikova et al. (2015) found relatively large cyt-*b*
125 divergences (7%) between two sublineages within Anatolian and Caucasian *T. levantis* s.l.,
126 suggesting that this taxon, as currently defined, constitutes a pair of cryptic species. Of these
127 sublineages, one occupies the majority of the Turkish range of *T. levantis* s. l., throughout the
128 Black Sea coast, westwards to the Marmara region in Anatolia, whilst the other is found in
129 Transcaucasia, and the adjacent parts of northeastern Anatolia. Bannikova et al., (2015)
130 recognized that these two sublineages likely constitute separate species, but refrained from
131 naming them since they lacked genetic data for moles from or close to the type locality of *T.*
132 *levantis*, and so could not determine whether this name applied to the eastern or western
133 sublineage.
134

135 Here we use a combination of nuclear and mitochondrial DNA sequence data to revisit the
136 systematics and phylogeography of *T. levantis* s. l., including material from the type locality,
137 in an attempt to stabilize the taxonomy of these moles and better understand their
138 evolutionary history and population expansion.

139 **Material and methods**

140 *Specimen collection*

141 Levant moles were collected from thirteen sites in northern Anatolia between 2007 and 2018
142 (Fig. 1), table 1 summarizes collection details. Standard voucher specimens (skins, skulls and
143 various tissues in ethanol) are deposited in the Department of Biology, Faculty of Sciences
144 and Arts, Ondokuz Mayıs University (OMUS), Samsun, Turkey for long term storage.

145

146 *Molecular analyses*

147 *DNA Extraction*

148 Total genomic DNA was extracted using Qiagen DNeasy tissue kits (QIAGEN Inc.) from tail
149 tips, kidneys or liver preserved in 95% ethanol. Extracted DNA was suspended in nuclease-
150 free water and DNA concentration quantified using a Nano-Drop spectrophotometer
151 (NanoDrop Technologies, Wilmington, DE), adjusted to 25–50 ng/μL, and stored at -20 °C
152 until used for PCR.

153 *PCR amplification and sequencing of the cyt-b gene*

154 A 1754 bp fragment encompassing a small part of tRNA-Glu (18 bp), the whole of the cyt-*b*
155 gene (1140 bp), tRNA-Thr, tRNA-Pro (277 bp) and the 5' end (the hypervariable region I) of
156 the D-loop (554 bp, excluding indels), spanning positions 14 163 to 15 913 of the *T. europaea*
157 mitochondrial genome (GenBank Y19192, Mouchaty et al., 2000) was amplified with primers
158 L14162 5'-GACATGAAAAATCATCGTTG-3' (modified from L14727-SP in Jaarola and
159 Searle, 2002) and H15917 5'-CCTGAAGTAAGAACCAGATG-3' (modified from H16498 in
160 Meyer et al., 1990). PCR amplifications were carried out in an S1000 thermal cycler
161 (BIORAD) using Platinium *Taq* DNA polymerase (INVITROGEN). The PCR protocol
162 consisted of an initial 2 min denaturation step at 95 °C, 35 cycles of denaturation at 94 °C for
163 40 s, annealing at 56 °C for 45 s and extension at 72 °C for 1.5 min, and a final 7-min
164 extension step at 72 °C. PCR products were purified using QIAquick kits (QIAGEN).
165 Negative controls were included in all PCRs to check for contamination. After amplification,
166 an aliquot was taken from each PCR reaction mix, and the amplified DNA fragment
167 quantified in agarose gel by comparison with known quantities of phage λ DNA.

168

169 The complete cyt-*b* gene was sequenced in both directions using one of the amplification
170 primers (L14162) plus three internal primers (H15351 5'-
171 TCTCCATTGCTGGTTTACAAGAC-3', modified from H15915 in Irwin et al., 1991 and two

172 newly designed Levant mole specific primers, L14711 5'-GGTAGACAAAGCCACACTCAC
173 -3' and H14935 5'-GAATGTAGTTGTCTGGATCTCC-3'). The position of the 3' end
174 oligonucleotide of each primer is given relative to the published sequence of the common
175 mole mtDNA (Mouchaty et al., 2000). Cycle sequencing reactions were carried out using
176 BigDye Terminator cycle sequencing kits (Applied Biosystems). Amplifications and
177 sequencing reactions were performed in an S1000 thermal cycler. Sequencing products were
178 purified using DyeEx 2.0 Spin Kits (QIAGEN) and run in an ABI 3100 automated DNA
179 sequencer (Applied Biosystems).

