Mitochondrial phylogeography of the common dormouse, Muscardinus avellanarius (Rodentia: Gliridae).

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38 ABSTRACT

39 This is the first mitochondrial phylogeography of the common dormouse, *Muscardinus avellanarius*, a

40 hibernating rodent strictly protected in Europe (Habitat Directive annex IV, Bern convention annex

41 III). The 84 individuals of *M. avellanarius*, sampled throughout the distributional range of the species,

42 have been sequenced at the mitochondrial DNA gene (cytochrome *b*, 704 base pairs). The results

43 revealed two highly divergent lineages with an ancient separation 7.7 Myr ago. Lineage 1 occurs in

44 Western Europe (France, Belgium, Switzerland) and Italy and Lineage 2 in Central-North Europe

45 (Poland, Germany, Latvia, Lithuania), on the Balkan Peninsula and in Turkey. These two lineages

46 were further subdivided into five allopatric sub-lineages.

47 Therefore, the Lineage 1 branches into two further sub-lineages (Western European and Italian) while

48 the Lineage 2 contained three sub-lineages (Central-North European, Turkish and a Balkan). These

49 different sub-lineages should be regarded as independently evolving units that have high conservation

50 significance.

51

52 ADDITIONAL KEYWORDS: Gliridae – Europe – Glacial refugia – conservation- cytochrome *b*.

74 INTRODUCTION

75 Climatic oscillations during the Pleistocene have greatly affected the pattern of distribution of many

76 species in the Western Palaearctic region, as well as their demographic history and patterns of

77 population genetic differentiation (Avise 2000; Hewitt 2004). Three main peninsular refugia have been

78 deduced from phylogenetic studies for most temperate species in Europe, namely Iberia, Italy, and the

79 Balkans (Hewitt 1999, 2001; Taberlet *et al.*, 1998; Michaux *et al.*, 2003; Krystufek, Bryja, Buzan,

80 2009). Increasing evidence suggests that the well-studied European southern and eastern refugia for

81 thermophilous animal and plant taxa were supplemented by cryptic refugia in more northern Europe

82 during the Late Pleistocene (Stewart & Lister, 2001). Since its publication, this hypothesis has

83 received significant support revealing northern refugia in small mammals (Brunhoff *et al.*, 2003;

84 Jaarola & Searle, 2002, 2004; Deffontaine *et al.*, 2005; Kotlik *et al.*, 2006). In addition, some studies

85 pointed out the role of Mediterranean refugia as sites of endemism (Bilton *et al.*, 1998; Stewart 2003;

86 Provan & Bennett, 2008; Bhagwat & Willis, 2008; Grill *et al.*, 2009; Krystufek *et al.*, 2009, Buzan *et*

87 *al.*, 2010).

88 Nonetheless all these studies concern and give detailed phylogeographic patterns of species within the

89 Muroid superfamily, whereas phylogeographic studies on Gliridae are almost inexistent despite the

90 interest of this group. Gliridae are one of the most ancient rodent family, emerging in the Eocene

91 (between 54-53 and 38-37 Mya) (Nadachowski & Daoud, 1995). They are small to medium size

92 rodents, mostly arboreal, and were restricted throughout their history to Europe, Asia and Africa

93 (Wilson & Reeder, 2005). The diversification of the Gliridae which began in the early Eocene

94 continued during the Oligocene and culminated in the Late Early Miocene of Europe, where they

95 appear to have occupied many ecological niches. The decline of this family becomes apparent during

96 the Late Middle Miocene (Vallesian). Casanovas - Vilar *et al.* (2005) suggested that the diversity of

97 forest-adapted rodents decreased significantly not only in coincidence with the climatic Vallesian

98 crisis, but also with the entry and widespread of Muridae. Most of the 28 species contained in the

99 family Gliridae (Holden, 2005) are now regarded as rare or endangered, attracting conservation-related

100 research and active habitat management to assist their survival (Morris, 2003).

101 A recent phylogeographical study on the edible dormouse, *Glis glis* (Hurner *et al.*, 2010) showed a

102 very low genetic variability throughout the northern part of its range which may have important

103 implication for conservation strategies of this species.

