

1 **Title: Evolutionary history of the European free-tailed-bat, a tropical affinity species**
2 **spanning across the Mediterranean Basin**

3 **Short running title: Evolutionary history of tropical affinity taxa**

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21

22 **Abstract**

23 The Mediterranean Basin is a global biodiversity hotspot, hosting a number of native species
24 belonging to families that are found almost exclusively in tropical climates. Yet, whether or
25 not these taxa were able to survive in the Mediterranean region during the Quaternary
26 climatic oscillations remains unknown. Focusing on the European-free-tailed bat (*Tadarida*
27 *teniotis*) we aimed to i) identify potential ancient populations and glacial refugia; ii) determine
28 the post-glacial colonization routes across the Mediterranean; and iii) evaluate current
29 population structure and demography. Mitochondrial and nuclear markers were used to
30 understand *T. teniotis* evolutionary and demographic history. We show that *T. teniotis* is
31 likely restricted to the Western Palearctic, with mitochondrial phylogeny suggesting a split
32 between an Anatolian/Middle East clade and a European clade. Nuclear data pointed to
33 three genetic populations, one of which is an isolated and highly differentiated group in the
34 Canary Islands, another distributed across Iberia, Morocco and France, and a third
35 stretching from Italy to the east, with admixture following a pattern of isolation by distance.
36 Evolutionary and demographic reconstruction supports a pre Last Glacial Maximum (LGM)
37 colonization of Italy and the Anatolian/Middle East, while the remaining populations were
38 colonized from Italy after the Younger Dryas. We also found support for demographic
39 expansion following the Iberian colonization. The results show that during the LGM *T.*
40 *teniotis* persisted in Mediterranean refugia and has subsequently expanded to its current
41 circum-Mediterranean range. Our findings raise questions regarding the physiological and
42 ecological traits that enabled species with tropical affinities to survive in colder climates.

43

44 1. Introduction

45 The Mediterranean Basin is a global biodiversity hotspot (Blondel, Aronson, Bodiou, &
46 Boeuf, 2010; Myers, Mittermeier, Mittermeier, da Fonseca, & Kent, 2000). Despite being
47 presently located in temperate latitudes, this region was mainly covered by tropical climates
48 during the Tertiary (Blondel & Mourer-Chauviré, 1998). Nowadays, Europe still hosts a
49 number of members belonging to several vertebrate groups that are almost exclusively
50 associated with the tropics (defined here as tropical affinities), including reptiles such as
51 geckos and chameleons, and birds such as rollers and bee-eaters (Ammerman, Lee, &
52 Tipps, 2012; Blondel & Mourer-Chauviré, 1998; Carranza & Arnold, 2006; Townsend &
53 Larson, 2002). However, the diversity of tropical species present in Europe is lower than that
54 of other Holarctic areas like North America or eastern Asia (Blondel & Mourer-Chauviré,
55 1998). The reason for such pattern is that both North America and eastern Asia remained
56 connected to the tropics over the whole Tertiary–Quaternary. In contrast, large geographical
57 barriers (mountain ranges, seas and desert-belts) prevented the Palearctic tropical biota
58 from expanding their range to tropical regions further south during glacial periods, and
59 tropical species from colonizing northern regions during inter-glacial periods (Blondel &
60 Mourer-Chauviré, 1998). Altogether, these led to a progressive decline of the tropical
61 species during the Pleistocene (Blondel et al., 2010). Under such circumstances, it is
62 remarkable that some of these species were able to persist in the western Palaeartic,
63 although mostly restricted to the circum-Mediterranean area. The population history of such
64 lineages during periods of glaciation is poorly understood and it is not known whether these
65 taxa were able to survive in the Mediterranean region during the climatic oscillations of the
66 Quaternary

67 Among non-flying mammals, only a small number of species in the western Palaeartic have
68 tropical affinities (Dobson, 1998). Although in some cases this was the result of a
69 longstanding human-mediated introductions across the Strait of Gibraltar, in others, such as
70 the Egyptian mongoose (*Herpestes ichneumon*), this was the result of natural dispersal into
71 the Iberian Peninsula during the Late Pleistocene (Gaubert et al., 2011). In bats, which are
72 likely to be able to disperse over greater distances, there is a higher number of species
73 shared between north-west Africa and Iberia (Dobson, 1998; García-Mudarra, Ibáñez, &
74 Juste, 2009), but even for these mammals the number of species with tropical affinities
75 occurring in temperate regions is relatively low. The European-free-tailed bat (*Tadarida*
76 *teniotis* Rafinesque, 1814) is the only European representative of the Molossidae family that
77 comprises more than 110 species (Ammerman et al., 2012). All the remaining molossids are
78 restricted to tropical regions, apart from the Mexican free-tailed bat (*Tadarida brasiliensis*)
79 and the Big free-tailed bat (*Nyctinomops macrotis*), which reach similar Northern latitudes in

80 the American continent. Molossidae is an ancient bat family that split into Old and New
81 World molossids ca. 29 million years ago (Ammerman et al., 2012), and fossil records of the
82 genus *Tadarida* in Europe date from the late Eocene ca. 25 million years ago (De Bonis et
83 al., 1973).

84 Understanding phylogeographic patterns shaping the distributions and expansion of species
85 is a powerful tool for predicting how future climatic changes will shape regional biodiversity
86 (Hickerson et al., 2010). During the Quaternary ice ages, Europe experienced dramatic
87 climatic fluctuations between glacial and interglacial cycles contributing to the contemporary
88 distribution and genetic composition of biodiversity (G. Hewitt, 2000). The distributions of
89 many animal species have been severely restricted to refugia to escape the harsh conditions
90 of the glacial periods. The Last Glacial Maximum (LGM 18–20 ka BP), and the Younger
91 Dryas (11.7–12.9 ka BP), correspond to the latest episodes where the ice sheets and cold
92 temperatures reached their extremes. The Mediterranean region encompasses a high
93 habitat diversity combined with topographic and geographic variability. Together with a
94 dynamic palaeogeographic and climatic history these features contributed to marked
95 environmental gradients (Blondel et al., 2010), strongly shaping current species and
96 biodiversity spatial patterns, population structure and demography (G. M. Hewitt, 1999).
97 Despite the increasing number of studies focusing on the phylogeography of species native
98 to temperate environments, to the best of our knowledge, representatives from tropical
99 families living in such environments have been seldom studied (but see Paulo, Pinto,
100 Bruford, Jordan, & Nichols, 2002; Rato, Carranza, & Harris, 2011).