180 *Nuclear mitochondrial pseudogenes (numts) detection*

181 It is crucial to avoid confusion of true mitochondrial sequences with copies in the nucleus
182 (pseudogenes – Mirol et al., 2000; Bensasson et al., 2001; Dubey et al., 2009). To screen for
183 pseudogenes, three different PCRs, producing overlapping fragments, were performed on five
184 randomly selected individuals from distinct localities. First, partial tRNA-Glu (42 bp), the
185 entire cyt-*b* (1140 bp) and partial tRNA-Thr (26 bp) were amplified with primers L14138 5'-
186 CCCACATGGAATTTAACCATGAC-3' (modified from Cb-M1 in Kurose et al., 2000) and
187 H15351; second, partial tRNA-Glu (42 bp), the entire cyt-*b* (1140 bp), complete tRNA-Thr
188 (69 bp), complete tRNA-Pro (70 bp) and the 5' end of the D-loop (554 bp) were amplified
189 with primers L14138 and H15933; and third, part of tRNA-Glu (18 bp), the entire cyt-*b* gene
190 (1140 bp), complete tRNA-Thr (69 bp), complete tRNA-Pro (70 bp) and the 5' end of the D-
191 loop (554 bp) were amplified with primers L14162 and H15933. These three PCR products
192 were then sequenced and aligned. Overlapping sequences were examined for any ambiguous
193 bases, stop codons or open reading frame shifts, which might have indicated the presence of
194 nuclear copies.

195 *PCR amplification and sequencing of breast cancer type 2 susceptibility protein (BRCA2)*
196 *gene*

197 Amplification of a 927 bp portion of exon 11 of *BRCA2* was performed using the primer pair
198 F1140a and R2050 (Bannikova et al., 2015). PCR conditions consisted of initial denaturing at
199 94 °C for 2 min; 30 cycles of 94 °C for 45 s, 64 °C for 45 s, and 72 °C for 1 min; and a final
200 extension at 72 °C for 6 min. PCR primers were used for sequencing in both directions.

201 *Sequence inspection and alignment*

202 All sequence traces were checked, aligned and ambiguous bases resolved by eye using
203 Sequencher v4.5 (Gene Codes Corp.). Nucleotide and amino acid composition were analyzed
204 using MacClade v4.08 (Maddison and Maddison, 2000).

205 *Phylogenetic analyses*

206 Nucleotide composition was analyzed and the frequency of each haplotype estimated using
207 MacClade v4.08. To evaluate the degree of differentiation between sublineages of *T. levantis*
208 s.l. compared to other species of the genus *Talpa*, and revisit the phylogenetic position of
209 these, we also downloaded 78 cyt-*b* sequences from GenBank, from fourteen *Talpa* taxa (*T.*
210 *altaica*, *T. aquitania*, *T. caeca*, *T. caucasica, T. davidiana, T. europaea*, *T. levantis* 'eastern'
211 *T. levantis* 'western' (sensu Bannikova et al., (2015)), *T. martinorum, T. occidentalis*, *T.*
212 *ognevi*, *T. romana*, *T. stankovici* and *T. talyschensis* – see Appendix). Phylogenetic
213 relationships amongst cyt-*b* haplotypes were inferred with Maximum Parsimony (MP) and
214 Maximum Likelihood (ML) algorithms implemented in PAUP v4.10b (Swofford, 2002) as
215 well as Bayesian inference of phylogeny (BI) as implemented in MRBAYES v3.1.2 (Ronquist
216 and Huelsenbeck, 2003). The Akaike information criterion (AIC) implemented in
217 jMODELTEST v1.0 (Posada, 2008) was used to establish the most appropriate model of
218 DNA substitution for our data, and this then employed in ML and BI analyses. The parsimony
219 analyses were replicated 10 times with the heuristic search approach using the TBR swapping
220 algorithm, steepest descent option and 10 random repetitions. Strict and 50% majority rule
221 consensus trees were constructed from equally parsimonious MP trees. Bootstrap analysis of
222 the MP tree was conducted with 1000 replications using 10 random repetitions of each
223 replication. ML analysis was conducted using the heuristic search, the 'as is' addition
224 replicate. Branch support was assessed using 1000 non-parametric bootstrap replicates. BI
225 analysis involved four Markov Chains of 20 million generations, with trees being sampled
226 every 100 generations and a burn-in of 25%. The software tool TRACER v1.6 (Rambaut et
227 al., 2014) was used to observe the parameters and to determine the number of trees needed to
228 reach stationarity (burn-in). After discarding burn-in trees and evaluating convergence,
229 remaining samples were retained for generating 50% majority rule consensus tree and
230 calculating posterior probabilities. Based on the existing supported phylogenetic hypothesis
231 (He et al., 2014), cyt-*b* sequences of four eastern Asian mole species of three genera (*Mogera*
232 *robusta* Nehring, 1891, *Euroscaptor mizura* (Günther, 1880), *Euroscaptor longirostris*
233 (Milne-Edwards, 1870) and *Parascaptor leucura* (Blyth, 1850)) were used as outgroups in
234 phylogenetic analyses (see Appendix).