104 In this context, we feel that a clear understanding of the evolutionary history of other members of the

105 Gliridae family, obtained by comparative phylogeography, would allow the identification of

106 biodiversity hotspots and increase awareness in conservation policies. In temperate regions glirids are

107 characterized by hibernation during winter, a behavior that has earned them a popular reputation for

108 perpetual sleepiness (Numone *et al.*, 2007). The thermal dependence during hibernation can constrain

109 the biogeography of species and therefore could imply other evolutionary history especially during the

110 Quaternary glaciations.

111   The target of our study is the common dormouse, *Muscardinus avellanarius* (Linnaeus, 1758), a

112   Gliridae protected in Europe (Habitat Directive Annex IV, Bern Convention Annex III) and included

113   in national Red Lists of many countries. The common dormouse occurs in Europe and northern Asia

114   Minor (Turkey) (Fig.1). In continental Europe, it is fairly widespread, although it is absent from Iberia,

115   South-West France, and boreal forests of majority of Fennoscandia and Russia. It is also absent from

116   steppic landscape in eastern Ukraine and southern Russia. Island populations occur in southern Britain

117   and on Corfu and Sicily (Morris, 1999; Rossolimo *et al.,* 2001). It is generally a lowland or mid-

118   mountain species although the highest record of *M. avellanarius* were at the altitude of 1980 m a.s.l. in

119   Macedonia (Kryštufek & Petkowski, 1990) and up to 1920 m a.s.l. in the Austrian Alps (Spitzenberger

120   & Bauer 2001; Juškaitis, 2008).

121   Population trends vary in different parts of the range: in some north-western areas (e.g., UK, the

122   Netherlands, Denmark, Belgium) populations are declining due to the interplay between the species'

123   complex biological requirements and habitat loss and fragmentation (Foppen, Verheggen, Boonman,

124   2002; Bright, Morris, Mitchell-Jones, 2006; Verbeylen 2006; Mortelliti *et al.*, 2010). In others parts of

125   the range, like Sweden and Lithuania, the species is considered stable (Wretenberg & Berglund, 2009;

126   Juškaitis, 2008). It is likely an excellent model for studying the effects of habitat fragmentation,

127   climatic shifts and climatic stochasticity (Bright & Morris, 1996). In addition, the common dormouse

128   exhibits differences in hibernation in different ecogeographical conditions confirming the peculiar link

129   between climate and hibernation length (Panchetti *et al.*, 2004; Sarà, Casamento, Spinnato, 2001).

130   Fossil data suggests that the Miocene/Pliocene boundary led to the diversification of the genus

131   *Muscardinus* into several lineages based on different body size and dental morphology (Garcia - Alix

132   *et al.*, 2008; Aguilar, 1982; Aguilar & Lazzari, 2006; Nadachowski & Daoud, 1995; Storch, 1978).

133   Toward the end of the Pliocene, most of these lineages vanished and since the Middle Pleistocene only

134   the extant species, *M. avellanarius* survived in the European faunas (Nadachowski & Daoud, 1995).

135   Incisive biological conservation of mammals or terrestrial vertebrates in Europe needs detailed data on

136   specie's phylogeography, the genetic diversity and structure of population, as well as on the dynamic

137   of past populations (Randi, 2003). The detection of phylogeographic structuring is important because

138   it helps identifying long-isolated populations that might have distinct gene pools and local adaptations;

139   thus the conservation concern for the common dormouse makes it an excellent candidate for such

140   studies. We therefore defined subsequently the phylogeographic structure of the species, never tackled

141   so far. In particular we tried to answer to the following questions: i) Is the common dormouse

142   structured phylogeographically? If so, ii) Are the mitochondrial sequences geographically structured,?

143   iii) How many distinct genetic lineages of *M. avellanarius* exist? Next issues concerned historical

144   process: iv) Did postglacial recolonization of Central Europe steams from the traditional of

145   Mediterranean refugia or from elsewhere? v) Did the Miocene/Pliocene boundary lead to the

146   diversification of the genus *Muscardinus*? vi) Our final issue was the implication of our results for the

147   species' conservation?

148   MATERIAL AND METHODS

149   SAMPLE COLLECTION AND DNA EXTRACTION

150   We gathered a total of 83 sample tissues of *Muscardinus avellanarius* from 28 localities (1-12 samples

151   per population) spread throughout the specie's geographical range (Table1; Fig.1). These specimens

152   were obtained from the authors and field collaborators (see acknowledgements). Tissues and hairs

153   were preserved in 96% Ethanol until DNA extraction. An additional sequence from Switzerland was

154   downloaded from the GenBank database (Bentz & Montgelard, 1999).Total DNA was extracted using

155   the DNeasy Tissue kit (Qiagen Inc., Valencia, California) following the manufacturer's instructions.