101 The European-free-tailed-bat is widespread throughout the Mediterranean and occurs in a
102 variety of environments and habitats from the colder Alps to the border of the Sahara desert
103 (Amorim, Jorge, Beja, & Rebelo, 2018; Arlettaz et al., 2000; Bendjeddou, Bakhouché, &
104 Bouslama, 2014). However, during the Late Glacial Maximum (LGM), large parts of Europe
105 had colder and drier habitats (Frenzel, Pécsi, & Velichko, 1992) with warmest month
106 temperature being 10 °C cooler than present, and coldest month temperature 20 °C colder
107 (Kageyama et al., 2006). These harsh conditions were likely unsuitable for most bat species
108 (e.g., Bilgin et al., 2016; Kerth et al., 2008; Razgour et al., 2013; Rossiter, Benda, Dietz,
109 Zhang, & Jones, 2007), thus raising the question of how species with tropical affinities were
110 able to survive. Here we focus on the evolutionary history of *T. teniotis*, which belongs to a
111 taxonomical family almost exclusively associated with the tropics and shows shorter duration
112 of torpor bouts, and higher minimal body temperature in torpor than other temperate bats
113 (Arlettaz et al., 2000). The high mobility and fast flight of these bats (Mata et al., 2016;
114 McCracken et al., 2008) allows them to respond fast to environmental changes by shifting to
115 more suitable areas. These features render *T. teniotis* a suitable model species to

116 understand how species with topical affinity reacted to the climatic oscillations of the
117 Quaternary in temperate and subtropical regions. Therefore, our main aims were to: i)
118 identify the location of potential ancient populations and glacial refugia; ii) determine the
119 post-glacial colonization routes across the Mediterranean; and iii) evaluate current
120 population structure and demography in light of the post-glacial colonisation history.

121

122 **2. Methods**

123 *2.1. Sample collection*

124 A total of 154 genetic samples collected across the Western and Central Palearctic were
125 obtained from researchers and museum collections. Samples spanned the entire range
126 although coverage was uneven with few samples available from some regions, particularly
127 from Asia, Eastern Mediterranean and North Africa. For a complete list of samples, origin
128 and providers see Appendix 1 (GenBank accession numbers MK817165 to MK817272).

129 *2.2. DNA extraction*

130 Due to the different nature of the samples obtained (old museum specimens and recently
131 collected wing tissue) we used different DNA extraction methods. For older museum
132 specimens we followed the ancient DNA extraction protocol described in Rohland &
133 Hofreiter, (2007) with modifications described in Dabney et al. (2013). For recent tissue
134 samples, we used DNA Micro Kits (QIAGEN) following the manufacturer's instructions.

135 *2.3. Validation of species identity and mitochondrial genotyping*

136 Given the poorly resolved taxonomic status of *Tadarida teniotis* (Mata, Amorim, Guillén-
137 Servent, Beja, & Rebelo, 2017), the identity of all samples were verified using mitochondrial
138 markers prior to microsatellite genotyping. Due to taxonomic uncertainties (Mata et al.,
139 2017), verification was considered to be especially important for putative *T. teniotis* samples
140 obtained from the eastern part of the distribution (Kyrgyzstan and China). Additionally,
141 samples from Laos previously identified as *T. latouchei* were also checked.

142 Four mitochondrial primer pairs were specifically designed using Geneious v9.1.7
143 (<http://www.geneious.com>, Kearse et al. 2012) based on an alignment of 37 mitogenomes
144 covering the species range. The primers were designed to amplify the most variable regions
145 of the mitogenomes (Supporting Information Table S1) and corresponded to three coding
146 regions (*COI* - *cytochrome c oxidase subunit I*, *ATP6* - *ATP synthase subunit 6*, and *CytB* -
147 *cytochrome b*) and one noncoding region (*D-loop*). While designing the primers took extra
148 precautions and carefully examined the mitogenomic data to avoid the amplification of

149 nuclear copies covering almost the entire mitogenome. We did this by comparing the
150 sequences containing nuclear copies (identified by the high prevalence of stop codons) to
151 those without nuclear copies and selecting the regions that did not amplify nuclear copies.
152 This way the primers designed assure that only the mitochondrial haplotype were amplified,
153 allowing the genotyping of samples through Sanger sequencing. For highly degraded
154 museum samples that did not amplify using the regular primers, we further developed
155 internal primers for the *COI* (*COI-mini*) and *D-loop* (*D-loop-mini*) regions targeting key SNPs
156 that enable to differentiate *T. teniotis* and its different haplogroups from *T. latouchei*
157 (Supporting Information Table S1).

158 The PCR reactions were carried in volumes of 10 μ L, comprising of 5 μ L of Multiplex PCR
159 Master Mix (QIAGEN), with 0.4 μ L of each 10 μ M primer, and 1 μ L of DNA extract. Cycling
160 conditions for *COI*, *ATP6*, *CytB*, and *D-loop* used initial denaturing at 95 °C for 15 min,
161 followed by 40 cycles of denaturing at 94 °C for 30 s, annealing at 59 °C for 45 s and
162 extension at 72 °C for 45 s, with a final extension at 72 °C for 10 min. For *COI-mini* and *D-*
163 *loop-mini* the cycling conditions were the same except the annealing temperature that was
164 52 °C and the number of cycles was increased to 45. Successful amplifications were
165 enzymatically purified, sequenced following the BigDye Terminator v3.1 Cycle sequencing
166 protocol (Applied Biosystems), and sequencing products were separated using an
167 automated Sequencer ABI3130xl Genetic Analyzer. Sequences were aligned and compared
168 in the software SEQSCAPE 3.0 (Applied Biosystems).