235

236 Relationships amongst cyt-*b* sequences of *T. levantis* s.l. were also investigated by
237 constructing a network using the median-joining (MJ) algorithm implemented in the software
238 NETWORK v4.6.1.2 (Bandelt et al., 1999; http://www.fluxus-engineering.com). We included
239 all newly sequenced specimens (24 individuals) and all specimens of *T. levantis* 'western'
240 (seven individuals) and *T. levantis* 'eastern' (five individuals) available in GenBank (Table 1).

241

242 As some of the *BRCA2* sequences in GenBank were shorter than ours or contained some
243 unresolved positions, the *BRCA2* alignment was limited to 656 bp to match newly obtained
244 haplotypes with published data, leading to some redundant sequences and some loss of
245 phylogenetic signal. Phylogenetic relationships between *BRCA2* sequences were also
246 investigated using MP (1000 bootstrap replicates), ML (1000 bootstrap replicates) and BI
247 approaches using the settings described above. The nucleotide substitution model selected
248 according to the AIC by jMODELTEST was employed in ML and BI analyses. We included
249 all newly described haplotypes (Hap.1-9) and those of *Talpa* species available in GenBank
250 (Table 1, Appendix). Unfortunately, two recently described species (*T. aquitania* and *T.*
251 *martinorum*) could not be included in the analyses since there are no *BRCA2* sequences
252 available. The full dataset contained 31 *Talpa* haplotypes. Again, *BRCA2* sequences of the
253 same four eastern Asian mole species were used as an outgroup in the phylogenetic analysis
254 (Appendix).

255 *Molecular diversity*

256 Genetic diversity estimates were calculated for cyt-*b* sublineage of interest (western).
257 Nucleotide diversity (π) and haplotype diversity (H_d) were calculated using ARLEQUIN v3.5
258 (Excoffier and Lischer, 2010). Another measure of nucleotide diversity, θw (computed from
259 the number of segregating sites; Watterson, 1975), was calculated using DNASP v5 (Librado
260 and Rozas, 2009). DNA net and mean divergences (*Da* and *D*xy; Nei, 1987) between
261 sublineages were estimated under the Kimura 2-parameter (K2P; Kimura, 1980) model using
262 MEGA X (Kumar et al,. 2018).

263 *Divergence time estimation and population demographics*

264 We obtained an approximate estimation of the divergence time between *T. levantis*
265 sublineages using the formula $T = Da/2\mu$, where 2μ is the divergence rate, using a substitution
266 rate for the cyt-*b* gene of 0.01407 changes per site per lineage per million years (Colangelo et

267 al., 2010). We performed a series of statistical tests to test the hypothesis of sudden
268 population expansion for the two sublineages. First, mismatch distributions (Rogers and
269 Harpending, 1992; Rogers, 1995) were calculated for each sublineage to examine historical
270 changes in population size with ARLEQUIN. To compare observed data with those expected
271 under a sudden expansion model we conducted goodness-of-fit tests based on the sum of
272 squared deviations (SSD) and Harpending's raggedness index (*rg*) (Harpending, 1994;
273 Schneider and Excoffier, 1999) using 10 000 parametric bootstrap replicates.
274