156   DNA samples were extracted and amplified in a separate room solely dedicated to DNA extractions.

157   PCR AMPLIFICATION AND mtDNA SEQUENCING

158   A fragment of 704 base pairs was sequenced from the cytochrome *b* of the mitochondrial DNA gene

159   (mtDNA). PCR amplifications were carried out using primers designed by Andrea Grill specifically

160   for *M. avellanarius* modified from Bentz & Montgelard (1999) : LMA14255 (5'-

161   TGGTGGAATTTCGGTTCTCT-3') and RMA15192 (5'-GTTGGCCTCCAATTCATGTT -3').

162   DNA isolated in some samples was highly degraded and therefore the amplifications of the entire

163   portion of the cyt *b* gene (>700 pb) was unsuccessful. In order to recover this material, two further

164   intern specific primers were designed by fragment alignment:

165   MUSCAR_RINTERN (5'-AAGGTGAACTATTACTAGGGC-3') and MUSCAR_LINTERN (5'-

166   ACCCTAGTAGAATGAATCTGA-3'). Those specific primers amplified two small overlapping cyt *b*

167   fragments (300-400 bp), which were then aligned to give a 704 bp sequence.

168   Amplifications were carried out following the protocol of Michaux *et al.* (2003) and performed in a

169   Labover PTC100 thermal cycler employing 40 cycles (30-45s/94°, 30-45s/50°, 45s-1min30s/72°) with

170   a final extension at 72°C (10 minutes). Products were visualized on an agarose gel to verify the

171   success of amplification. All the sequencing procedures were performed by Macrogen Inc. (Seoul,

172   Korea). The sequences were edited and then aligned using ClustalW algorithm with the 7.0.9.0 version

173   BIOEDIT program (Hall, 1999).

174   PHYLOGENETIC AND PHYLOGEOGRAPHIC ANALYSIS

175   The model of nucleotide substitution that best fits the data set was identified with the web application

176   FindModel developed from ModelTest (Posada & Crandall, 1998) and Weighbor (Bruno, Socci,

177   Halpern, 2000) with the initial tree built with PHYML program (Guidon & Guascuel, 2003). The

178   robustness of the trees was assessed by bootstrap resampling (Felsenstein, 1985).

179   The Bayesian phylogeny reconstruction (Yang & Rannala, 1997) was implemented in MRBAYES

180   3.1.1 (Huelsenbeck *et al.*, 2001). Bayesian posterior probabilities were picked from the 50% majority

181   rule consensus of trees sampled every 1000 generations, discarding the trees obtained before the

182   chains reached stationary distribution ("burn in", empirically determined by checking of likelihood

183   values). A 50% Majority-rule consensus tree was generated in PAUP v4.0b10 (Swofford, 2000).

184 A minimum spanning haplotype network was constructed using the MINSPNET algorithm available

185 in the ARLEQUIN 3.0 software (Schneiders, Roessli, Excoffier, 2000) in order to more effectively

186 portray the relationships among sequences for populations with low sequence diversity (Crandall &

187 Templeton, 1993). To infer the relationships between haplotypes a Median-Joining Network was also

188 constructed with the same combined sequence dataset using the software NETWORK v 4.5 (Bandelt,

189 Forster, Röhl,1999).

190 ANALYSIS OF GENETIC DIVERSITY AND DIFFERENTIATION

191 Haplotype (h), nucleotide (π) diversities (Nei, 1987), and their standard deviations (Tajima, 1989),

192 gene flow and genetic differentiation (using population pairwise Fst) between the two major lineages

193 and sub-lineages were estimated using DnaSP v. 5 (Librado & Rozas, 2009). Net distance between

194 groups and average distances within groups were calculated in MEGA 4 (Tamura *et al.*, 2007).

195 The genetic structure of populations was examined using an analysis of molecular variance (AMOVA)

196 performed in ARLEQUIN 3.0. AMOVA was conducted at three hierarchical levels of population

197 subdivisions: among genetic groups (corresponding to the two lineages), among populations within

198 each genetic group (corresponding to the sub-lineages) and within each population. The significance

199 of these parameters was estimated by 10 000 random permutations of the distance matrix.

200 DIVERGENCE TIME

201 Relative-rate tests and an approximate time of divergence between the observed mtDNA lineages were

202 calculated as explained in Michaux *et al.* (2003). The divergence time between *Eliomys quercinus* and

203 *E. melanurus* (7 ± 0.9 Myr; Montgelard, Matthee, Robinson, 2003) was used as a calibration point.