169 2.4. Microsatellite genotyping

170 A custom microsatellite library was developed through 454 GS-FLX Titanium
171 pyrosequencing of enriched DNA libraries based on 12 individuals along the distribution
172 range of *T. teniotis* (Malausa et al., 2011). This process was developed by GenoScreen
173 (http://www.pasteur-lille.fr/fr/recherche/plateformes/tordeux_plat.html) and included
174 sequence data quality control, assembly and analyses, and primer design.

175 From the 159 candidate microsatellite loci, we selected 26 microsatellites with different
176 numbers of repeat units, compatible allelic ranges and melting temperatures for multiplexing.
177 We first tested the genotyping performance on four *T. teniotis* samples and discarded
178 microsatellites that: i) showed no amplification, ii) had multiple bands and iii) had excessive
179 slippage (many stutter bands). Those remaining were combined into two multiplex panels
180 according to their allele size range and compatibility among primers, which was checked
181 using Auto-Dimer (Vallone & Butler, 2004).

182 The optimisation of PCR conditions for multiplex loci and polymorphism detection was
183 performed using 16 samples. From the 26 loci initially checked a total of 12 di and 2 tetra-

184 nucleotides polymorphic markers (with more than 2 alleles) were selected and genotyped for
185 129 individuals in two multiplex panels with seven markers each. PCR fragments were
186 fluorescent labelled following Schuelke (2000) but with FAM, VIC, NED, and PET dyes. A pig
187 tail (GTTT) was added to the 5' end of the primer reverse in order to reduce stutter and drive
188 the reaction to the "plusA" band (Brownstein, Carpten, & Smith, 1996). For additional details
189 on microsatellite primers, see Supporting Information Table S2.

190 PCR amplifications were conducted as for mitochondrial fragments except that 1 μ L of primer
191 mix was used per reaction. The PCR cycling profile was divided in four main steps:
192 denaturation at 95 °C for 15 min; 13 cycles with denaturation at 95 °C for 30 s, annealing at
193 58 °C for 90 s with a touchdown of 0.5 °C per cycle and extension at 72 °C for 45 s; 27
194 cycles with denaturation at 95 °C for 30 s, annealing at 52 °C for 60 s and extension at 72 °C
195 for 45 s; and a final extension at 60 °C for 30 min. PCR products were later separated by
196 capillary electrophoresis on the same automatic sequencer ABI3130xl Genetic Analyzer (AB
197 Applied Biosystems). Fragments were scored using GENEMAPPER V4.0 (Applied Biosystems)
198 and checked independently by two people.

199 2.5. Genetic data analysis

200 2.5.1. Mitochondrial data

201 Sequences from the four mitochondrial markers were concatenated and standard molecular
202 diversity statistics calculated in ARLEQUIN 3.5 (Excoffier & Lischer, 2010). To test for
203 geographical genetic structure, analyses of molecular variance (AMOVA) were carried out
204 with 10,000 permutations and diversity measures were reported for geographic groups and
205 assessed according to the degree of differentiation between regions (Φ CT), between
206 populations within regions (Φ SC) and between all populations (Φ ST). A median-joining (MJ)
207 haplotype network was build using POPART (Leigh & Bryant, 2015) for each marker and for
208 the concatenated sequences. Mitochondrial diversity was assessed considering seven
209 geographic populations based on the common population structure of European bats (e.g.
210 Bilgin et al., 2016; Razgour et al., 2013): 1) Canary Islands; 2) Iberian Peninsula (Portugal
211 and Spain, excluding Canary Islands); 3) Morocco; 4) France; 5) Italy; 6) Greece; 7) Anatolia
212 and 8) Middle East (Lebanon, Israel and Palestine).

213 Phylogenetic reconstruction was performed on the CIPRES Science Gateway V. 3.3 (Miller,
214 Pfeiffer, & Schwartz, 2010) using Bayesian inference implemented in BEAST v1.8.4
215 (Drummond, Suchard, Xie, & Rambaut, 2012) considering unique haplotypes only (n = 65)
216 from the concatenated sequences and with inclusion of *T. latouchei* as outgroup (Mata et al.,
217 2017, GenBank Accession numbers: NC_036331 and KY581662). The best substitution

218 model scheme was determined using PARTITIONFINDER v2.1.1 (Lanfear, Frandsen, Wright,
219 Senfeld, & Calcott, 2016). We used a coalescent tree prior under constant population. Three
220 independent runs of 10^8 generations sampled every 1000 were combined in TRACER V1.7
221 (Rambaut, Drummond, Xie, Baele, & Suchard, 2018) to confirm convergence on the same
222 posterior distribution in the MCMC runs. The first 10^7 runs (10%) were discarded as burn-in.

223 2.5.2. Microsatellite data

224 To test for departures from Hardy–Weinberg and linkage equilibrium, both across the whole
225 samples and within populations, we used the ‘pegas’ R package (Paradis, 2010). Loci that
226 violated Hardy–Weinberg equilibrium in more than two populations were excluded from
227 further analysis (Supporting Information Table S2). Allele frequencies and number of private
228 alleles were estimated in GENETIX v4.05 (Belkhir, Borsa, Chikhi, Raufaste, & Bonhomme,
229 2004) and `mean` allele frequency across all loci was calculated for each population.
230 Estimates of expected heterozygosity (H_e), observed heterozygosity (H_{obs}) and allelic
231 richness within populations, and differentiation (F_{st}) among populations, were all calculated
232 using the ‘PopGenReport’ R package (Adamack & Gruber, 2014). Relatedness among
233 individuals was measured using the triadic maximum likelihood estimator (TrioML; Wang,
234 2007) implemented in ‘related’ R package (Pew, Muir, Wang, & Frasier, 2015). This
235 estimator was chosen because it allows for inbreeding and accounts for genotyping errors in
236 the data.