275 The mismatch distribution of pairwise genetic differences was also used to calculate
276 expansion times (Rogers and Harpending, 1992). The change of effective female population
277 size (N) since population expansion is used to estimate the time since expansion in
278 generations. A population at equilibrium with N_0 changes to N_1 at τ units of mutational time.
279 The modal value τ is determined from the distribution of pairwise genetic differences in the
280 extant population. The parameters of the model are given by: $\theta_0 = 2N_0u$, $\theta_1 = 2N_1u$ and $\tau =$
281 $2ut$. Here, t is the time since expansion in generations and u is the mutation rate of the entire
282 DNA fragment (expressed as $u = 2\mu k$, where μ is the mutation rate per nucleotide per
283 generation and k is the length of the sequence). Population expansion times were estimated
284 assuming a mean clock rate of 1.407×10^{-8} substitution/site/year, as observed in the genus
285 *Talpa* (Colangelo et al., 2010). Second, we conducted tests of neutral equilibrium assumptions
286 using two widely used statistics, Tajima's D (Tajima, 1989) and Fu's F_S (Fu, 1997), as
287 additional assessments of possible population expansion. Tajima's D statistic tests the null
288 hypothesis that the average number of pairwise nucleotide differences and number of
289 segregating sites in the sample are equal (Tajima, 1989). This method explicitly tests for
290 selective neutrality and is based on an infinite-sites model assuming no recombination. Fu's
291 *Fs* statistic is also a selective neutrality test based on the infinite-sites model, assumes no
292 recombination and tests the probability of having no fewer haplotypes than the observed
293 number in the sample (Fu, 1997). Negative values for these two statistics are most often
294 attributed to positive selective sweeps, population size expansion or background selection.
295 Used in combination, the tests can provide evidence for or against particular evolutionary
296 mechanisms. As population size expansion leads to changes in the frequency distribution of
297 haplotypes (i.e. an excess of haplotypes or an excess of singleton mutations), we also
298 calculated Strobeck's S statistic (Strobeck, 1987) and R_2 (Ramos-Onsins and Rozas, 2002).
299 Strobeck's S compares the observed number of haplotypes to that expected based on the

300 frequency distribution derived from the inferred mutation rate, whereas R_2 is based on the
301 difference between the number of singleton mutations and the average number of nucleotide
302 differences, where lower R_2 values are expected under population expansion. The R_2 test is
303 very powerful in detecting population expansions in small sample sizes (Ramos-Onsins and
304 Rozas, 2002). Significance values for Tajima's D, Fu's F_S and R_2 were obtained from 10 000
305 coalescent simulations conditioned on theta as implemented in DNASP. The cut-off level for
306 statistical significance was 0.05. For Fu's F_S, significance at the 0.05 level was indicated
307 when P values were < 0.02 (Excoffier and Lischer, 2010).

308 **Results**

309 *Sequence analysis and phylogenetic reconstructions*

310 *Mitochondrial DNA*

311 The entire cyt-*b* gene (1140 bp) was sequenced from 24 Levant moles from 13 localities,
312 revealing 15 unique haplotypes (Hap.1-15) with K2P-distance values 0.09-3.34% (overall
313 mean K2P distance of 1.54%). Eleven of these haplotypes were found in one individual, four
314 in more than one animal (Table 1). No internal stop codons or insertion/deletions were
315 detected. Sequences with the same properties were also obtained using the alternative primer
316 pairs for amplification and sequencing, making it reasonable to assume that no mitochondrial
317 nuclear mitochondrial insertions (numts) were sequenced. The full dataset, including novel
318 and published *Talpa* sequences contained 425 variable sites, of which 381 were parsimony
319 informative, with 293 synonymous and 32 non-synonymous changes across all taxa. The
320 substitution model supported was the General Time Reversible, with specified substitution
321 types (AC-1.0052, AG-31.6171, AT-0.7223, CG-0.1456, CT-18.9234, GT-1.0), proportion of
322 invariable sites (0.5570), gamma shape parameter (1.1580) and nucleotide frequencies
323 (A-0.3572, C- 0.3041, G-0.0941, T-0.2446).
324

325 The phylogenetic hypothesis for species of *Talpa* based on cyt-*b* gene sequences from ML is
326 shown in Fig. 2. The different phylogenetic methods used (BI, ML and MP) all produced
327 similar topologies. Our tree broadly agrees with previous phylogenetic studies of the genus
328 (Colangelo et al., 2010; Bannikova et al., 2015; Kryštufek et al., 2018), including the
329 separation of Levant moles into an eastern and western sublineage. Specimens from the type
330 locality of *T. levantis* (possessing Haplotype 1), fall into the western sublineage (see Fig. 2).
331 The network profile of 26 haplotypes of *T. levantis* is shown in Fig. 3A. This haplotype