204 Another estimate of the divergence time of the main lineages of *M.avellanarius* used a Bayesian

205 approach implemented in the software BEAST v. 1.5.4 (Drummond & Rambaut, 2007). We used the

206 *Eliomys quercinus/E. melanurus* divergence as a fossil point calibration. We included the entire set of

207 mitochondrial sequence for the *Muscardinus* group as well as two sequences of *Eliomys melanurus*

208 (GenBank Accession number xxxx-xxxx) and two sequences of *Eliomys quercinus* (GenBank

209 Accession number xxxx-xxx). Analyses were performed under the GTR + G substitution model

210 parameter (previously estimated by FindModel), an uncorrelated lognormal molecular clock and a

211 Bayesian Skyline plot demographic model (Drummond *et al.*, 2005). All other settings were the

212 default provided by BEAST. Two independent runs were performed, with 80 000 000 Markov Chain

213 Monte Carlo (MCMC) sampling every 1000th generation. Results were visualized using TRACER

214 v1.5.

215 ANALYSIS OF DEMOGRAPHIC HISTORY

216 The hypothetical presence of glacial refugia was checked by searching for the possibility of population

217 expansion. To avoid biased conclusion, we examined only the sub lineages including more than 15

218 samples. We inferred past demographic trend for three sub lineages: the Western, the Central-North

219 and the Italian. As the Italian sub-lineage is well structured in three groups, we decided to infer also

220 the past trend for the Central Italian group (n >15). Coalescent-based Tajima's *D* (Tajima, 1989) was

221 calculated to test for selective neutrality (calculation using the total number of mutations) and

222 population history was also inferred by testing departure from neutrality using R_2 (Ramos-Onsins &

223 Rozas, 2002) and Fu's *Fs* (Fu, 1996) in DnaSP v.5 (Librado & Rozas, 2009). Strobeck's S statistic

224 (Strobeck, 1987) is the probability of having an equal number or fewer haplotypes than observed

225 based on the gene frequency distribution derived from the inferred mutation rate θ. High S probability

226 values (0.9-1.0) indicate deviation from neutrality due to either selection or population expansion.

227 Strobeck's S statistic was also calculated using DnaSP v. 5 (Librado & Rozas, 2009). A Bayesian

228 Skyline reconstruction performed in Tracer v1.5. allowed us to examine the historical demography of

229 each lineage. We checked that the settings were able to capture well the model parameters. For

230 example, an estimate of effective sample size (ESS) higher than 200 would indicate a good

231 convergence of MCMC within chains as suggested by Drummond & Rambaut (2007). The

232 demographic histories of the sub-lineages of *M. avellanarius* were examined also using mismatch

233 distribution of pairwise nucleotide differences estimated in DnaSP v.5 (Librado & Rozas, 2009) only

234 for populations including more than 15 samples. Multimodal mismatch distributions would correspond

235 to a condition of demographic stability, whereas sudden population expansions would generate

236 unimodal patterns (Slatkin & Hudson, 1991). The overall validity of the estimated demographic model

237 was tested by obtaining the distribution of a test statistic SSD (the sum of squared differences)

238 between observed and expected mismatch distributions. A significant SSD value is considered as the

239 evidence of departure from the estimated demographic model of a sudden population expansion using

240 Arlequin 3.0. Furthermore, we calculated the raggedness index (Harpending, 1994) of the observed

241 mismatch distribution for each of populations according to the population expansion model

242 implemented in Arlequin 3.0. Small raggedness values represent a population which experienced

243 sudden expansion whereas higher values of the raggedness index suggest stationary or bottleneck

244 populations.

245

246 RESULTS

247 PHYLOGENETIC AND PHYLOGEOGRAPHIC ANALYSIS

248 A total of 33 haplotypes was identified among the 84 common dormouse specimens (Table 1).The

249 total data matrix comprised 33 *Muscardinus* haplotypes plus three other sequences (2 *E. quercinus* and

250 1 *G. glis*) chosen as outgroups according to a molecular phylogenetic study of Gliridae (Montgelard *et*

251 *al.*, 2003). This matrix provided, without the outgroups, 704 base pairs, 120 of which were variable,

252 102 were parsimoniously informative and 18 were singleton-variable sites. The average

253 transitions/transversions ratio was 5,935 and the nucleotide frequencies were: 28.2%, 26.6%, 13.7%

254 and 31.4% for A, C, G and T respectively. The best model of sequence evolution identified in the

255 program FindModel by the Akaike information criterion was the GTR + G model. The shape

256 parameter of Gamma distribution equaled 0.250.