237 Population genetic structure was first examined using the principal component analysis in
238 ‘PopGenReport’ R package (Adamack & Gruber, 2014) followed by the Bayesian clustering
239 analysis implemented in STRUCTURE 2.3.4 (Pritchard, Stephens, & Donnelly, 2000) with all
240 genotyped samples. We performed 10 replicate runs of structure for each value of K, from K
241 = 1 to 10, and we applied the admixture model with a burn-in of 5×10^5 and a run length of
242 10^6 with and without the prior population information (LOCPRIOR). The latter can often
243 provide accurate inference of population structure and individual ancestry in datasets where
244 the signal of structure is too weak to be found using the standard models (Hubisz, Falush,
245 Stephens, & Pritchard, 2009). We used STRUCTURE HARVASTER v0.6.94 to visualize
246 likelihood and detect the number of genetic groups that best fit the data (Earl & VonHoldt,
247 2012). The Greedy algorithm of CLUMPP (Jakobsson & Rosenberg, 2007) was used to derive
248 symmetric similarity coefficients (SSC) among replicate runs within each value of K. Groups
249 of runs with an $SSC \geq 0.8$ were then combined and their outputs for each value of K were
250 graphically displayed.

251 Spatial structuring was further analysed using multivariate analyses of spatial genetic
252 patterns in ‘adegenet’ (Jombart, 2008). Spatial Analysis of Principal Components (sPCA)

253 allows to find the individual scores that maximize the product of variance and spatial
254 autocorrelation (Jombart, Devillard, Dufour, & Pontier, 2008). Isolation by Distance (IBD)
255 across all individuals within the species range was tested for in the R using the package
256 'ade4' (Bougeard & Dray, 2018) and using a Mantel test.

257 *2.6. ABC inference of evolutionary and demographic history*

258 2.6.1. General overview

259 The evolutionary and demographic history of *T. teniotis* was reconstructed using
260 Approximate Bayesian Computation (ABC) approach implemented in DIYABC v2.1 (Cornuet
261 et al., 2014). We carried out two sets of analyses, aimed to: 1) infer the source population
262 and patterns of range colonisation from putative refugia in the Western Palearctic; and 2)
263 infer demographic history in the western range (Iberia, Morocco and France). In the first
264 step, we modelled the probability of different scenarios considering 122 individuals from six
265 populations (Iberia, Morocco, France, Italy, Greece and Anatolia/Middle East) and combining
266 information from 12 microsatellites loci and two mitochondrial sequences (*COI* and *D-loop*).
267 Multiple scenarios were compared representing a comprehensive range of alternative
268 phylogeographic hypothesis and permuting the six geographic groups at the tips (Supporting
269 Information Fig. S1 and Table S3).

270 Using the scenario topology identified in the first step, we carried out a demographic history
271 analysis of the western range to determine changes in population size during colonisation.
272 We compared a null model of no change in population size (Scenario 1) to a model of
273 colonization and expansion in all populations (Scenario 2), and two models of recent change
274 with increase or decrease in Iberian population size (Scenario 3 and Scenario 4
275 respectively). For a schematic representation of the different scenarios, see Supporting
276 Information Fig. S2.

277 Each scenario was tested using the combined microsatellite and mtDNA datasets and
278 running 10^6 simulations. The posterior probability of scenarios was then estimated using a
279 weighted polychotomous logistic regression. Due to the criticism of ABC model choice
280 outlined in Robert, Cornuet, Marin, & Pillai (2011) we empirically evaluated the power of the
281 model to discriminate among scenarios by simulating pseudo-observed datasets and
282 calculating false allocation rates (type1 and 2 errors, Cornuet, Ravigné, & Estoup, 2010).
283 Further details on the methods, model specifications and run parameters are presented in
284 the following sections and in Supporting Information Table S3.

285 2.6.2. Specific model parameters

286 Microsatellite loci were assumed to follow a Generalized Stepwise Mutation model (GSM)
287 and mean mutation rate was bounded between 10^{-3} and 10^{-4} (Balloux & Lugon-Moulin, 2002;
288 Storz & Beaumont, 2002). For mtDNA we only considered *COI* and *D-loop* due to
289 computational requirements and sequence completeness. We used the best substitution
290 model scheme determined using PARTITIONFINDER v2.1.1 (Lanfear et al., 2016) as follow:
291 HKY for the coding region (*COI*) and K80 for non-coding region (*D-loop*). Generation time
292 was set at three years, a value in between the age of first breeding for different bat families
293 that can go from one to five years (Crichton & Krutzsch, 2000) which meets our expectations
294 for *T. teniotis*. We considered a mean mutation rate (per site per generation) between $5.25E^{-8}$
295 ⁸ and $7.2E^{-8}$ for *COI* (Juste et al., 2004; Ruedi & Mayer, 2001) and between $9.45E^{-8}$ and
296 $3.75E^{-7}$ for *D-loop* (Petit, Excoffier, & Mayer, 1999).

297 In the colonization analysis, uniform priors were assumed for all demographic parameters.
298 Effective population size (N_e) was kept as equal for all populations, ranging between $1E^3$
299 and $1E^6$. Population divergence time priors were bounded between $1E^3$ and $2E^5$ generations
300 and varied depending on model analysis. Divergence times between source populations
301 were set at either pre-LGM ($1E^4$ - $2E^5$) or flexible pre-post LGM ($1E^3$ - $2E^4$). Priors for
302 admixture rates were bounded between 0.01 and 0.99. In the demographic history analysis,
303 we used variable Effective population size ranging from 10 to $1E^6$. Population divergence
304 time priors were bounded to post-LGM (10 and $1E^4$) and varied depending on model
305 analysis.