332 network was consistent with the results of the phylogenetic analyses, providing an enhanced
333 visualization of intraspecific genetic variation, with many substitutions accumulated
334 between the western and eastern sublineages of *T. levantis* s.l. The two sublineages were
335 connected via two median vectors, separated by 45 mutational steps. There were no shared
336 haplotypes between the two sublineages, suggesting complete lineage sorting. The majority
337 of the haplotypes in the western (Hap.5-15, KP717339, KP717340 and FN640572)
338 sublineage belonged to one group with short to long branches between the haplotypes,
339 connected via median vectors. Three smaller group of haplotypes formed more distant sub-
340 clusters within the western sublineage (Hap.1-3; FN640571; KP717336 and KP717338),
341 suggesting additional subdivisions within this sublineage. One sub-cluster, comprising
342 haplotypes Hap.1-3 is restricted to north-east Anatolia, including the locality of topotypes of
343 *T. levantis* (localities 5 and 6), another sub-cluster comprising haplotypes FN640571,
344 KP717336 and KP717338 is restricted to north-west Anatolia (localities 21-23), whilst the
345 sub-cluster comprising the remaining 18 haplotypes is distributed across north-central
346 Anatolia (localities 7-20) (Fig. 1, Table 1). The eastern sublineage is represented by fewer
347 sequences than the western, but it is nevertheless clear that the haplotype (FN640570) from
348 Çam geçidi, Ardahan (locality 4) in northeastern Anatolia is rather strongly differentiated
349 from the four relatively closely related haplotypes (KP717334, KP717335, KP717337 and
350 FN640574) from Russia (Nalchik, locality 1) and Armenia (Fioletovo and Margahovit,
351 localities 2 and 3).
352
353 *Nuclear DNA*
354 Sequences of a 927 bp portion of exon 11of *BRCA2* were generated from 24 moles from 14
355 localities, revealing nine unique haplotypes (Hap.1-9), six of which were found in one
356 individual, three in more than one (Table 1). When combined with available (shorter – see
357 Materials and methods) published *Talpa BRCA2* sequences, the dataset contained 31 *Talpa*
358 haplotypes of 656 bp long (see Appendix). This alignment contained 43 variable sites, 22 of
359 which were parsimony informative. jMODELTEST supported the Hasegawa-Kishino-Yano
360 (HKY) substitution model for these data, maximum likelihood analysis resulting in an optimal
361 which was only partly resolved due to the relatively low number of polymorphic sites (Fig. 4).
362 BI and MP analyses both produced topologies similar to that of ML. As with the cyt-*b*
363 phylogeny, the *BRCA2* data separated *T. levantis* s.l. into a geographically widespread
364 western sublineage represented by nine haplotypes (Hap.1-9, including Hap.1 from the
365 topotypes of *T. levantis*, locality 5 in Fig. 1) from this study, plus KP717122 from Uzungüney

366 Köyü, Zonguldak (locality 25 in Fig. 1), and a more restricted eastern sublineage represented
367 here by one haplotype only, KP717115 from the Nalchik region in Russia (locality 1 in Fig.
368 1).

369 *Diversity, divergence time and demographic history*

370 The western sublineage of *T. levantis* contained 21 cyt-*b* haplotypes (15 new in the present
371 study, six additional haplotypes from GenBank), resulting in a haplotype diversity (H_d) of
372 0.996, nucleotide diversity (π) of 1.894% and a θw of 2.447%. Mean and net divergence
373 estimates between cyt-*b* sequences in eastern and western sublineages of *T. levantis* were
374 7.28% and 5.75%, respectively. This net divergence estimate equates to a separation time of
375 ~1.91 Mya, suggesting that the two sublineages diverged in the early Pleistocene. For the
376 western sublineage of *T. levantis*, neutrality tests for cyt-*b* data revealed no significant deviation
377 from neutrality and the expected equilibrium. Both Tajima's *D* (D = –0.9346, P = 0.1765) and
378 Fu's *Fs* (Fs = –0.8062, P = 0.3982) yielded negative but nonsignificant values. Strobeck's *S*
379 was low (0.816) and Ramos-Onsins and Rozas' R_2 test was not significant (R_2 = 0.0894, P =
380 0.1816). These results were corroborated by a mismatch distribution that showed a wave signal
381 (multimodal distribution) consistent with constant population size or demographic equilibrium
382 (Fig. 3B). Conversely, the goodness of fit test was consistent with a hypothesis of
383 demographical expansion (SSD = 0.0239, P = 0.1241; rg = 0.0165, P = 0.1812). Demographic
384 analyses were not possible for the eastern sublineage due to low sample size (n = 5).