257   The Maximum Likelihood (ML) and the Bayesian Inference (BI) phylogenetic trees showed the same

258   tree topology. The haplotypes segregated into two lineages which gained strong support in ML (97%)

259   but remained unsupported in BI. The cyt-*b* net genetic distance between these is very high (7.7%)

260   (Table 2).

261   The first lineage (hereafter Lineage 1) split into two well supported (BS: 99%; BP: 100%) sub-lineage,

262   the first of which encompassed individuals from Western Europe (Belgium, Switzerland, France)

263   whereas the second comprised all the haplotypes from Italy (including Sicily). The Italian sub-lineage

264   further diverged into two supported groups (BS: 96%; BP: 100%), the first comprising specimens

265   from Central Italy and the second specimens from Southern Italy. The latter was further split into two

266   sub-groups (a Sicilian and a Calabrian), which found support only in BP: 100%.

267   The second major lineage (hereafter Lineage 2) gathers populations from the remainder of the species

268   range, in Central-North Europe (Lithuania, Latvia, Germany, Poland), the Balkan Peninsula

269   (Macedonia, Slovenia, Serbia) and Turkey. A further substructuring was poorly evident in Lineage 2,

270   however the Balkan samples may hold a sister position against those from Central-North Europe. The

271   Minimum Spanning Network (MSN) (Fig 3) and the Median Joining Network revealed a clear

272   geographical partitioning of the haplotypes with a considerable divergence between genomes

273   occurring in different regions of the species range. They also reproduced exactly the same topology

274   than ML and BI trees. The two major lineages are separated by 68 mutational steps.

275   Within the Lineage 1, the Western sub-lineage and the Italian sub-lineage are highly separated with 28

276   mutational steps and have a genetic net distance of 3.2%.The latter split into three groups

277   corresponding to Central Italian, Calabrian and Sicilian groups respectively. Identical substructuring

278   was also recognized in the ML and BI analysis. These groups were separated by 6 mutational steps

279   between each of them and a net distance of 0.8% and 0.7%, respectively between Central Italian and

280   Calabrian groups and between Calabrian and Sicilian groups.

281   Despite the BI analysis did not support the further branching within the Lineage 2,the MSN analysis

282   evidenced that the Balkans sub-lineage and the single Turkish haplotype steamed from the Central

283   North European sub-lineage being separated by 17 and 28 mutational steps, respectively (net distances

284   of 2.6% and 3.3%, respectively).

285   ANALYSIS OF GENETIC DIVERSITY AND DIFFERENTIATION

286   The AMOVA analysis showed that 70.43% (p=0.000) of the total mtDNA variation was distributed

287   among the 2 genetic groups, 26.30% (p=0.000) among populations within groups and 3.26%

288   (p=0.000) within populations.

289   Results are summarized in Table 3 and indicate in general a very low level of diversity for the two

290   lineages (Lineage1 and Lineage 2) with a π value of 0.02. The highest nucleotide diversities were

291   found in the Balkan and the Italian sub-lineages with π values of 0.0067 and 0.006 respectively. Fst

292   values (Table 4) are very high and significant among all the sub- lineages evidencing low gene flow

293   among them.

294   DIVERGENCE TIME

295   Relative rate did not show any difference of evolutionary rate among the observed lineages. This

296   allowed us to apply a molecular clock therefore the approximate time of divergence between the

297   observed mitochondrial DNA sub-lineages was calculated.

298   According to the Mean K2P distance between *E. quercinus* and *E. melanurus,* the gross estimate of the

299   evolutionary rate for the Gliridae is around 1% per Myr. The application of this rate to the different

300   dichotomies obtained within the *Muscardinus avellanarius* tree, resulted in the following approximate

301   molecular dating: 7 Mya for the split between Lineage 1 and Lineage 2; 3.2 Mya between the Western

302   sub-lineage and the Italian sub-lineage and between the Central- North sub-lineage and the Turkish

303   sample; 2.6 Mya between the Balkan sub-lineage and the Central- North sub-lineage. Finally, the

304   separation between the Central Italian and the Calabrian group and between the Calabrian and the

305   Sicilian group should have taken place around 0.8 and 0.7 Mya respectively.

306   Divergence times calculated using the coalescent approach were quite similar to the K2P corrected

307   distance and are summarized in Fig 4.