306 In each ABC analysis we used 269 summary statistics. For the microsatellite loci we used
307 three single sample statistics (mean number of alleles, mean Nei's genetic diversity index
308 and mean allele size variance), and five between-sample statistics (F_{ST} , mean number of
309 alleles, mean genic diversity, mean allele size variance and shared allele distance). For the
310 mtDNA sequence we used seven single sample statistics (number of distinct haplotypes,
311 number of segregating sites, mean pairwise differences, variance of pairwise distance,
312 Tajima's D statistics, private segregating sites, mean of numbers of the rarest nucleotide at
313 segregation site) and four between-sample statistics (F_{ST} , number of haplotypes, number of
314 segregating sites, mean within sample pairwise differences and number of segregating
315 sites). The demographic history analysis included only 47 summary statistics due to the
316 small number of groups compared.

317 The complete list of parameters used in the ABC analysis, respective priors and estimated
318 results for the most supported colonization scenario (SC2) and the most supported
319 demographic history scenario (SC2) can be found in Supporting Information Table S4.

320 2.6.3. Colonization analysis

321 This analysis included the potential range colonization from an ancient unsampled
322 population with unknown origin. For a schematic representation of the different scenarios,
323 see Supporting Information Fig. S1.

324 *Scenario 1* considered an Iberian colonization from an ancient unsampled population before
325 the LGM, and a long-range colonization of the Eastern Mediterranean through an admixture
326 event from Iberia and the ancient unsampled population. The Iberian population later
327 colonized Morocco and the later colonized Italy. Admixture events between Iberia and Italy
328 and between the Eastern Mediterranean and Italy resulted in the French and Greek
329 populations, respectively.

330 *Scenario 2* considered an Italian colonization from an ancient unsampled population before
331 the LGM, and a colonization of the Eastern Mediterranean through an admixture event from
332 Italy and the ancient unsampled population. The Italian population then colonized Morocco
333 and France, while Iberia and Greece were colonized through admixture events between
334 France and Morocco and Italy and Eastern Mediterranean, respectively.

335 *Scenario 3* considered a colonization of the Eastern Mediterranean from an ancient
336 unsampled population before the LGM, and a colonization of the Greek population through
337 an admixture event between the Eastern Mediterranean population and the ancient
338 unsampled population. Italy was later colonized from Greece, while Morocco and France
339 were both be colonized from Italy. Finally, Iberia was colonized through an admixture event
340 between the Moroccan and French populations.

341 *Scenario 4* considered an Italian colonization from an ancient unsampled population before
342 the LGM, and a colonization of the Greek population from an admixture event between the
343 Italian population and the ancient unsampled population. Eastern Mediterranean was then
344 be colonized from Greece, while Italy colonized both Morocco and France. Finally, Iberia
345 was colonized through an admixture event between the French and Moroccan populations.

346 *Scenario 5* considered a colonization of the Eastern Mediterranean from an ancient
347 unsampled population before the LGM, and an Italian colonization through an admixture
348 event between the Eastern Mediterranean and the ancient unsampled populations. Greece
349 was also colonized through an admixture event, this time between the Italian and the
350 Eastern Mediterranean populations. The Italian population then colonized France, while the
351 Eastern Mediterranean population colonized Morocco. Finally, Iberia was colonized through
352 an admixture event between the Moroccan and French populations.

353

354 **3. Results**

355 3.1. Mitochondrial data

356 We were able to amplify DNA from 136 samples. Samples from Kyrgyzstan were sequenced
357 using *COI*-mini marker and showed a high mitochondrial divergence from *T. teniotis* (ca.
358 13%) and aligned with sequences belonging to *T. latouchei* from Laos (99% similarity, Mata
359 et al., 2017). Additionally, from the four samples from China identified as *T. teniotis* in
360 museum collections (Appendix 1), we were able to sequence two, both aligning with
361 *Chaerephon plicatus* (vouchers: MVZ:Mamm:192571 and MVZ:Mamm:193379). According
362 to the available information, the four samples were collected in the same event at a bat cave
363 in Southern China, and thus assumed to belong to the same species. According to the
364 International Union for Conservation of Nature (IUCN) the species has a highly fragmented
365 distribution in central and eastern Asia (Benda & Piraccini, 2016) and our results suggest
366 that *T. teniotis* could be absent or rare in this region. Therefore samples from Kyrgyzstan
367 eastwards were excluded from further analysis.

368 A total of 120 samples belonging to *T. teniotis* were successfully sequenced for *COI* (566 bp
369 final alignment) and *D-loop* (307 bp final alignment), 114 for *CytB* (509 bp final alignment)
370 and 109 for *ATP6* (639 bp final alignment). The number of unique haplotypes ranged from
371 17 for *ATP6* to 33 for *D-loop*. After concatenation the length of the resulting sequences was
372 between 873 and 2020 bp (average = 1937 bp, Alignment in Supporting Information) and
373 included 56 unique haplotypes (N = 109, 2020 bp). The Bayesian phylogenetic tree showed
374 maximum posterior probability support (> 0.9) for the split of two main lineages,
375 Anatolian/Middle East clade (AMh) and a European clade (EUh) further splitting into two
376 subgroups but in this case with low support (EUh-A and EUh-B) (Fig. 1).

377 The haplotype network divided the haplotypes into three separate groups, of which one was
378 exclusive to Iberia and Morocco (EUh-A) and one was distributed elsewhere in central and
379 western Mediterranean (EUh-B) (Fig. 1 and Supporting Information Fig. S3). The third group
380 comprised all the haplotypes from Anatolia and Middle East and one additional haplotype
381 from eastern Crete, broadly supporting the phylogenetic tree. The most common haplotypes
382 from EUh-A and EUh-B were separated by only one mutational step (percent differences
383 <0.05 %), while AMh shows a divergence of 0.70% from EUh-A and 0.59 % from EUh-B.

384 Despite the split between the eastern and western clades, the phylogenetic tree and
385 haplotype network based on mtDNA showed low levels of geographic structuring within each
386 haplogroup. Mitochondrial haplotype diversity was highest and equal to one in the Middle
387 East (N = 7), France (N = 4) and Morocco (N = 6), while nucleotide diversity was highest in
388 Anatolia (Pi = 0.0040, N = 3) and the Middle East (Pi = 0.0036, N = 7) (Table 1). The lowest
389 values for both haplotype and nucleotide diversity were found in the Canary Islands.