385 **Discussion**

386 Our data confirm that *Talpa levantis* s.l. is divisible into distinct eastern and western
387 sublineages, which apparently diverged from each other in the early Pleistocene. Given the
388 degree of DNA sequence divergence observed between sublineages, and the fact that these
389 remain robust and geographically coherent in light of our more extensive sampling, we agree
390 with Bannikova et al. (2015) that these should be considered as separate species. Mean cyt-*b*
391 K2P divergences observed between most mole species examined previously range from 8.6%
392 (*T. europaea* vs. *T. occidentalis*) to 15.6% (*T. altaica* vs. *T. romana*) (Colangelo et al., 2010;
393 Feuda et al., 2015). However, slightly lower genetic divergence (7.7%) was observed between
394 *T. occidentalis* and its recently described relative *T. aquitana* (Nicolas et al., 2017a,b). The
395 7.28% cyt-*b* divergence between sublineages revealed here is very close to this value.

396 The inclusion of *T. levantis* specimens from the type locality allows us to fix the
397 nomenclature of Levant moles for the first time. Topotypical *T. levantis* belong to the western
398 sublineage, on both mitochondrial and nuclear data and it is therefore this taxon which is the

399 true *T. levantis* sensu Thomas, 1906. As discussed by Bannikova et al. (2015) this therefore
400 means that the oldest name available for the eastern clade is *T. transcaucasica* Dahl, 1944.
401 Traditional delineation of species-level entities in morphologically constrained subterranean
402 mammals such as moles is intrinsically difficult (see Nevo, 1979), and there are, to date, no
403 known diagnostic morphological characters on which *T. levantis* and *T. transcaucasica* can be
404 separated. Selçuk et al. (2018) examined skull and mandible morphometrics of selected
405 Turkish mole species, including specimens of *T. levantis* s.l. from both eastern and western
406 areas; i.e. both *T. levantis* s. str. and *T. transcaucasica* as defined here. Their results reveal
407 relatively high morphological variability in *T. levantis* s.l. compared to the other taxa studied,
408 suggesting that morphometric separation between *T. levantis* and *T. transcaucasica* may be
409 possible. Future studies exploring this, together with additional nuclear markers, would
410 clearly be useful to confirm our taxonomic hypothesis. There is no clear geographical barrier
411 currently separating *T. levantis* s. str. and *T. transcaucasica*, although the species appear to
412 occur either side of the Anatolian Diagonal (sensu Davis, 1971), a biogeographical break
413 between central and eastern Anatolia which is observed in a wide variety of organisms
414 (Çıplak, 2003; Gündüz et al., 2007; Mutun, 2010; Kapli et al., 2013; Stümpel et al., 2016; van
415 Riemsdijk et al., 2017). As discussed by Gür (2016), this seems to result largely from
416 differences in temperature seasonality, which apparently also existed during the Last Glacial
417 Maximum and possibly earlier. The identity of *T. levantis* s.l. reported from southeastern
418 Bulgaria and Turkish Thrace remains unclear, due to the absence of molecular data from these
419 regions. A number of small mammal species are known to span the Bosphorus (e.g. Dubey et
420 al., 2007, Helvacı et al., 2012) and it is possible that European populations are indeed
421 conspecific with Anatolian ones. Alternatively, what has been considered as *T. levantis* in
422 Europe may belong to yet another distinct taxon, something which should be tested in the
423 future. It is clear, however, that Turkey is a very important centre for mole speciation and
424 diversity, now being home to six distinct species, more than any other comparable
425 geographical region.
426
427 Compared with other mole species, *T. levantis* s. str. has relatively high haplotype and
428 nucleotide diversity (H_d = 0.996, π = 1.894%). Based on cyt-*b* data, Canestrelli et al. (2010)
429 estimated haplotype and nucleotide diversities for the six haplogroups of *T. romana* to be
430 0.81-0.98 and 0.18-0.77%, respectively. Nicolas et al. (2017b) found almost equally high
431 levels of haplotype diversity but relatively low nucleotide diversity values for three mole
432 species: H_d = 0.932, π = 0.371% in *T. europaea*; H_d = 0.908, π = 0.830% in *T. aquitania* and