390 Genetic differentiation at mitochondrial DNA was seen between all populations ($\chi^2 = 532.49$,
391 $P < 0.001$, overall $\theta_{ST} = 0.57$), with Anatolia and Middle East being genetically differentiated
392 from all populations except for each other (Supporting Information Table S4). The general
393 pattern showed a higher mitochondrial diversity in Anatolia/Middle East and equally low
394 diversity in all the three peninsula.

395 *3.2. Microsatellite data*

396 A total of 128 individuals were successfully genotyped. Of the 14 microsatellite loci, two
397 markers (TAD5 and TAD9) were removed due to violation of Hardy-Weinberg equilibrium
398 (Supporting Information Table S2). After removing these markers all populations and
399 markers were overall in Hardy-Weinberg. Our final dataset contained a total of 146 alleles,
400 with an average number of 12.17 ± 2.44 alleles per locus (range 7-15) and 24 private alleles.

401 Genetic diversity in terms of allelic richness was highest in Anatolia and the Middle East,
402 followed by Italy and the Iberian Peninsula (Table 1 and Supporting Information Fig. S4).
403 Expected heterozygosity was high in all populations with the exception of the Canary
404 population, where the relatedness was particularly high (mean TrioML = 0.40). Overall
405 population differentiation was low, suggesting a meaningful gene flow. Canaries showed the
406 highest F_{ST} values with some degree of differentiation with Greek and Anatolian populations
407 (Supporting Information Table S5).

408 Model-based clustering method implemented in STRUCTURE without prior population
409 information did not identify any population structure (Supporting Information Fig. S5).
410 However, when using this prior, models revealed three main genetic populations (Supporting
411 Information Fig. S6 and Table S6). Individuals from the Canary Islands formed a separate
412 population, while all individuals from the Iberian Peninsula, Morocco and France showed a
413 higher estimated membership fraction to a second inferred cluster, and individuals from Italy
414 eastwards consistently showed higher estimated membership fraction to a third inferred
415 cluster (Fig. 2). The three clusters topology was further supported by the Spatial Analysis of
416 Principal Components (sPCA), although the pattern was not significant (Monte-Carlo test,
417 $p=0.082$) (Fig. 3). Both analyses showed that, except for the Canary population, most
418 individuals had high levels of admixture, and only a west to east geographic gradient was
419 evident. An overall observed pattern of isolation by distance was significant (Monte-Carlo
420 test, $p = 0.001$) (Supporting Information Fig. S7).

421 *3.3. ABC inference of evolutionary and demographic history*

422 Model-based inference showed high support (86 %) for a pre-LGM colonization of Italy from
423 an unsampled population (Supporting Information Fig. S1), while the Anatolian/Middle East

424 population was also colonized pre-LGM from an admixture event between Italy and the
425 unsampled population, with a similar contribution from both (proportion of admixture from
426 unsampled population 0.46). The remaining European populations were colonized from Italy
427 after the Younger Dryas, either directly or via a stepping stone manner with admixture (Fig.
428 4). However, the Greek population showed some level of admixture between Italy and
429 Anatolia/Middle East (Fig. 4 and Supporting Information Table S3). Overall, our models
430 identified two glacial refugia, in Italy and the Anatolia/Middle East with high confidence and
431 low error rates (type I = 0.04; type II = 0.05).

432 Within the western edge of the range, ABC inference indicated a colonization and population
433 expansion in Iberia with a generation time similar to that of the colonization analysis
434 (Supporting Information Table S4). This scenario received high support (99 %) (Supporting
435 Information Fig. S2) and error rates were estimated at 0.19 and 0.17 for type I and II errors
436 respectively.

437

438 **4. Discussion**

439 We reconstructed the evolutionary history of a European bat species with tropical affinities.
440 We show that *T. teniotis* populations were able to survive in Italy and Anatolia/Middle East
441 during the LGM, and have subsequently colonized the current species range. The species
442 has experienced a strong population expansion during the post-glacial colonization of its
443 western range. Our results also point to the occurrence of another population in the
444 Anatolian/Middle East area. Yet, the high haplotype diversity and network pattern found
445 suggests that our samples did not cover the eastern refugium, which is likely located further
446 east (Rossiter et al., 2007) or perhaps towards the Caucasus as suggested for the bat
447 *Myotis bechsteinii* (Kerth et al., 2008).

448 *4.1. Postglacial colonization and demographic expansion*

449 Our inferences of demographic history indicate two main refugia during the LGM, one in the
450 Italian Peninsula and another further east in the Anatolian/Middle East region. During this
451 period, the species may have been extinct throughout the rest of southern Europe, with
452 subsequent recolonization from the Italian Peninsula. Although the origin of the ancestral
453 population is unclear, ABC indicates some degree of gene flow between Europe and
454 Anatolia/Middle East before the LGM. Central and western Mediterranean areas were
455 subsequently colonized in a stepping-stone manner, and through gene flow between
456 populations originating from North Africa and France leading to an admixed population in the
457 Iberian Peninsula. Although samples obtained provided a good coverage of the species

458 range in the western Palaearctic, only a limited number of samples were available from
459 North Africa. This is a common caveat of phylogeographic studies (Husemann, Schmitt,
460 Zachos, Ulrich, & Habel, 2014) and we stress that our models do not negate the possibility of
461 north African or Asian glacial refugium. While such a refugium could be the origin of the
462 unknown ancestral population inferred in this study, our evolutionary history models show
463 that a species with tropical affinities was able to survive in Italy during the LGM, from where
464 it expanded across its current European range.

465 The inferred scenario of an Italian refugium and post-glacial European recolonization
466 concurs with the widely accepted phylogeographic paradigms for the western Palearctic (G.
467 M. Hewitt, 1999). Among bats, Italy has been identified as a glacial refugium for *Myotis*
468 *myotis* (Ruedi et al., 2008) and a possible refugium for *Rhinolophus ferrumequinum*
469 (Rossiter et al., 2007). In a recent paper, Bogdanowicz et al. (2015) suggested that this
470 pattern might be widespread among bat species. Focusing on *Miniopterus schreibersii*,
471 Bilgin et al. (2016) suggested a new paradigm of European colonization from Anatolian
472 populations, and although we identified an ancient population in Anatolia/Middle East, our
473 results do not support the hypothesis of a European recolonization from this region, a similar
474 pattern to *R. ferrumequinum* (Rossiter et al., 2007). In fact, samples from Anatolia and the
475 Middle East formed a distinct clade at the mitochondrial level (AMh), with no haplotypes
476 shared with Europe. Interestingly, the high haplotype diversity (nine haplotypes in 10
477 samples) and the absence of a star-like pattern in the haplotype network for this region,
478 suggests that the eastern refugium could be located further east.

479 High levels of relatedness and reduced genetic diversity in the Canary Islands likely reflect
480 inbreeding in an isolated population. Increased inbreeding relative to mainland populations
481 has been described for different taxa in insular populations (Frankham, 2008), including
482 bats. Our results suggest that Canary Islands were colonized following a model of long-
483 distance dispersal and establishment with limited subsequent gene flow from the parent
484 population (Crisp, Trewick, & Cook, 2011). A general pattern of continental dispersion to the
485 Canary Islands driven by stochastic events such as storms was described by Juan,
486 Emerson, Oromí, & Hewitt (2000).

487 The star-like topology in the European mitochondrial groups (EUh-A and EUh-B) indicates
488 population expansion (Slatkin & Hudson, 1991). This hypothesis was further supported by
489 the ABC inference, which shows a demographic expansion following the Iberian
490 colonization. Such expansion could be the result of a natural process (e.g., Bilgin et al.,
491 2016; Razgour et al., 2013) or might be mediated by human activity, such as through
492 increased roost availability from tall buildings and other structures including bridges, many of

493 which were built during the 20th century (Amorim, Alves, & Rebelo, 2013; Russo &
494 Ancillotto, 2014).

495 Post-glacial population growth appear to be common in taxa with that underwent the same
496 climatic changes since the LGM (Branco, Monnerot, Ferrand, & Templeton, 2002; Korsten et
497 al., 2009), and was also suggested for another fast flying bat species, *Nyctalus noctula* (Petit
498 et al., 1999). Microsatellites have a fast mutation rate when compared to other molecular
499 markers, but it has been questioned whether this rate is fast enough to detect recent
500 population changes (Barrett & Schluter, 2008). Therefore, it is difficult to ascertain if these
501 populations, especially the ones located in the western edge of the species' range are still
502 expanding.

503 4.2. Barriers to gene flow

504 Our results show high differentiation at mitochondrial markers between the populations from
505 the Anatolia and Middle East region and those from central and western Mediterranean. We
506 also found evidence of genetic differentiation within the European clade, whereby
507 populations from Canary Islands, Morocco and Iberia seemed to form a distinct group from
508 Central Mediterranean populations (Italy, France and Greece). Genetic structuring at the
509 mitochondrial level suggests that, once established, females will not disperse freely,
510 supporting some degree of philopatry, a common trait among several bat species (reviewed
511 in Burland & Worthington Wilmer, 2001). In fact, the Iberian Peninsula seems to have been
512 colonized following a first-come, first-served pattern, as indicated by the presence of
513 haplotypes from both the central Mediterranean and North African haplogroups. Even though
514 *T. teniotis* females are physically capable of crossing geographical barriers (e.g. mountain
515 ranges and large bodies of water), philopatric behaviour may have a strong effect on female
516 dispersal, thus explaining the absence of Iberian/north African haplotypes in central
517 Mediterranean. Contrary to mtDNA, at the nuclear level we confirmed some degree of gene
518 flow between Europe and the Anatolia/Middle East. We also found high levels of gene flow
519 within the European range and North Africa, whereas the Gibraltar strait does not act as a
520 barrier to current or even past gene flow (García-Mudarra et al., 2009). Yet, the Canaries
521 show high levels of isolation from mainland Africa. Combined, these results reflect a typical
522 pattern of male-mediated gene flow (Castella, Ruedi, & Excoffier, 2001).

523 Gene flow inferred from nuclear markers seemed to be solely restricted by geographic
524 distance, showing a clear pattern of isolation by distance and the absence of strong
525 geographic barriers to dispersal. *T. teniotis* performs fast and direct flights while foraging
526 with median speeds of 50 km.h⁻¹ and covering linear distances of up to 70 km (Marques,
527 Rainho, Carapuço, Oliveira, & Palmeirim, 2004). Although flight altitudes have not been

528 reported for *T. teniotis*, the species is known to prey on large moths that migrate at high
529 altitudes (Mata et al., 2016). Indeed the smaller congeneric species, *T. brasiliensis* (approx.
530 12 g compared to 30 g of *T. teniotis*), can fly up to 1 km above ground level (McCracken et
531 al., 2008). Thus, the absence of geographic barriers to gene flow in our focal taxa is not
532 surprising.

533 4.3. Implications for the phylogeography of Western Palearctic species with tropical affinity

534 The importance of refugia for conservation planning has been widely recognized because
535 they can facilitate the persistence of biodiversity under changing climates (Keppel et al.,
536 2012), and their relevance is even greater in the face of anthropogenic climate change.
537 Common refugia in the Western Palearctic have been widely acknowledge for a number of
538 species (G. M. Hewitt, 1999; Husemann et al., 2014), however of the 914 studies focusing on
539 taxa that occur in the western Palearctic (Keppel et al., 2012) only very few focus on species
540 with tropical affinities (but see Rato et al., 2011). The location of refugia are often similar
541 between species sharing climatic and environmental requirements, though it has been
542 shown that species may respond differently to changes in habitat availability resulting from
543 climatic changes at the end of the LGM (Taberlet, Fumagalli, Wust-Saucy, & Cosson, 1998).
544 In a recent paper, Carstens, Morales, Field, & Pelletier (2018) showed that species' traits in
545 bats can influence the response to climatic oscillations. Most importantly, they found that
546 heavier bat species and those with longer wings were more likely to suffer a bottleneck at
547 the LGM, and although this was mostly driven by frugivorous species from the neotropics, it
548 highlights the importance of phylogeographic studies on species showing different traits in
549 similar environments.