H_d = 0.892, π = 0.724% in *T. occidentalis.* The high diversity values observed in *T. levantis* s. str. suggest either the maintenance of relatively large populations throughout the species evolutionary history, and/or its survival in a number of separate refugia during the Pleistocene (Grill et al., 2009; Vega et al., 2010; Nicolas et al., 2017b). Our understanding of Pleistocene palaeoenvironmental history in northern Anatolia remains incomplete, but it is clear that the region hosts a large number of microendemics, in a variety of plant and animal taxa (e.g. Davis, 1971; Wielstra et al., 2010; Trizzino et al., 2013), which strongly suggests the occurrence of multiple, long-term refugial areas in the region, as has been hypothesised elsewhere (e.g. Lindell et al., 2006; Soltis et al., 2006; Byrne et al., 2008; Canestrelli and Nascetti, 2008; Ursenbacher et al., 2008; Gonçalves et al., 2009; Wang et al., 2009; Vega et al., 2010; Bidegaray-Batista et al., 2016; van Riemsdijk et al., 2017; Wielstra et al., 2017). Genetic substructuring and mutation rate heterogeneity may produce a multimodal pattern, even in demographically unstable populations (Marjoram and Donnelly, 1994; Aris-Brosou and Excoffier, 1996). The multimodality of the mismatch distribution of *T. levantis* s. str. seems to result from the presence of different haplogroups, rather than demographical stability. This interpretation is supported by the phylogenetic and network analyses of cyt-*b* data, which suggest three distinct sub-clusters within this taxon (see Fig. 2 and 3A), perhaps corresponding to populations which have persisted in separate refugial areas which retained suitable habitat during much of the species evolutionary history. If we assume a hypothesis involving some degree of population growth for *T. levantis* s. str., demographic expansion for this taxon, based on our estimated values of τ, occurred approximately 296,000 years BP in the late Pleistocene.

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872

873 **Figure legends**

874 **Figure 1.** Sampling locations and habitat of *T. levantis* s.l. in Anatolia. **A)** Map of sampling
875 locations (see Table 1). ● *T. levantis* s.l. 'western', this study; ○ *T. levantis* s.l. 'western';
876 previous studies and ▲ *T. levantis* s.l. 'eastern', previous studies. Western and Eastern
877 sublineages follow Bannikova et al. (2015). **B)** Typical habitat of *T. levantis* s.l. at the type
878 locality (Altındere, locality 5); 1650 m a.s.l., Trabzon, Anatolia. Photograph S. Demirtaş.

879

880 **Figure 2.** Results of BI, ML and MP analyses combined on a ML tree based on cyt-*b* sequences
881 of *Talpa* and outgroup species. Numbers at nodes indicate posterior probabilities (BI) and
882 bootstrap support values (ML and MP) which are reported only for the key nodes. Bayesian
883 posterior probabilities (≥0.90) and bootstrap supports (≥70%) are shown. The locality numbers
884 for the haplotypes of *'western'* and *'eastern'* sublineages are given in parentheses just after the
885 haplotype IDs according to the numbering on map on the Figure 1 and Table 1. For the
886 geographical origins of the published sequences see Table 1 and Appendix.

887

888 **Figure 3. A**) Median-joining network constructed using cyt-*b* sequences of *Talpa levantis* s.l.
889 The size of each circle is proportional to the frequency of the particular haplotype in the sample.
890 Median vectors are indicated by blank circles. For the geographical origins of the published
891 sequences see Appendix. **B**) Mismatch distribution of cyt-*b* sequences for the western
892 sublineage of *Talpa levantis* s.l., showing observed and expected values (see text).