550 In this study we show that a species with tropical affinities was able to survive in the harsh
551 environments of glacial Europe when a large area of the Western Palearctic was covered in
552 ice sheets and permafrost, and temperatures were 10-20 °C cooler than today (Kageyama et
553 al., 2006). Yet, these results raise new questions regarding how these species survived in
554 colder climates where the environment carrying capacity was lower (Frenzel et al., 1992).
555 Moreover, free-tailed bats, such as *T. teniotis*, are thought to be poor hibernators. Although
556 Arlettaz et al. (2000) found that in the Swiss Alps *T. teniotis* can go through torpor bouts that
557 can last up to 8 days, average body temperature during hibernation and mean arousal
558 frequency was much higher than in other temperate bat species.

559 This study contributes to understanding the evolutionary history of species with tropical
560 affinities living in temperate regions, and raises questions regarding the physiological,
561 behavioural and ecological traits that enabled them to survive in colder climates. The lack of
562 phylogeographic studies focusing on these species highlights the importance of such studies

563 for informing their population management and conservation, in particular under future
564 environmental changes.

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581

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843

844 **Figure 1** – Map showing the study area, with colour representing geographic origins of
845 samples. Grey lined filled area represents IUCN species range within the study area.
846 Bayesian phylogenetic tree and Median-joining haplotype network for *T. teniotis* based on
847 2020 bp of mtDNA (concatenated genes *COI*, *ATP6*, *CytB*, and *D-Loop*). Bayesian posterior
848 probabilities (BPP) equal to 1 (*) and greater than 0.9 (†) are marked above branches.
849 Proportional geographic origin of shared haplotypes is indicated in colour at the branch tips
850 along with total number of samples. Major supported clades (EUh and AMh) and subgroups
851 (EUh-A and EUh-B) are indicated (EUh and AMh). Median-joining haplotype networks for
852 each supported clade as well as the European subgroups (EUh-A and EUh-A) are shown
853 below where branch lengths are not proportional to base-pair changes. Sampling locations
854 and haplotype frequency scale are shown in inset. The Bayesian phylogeny used unique
855 haplotypes only (n = 56) and is shown with out-group (*T. latouchei*). For the median-joining
856 network all concatenated mtDNA sequences (n = 109) were used.

857

858 **Figure 2** – *Tadarida teniotis* population structure based on the microsatellite dataset. Cluster
859 membership plots from STRUCTURE analysis using prior population information (LOCPRIOR)
860 including all samples. Results from 3 to 5 cluster are presented (K = 3 gets the highest rank
861 according to the Evanno method, Supporting Information Fig. S6 and Table S6).

862

863 **Figure 3** – Spatial Analysis of Principal Components (sPCA) showing the spatial genetic
864 pattern of *Tadarida teniotis* population based on the microsatellite dataset. The Canaries
865 form a separate cluster in the left down part, and with less support Greece, Anatolia and the
866 Middle East also cluster together (top left). The two PCs explain 55.4% of the spatial genetic
867 pattern. See also the sPCA Eigenvalues histogram in the inset. Dots indicate individual
868 genotypes.

869

870 **Figure 4** – Colonization patterns across the range of *T. teniotis* according to the best
871 supported scenario (86 %) based on Approximate Bayesian Computation model inference
872 (presented in the inset). The geographical location of *T. teniotis* genetic samples included in
873 the study are plotted over an elevation map, with the location of the six populations marked
874 and colour coded following the inset. Arrows indicate patterns of pre and post-Last Glacial
875 Maximum range colonisation. Map coordinate system: Aitoff (sphere-based).

876

877 **List of Supporting Information**

878 Table S1
879 Table S2
880 Table S3
881 Table S4
882 Table S5
883 Table S6
884 Figure S1
885 Figure S2
886 Figure S3
887 Figure S4
888 Figure S5
889 Figure S6
890 Figure S7
891

892 **Table 1** – Genetic diversity of *T. teniotis* populations based on microsatellite (first five
 893 columns) and mtDNA (last two columns) datasets. Sample sizes in brackets. Mean allelic
 894 richness and mean allele frequency across all loci (\pm SD). H_e – Expected Heterozygosity;
 895 H_{obs} – Observed Heterozygosity.

	Mean Allele frequency	Mean Allelic richness	Number of private alleles	H_e	H_{obs}	Haplotypic diversity	Nucleotide diversity (Pi)
Canary (5)	0.34 \pm 0.10	2.62 \pm 0.45	0	0.58	0.63	0.40	0.0004
Morocco (6)	0.19 \pm 0.04	3.74 \pm 0.45	1	0.76	0.81	1.00	0.0022
Iberia (60)	0.1 \pm 0.020	3.91 \pm 0.37	14	0.80	0.78	0.92	0.0013
France (7)	0.19 \pm 0.03	3.72 \pm 0.31	1	0.76	0.78	1.00	0.0011
Italy (16)	0.12 \pm 0.03	4.00 \pm 0.40	3	0.80	0.77	0.83	0.0010
Greece (5)	0.22 \pm 0.06	3.55 \pm 0.47	3	0.73	0.73	0.90	0.0011
Anatolia (3)	0.25 \pm 0.08	3.56 \pm 0.57	0	0.71	0.83	0.67	0.0040
Middle East (7)	0.15 \pm 0.03	4.00 \pm 0.44	2	0.79	0.78	1.00	0.0036

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