893

894 **Figure 4.** Results of BI, ML and MP analyses combined on a ML tree based on *BRCA2*
895 sequences of *Talpa* and outgroup species. Numbers at nodes indicate posterior probabilities
896 (BI) and bootstrap support values (ML and MP). Bayesian posterior probabilities (≥ 0.90) and
897 bootstrap supports (≥70%) are shown. The locality numbers for the haplotypes of '*western*' and
898 '*eastern*' sublineages are given in parentheses just after the haplotype IDs according to the
899 numbering on map on the Figure 1 and Table 1. For the geographical origins of the published
900 sequences see Table 1 and Appendix.

Table 1. Populations of *T. levantis* s.l. sampled in this and previous studies (Colangelo et al., 2010; Bannikova et al., 2015), with details on the distribution of haplotypes (if more than one haplotype is present in a population the frequency is stated in parentheses). Cyt-*b* haplotypes (Hap.1-15, GenBank accession numbers: XXXXXXXX–XXXXXXXX) and *BRCA2* haplotypes (Hap.1-9, GenBank accession numbers: YYYYYYYY–YYYYYYYY) were new to this study. Locality codes correspond to Fig. 1. See Appendix for sequences from other taxa used in phylogenetic analyses.

Locality	Locality code	Latitude/longitude	*n*	GenBank Accession No/Haplotype ID		Reference
				Cyt-*b*	*BRCA2*	
RUSSIA						
Nalchik	1	43º46' N, 43º66' E	2	KP717334	KP717115	Bannikova et al., 2015
				FN640574		Colangelo et al., 2010
ARMENIA						
Fioletovo	2	40º71' N, 44º71' E	1	KP717337		Bannikova et al., 2015
Margahovit	3	40º74' N, 44º70' E	1	KP717335		Bannikova et al., 2015
TURKEY						
Çam geçidi, ARDAHAN	4	41º20' N, 42º55' E	1	FN640570		Colangelo et al., 2010
Taşköprü, Altındere, TRABZON	5	40º64' N, 39º67' E	2	Hap.1	Hap.1	
Görele, GİRESUN	6	41º02' N, 39º02' E	2	Hap.2	Hap.1	
				Hap.3		
Batlama Deresi, GİRESUN	7	40º88' N, 38º34' E	1	Hap.4	Hap.2	
Ulubey, ORDU	8	40º85' N, 37º76' E	2	Hap.5	Hap.3	
				Hap.6	Hap.4	
Geyikçeli Köyü, Fatsa, ORDU	9	40º93' N, 37º37' E	1	Hap.7	Hap.5	
Kurupelit, SAMSUN	10	41º37' N, 36º19' E	3	Hap.8	Hap.6 (2)	

					Hap.7	
İncesu Köyü, SAMSUN	11	41º38' N, 36º18' E	1	Hap.9	Hap.8	
Erikli Köyü, SAMSUN	12	41º37' N, 36º05' E	1	Hap.10	Hap.6	
Karakavuk, SAMSUN	13	41º38' N, 36º11' E	1	Hap.10	Hap.6	
Kürtler, SAMSUN	14	41º48' N, 36º09' E	1	FN640572		Colangelo et al., 2010
Dereköy, Ondokuzmayıs, SAMSUN	15	41º45' N, 36º11' E	4	Hap.11	Hap.3 (2)	
				Hap.12		
				Hap.13 (2)	Hap.6 (2)	
Kızılırmak deltası, Bafra, SAMSUN	16	41º67' N, 35º98' E	4	Hap.12 (2)	Hap.6 (3)	
				Hap.13	Hap.1	
				Hap.14		
Uluağaç Köyü, Bafra, SAMSUN	17	41º26' N, 35º58' E	1	Hap.13	Hap.3	
Abalı Köyü, SİNOP	18	42º02' N, 34º98' E	1	Hap.15	Hap.9	
Sefercik, Filyos, ZONGULDAK	19	41º55' N, 32º03' E	2	KP717339		Bannikova et al., 2015
				KP717340		Bannikova et al., 2015
Çaycuma, ZONGULDAK	20	41º43' N, 32º15' E	1	KP717339		Bannikova et al., 2015
Uzungüney Koyu, ZONGULDAK	21	41º37' N, 31º70' E	1	KP717338	KP717122	Bannikova et al., 2015
Alapli, ZONGULDAK	22	41º18' N, 31º40' E	1	KP717336		Bannikova et al., 2015
Uludağ, BURSA	23	40º10' N, 29º24' E	1	FN640571		Colangelo et al., 2010