308   ANALYSIS OF DEMOGRAPHIC HISTORY

309   Neutrality tests of Tajima's *D* revealed non-significant negatives values in all populations (Table 5).

310   Furthermore, the R_2 values fall within the range expected under the model of constant population size,

311   so accepting the null hypothesis of constant population size in all populations. Non-significant

312   Strobeck's S and negative Fu's *Fs* values were obtained for the Western, Italian and Central Italian sub

313   lineages. However, Fu's test for neutrality (Fu 1996) and the Strobeck's S indicated a deviation from

314   neutrality due to either selection or population expansion for the data set of the Central-North

315   European lineage (Fu's Fs= -6,318, p=0.000; S=1, p=0.001).

316   The Bayesian skyline plot reconstruction showed that the four lineages appear to have experienced a

317   long period of constant population size, followed by a decline which started around 1 Mya. The

318   mismatch distribution for the four above clusters also did not show the bell-shaped curve consistent

319   with the hypothesis of rapid population expansion (Fig.6). The mismatch population test

320   statistics SSD and raggedness index values were also consistent with constant population sizes (Table

321   5).

322

323   DISCUSSION

324   GENETIC STRUCTURE AND TIME OF DIVERGENCE OF *MUSCARDINUS AVELLANARIUS*

325   Consistently to paleontological data and the resulting 7 Myr time of divergence, we might hypothesize

326   that the putative ancestor of *Muscardinus avellanarius* (*M hispanicus - M pliocaenicus;* Garcia-Alix *et*

327   *al.,* 2008*)* would have split very early and different subsequently by allopatry into two highly

328   divergent genetic lineages in Europe.

329   Our mtDNA study suggests that the Late Miocene/Early Pliocene was an important period for the

330   diversification of European mammals. Several other western and eastern European mammalian taxa

331 are known to have diverged at about the same time, such as *Talpa* spp (Colangelo *et al.*, 2010),

332 *Erinaceus europaeus* and *E. roumanicus* (Santucci, Emerson, Hewitt, 1998), or the eastern and

333 western clade of *Cervus elaphus* (Ludt *et al.*, 2004). A closer sampling in the possible zones of overlap

334 between the two ancient lineages could reveal a contact zone as shown in previous studies, e.g.

335 yellow-bellied and fire-bellied toads (*Bombina variegate* and *B. bombina*; Szymura 1993), oaks

336 (*Quercus robur* group; Ferris, Oliver, Davy, 1993), shrews (*Sorex araneus* group; Taberlet, Fumagalli,

337 Hausser, 1994).

338 During the Late Pliocene and throughout the Quaternary, a substantial subdivision of extant common

339 dormouse lineages into more sub-lineages seems to have occurred. Based on the results and on our

340 estimation of divergence time, Lineage 1 split into a Western European and an Italian sub-lineage

341 around 3.2 Mya. Lineage 2 split into a Central - Northern European, a Turkish and a Balkanic sub-

342 lineage around 3.2 Mya and 2.7 Mya respectively. In coincidence with those divergence times, around

343 3 Mya, at the Pliocene-Pleistocene boundary a further strong climatic deterioration occurred with the

344 intensification of glaciations and the establishment of the great northern ice sheets in America and in

345 Europe (Santucci *et al.*, 1998). The Early Pleistocene saw the definitive decline of Tertiary forests in

346 northwestern Europe (West, 2000) and the disappearance of such rich floristic habitats might have

347 promoted further isolation of the different *M. avellanarius* lineages.

348 PAST DEMOGRAPHY

349 The generalized Bayesian Skyline reconstruction showed that the sub-lineages have experienced a

350 long period of constant population size, followed by a general decline which started around 1 Mya.

351 Contemporary population fragmentation which primarily steamed from the intensification of

352 glaciations, but possibly also from the accelerated competition with the Muridae family lead to

353 contraction of the effective size and therefore could explain the demographic decline observed since

354 Early Pleistocene (1 Mya). This is further evidenced by the neutrality tests, the mismatch analysis, as

355 no one of the mismatch graphics showed a bell-shaped curve confirming a population expansion. The

356 straightforward explanation of our results can be misleading, however. The only exception concerns

357 the central north European sub lineage where the Strobeck'S test, the star-like topology of the network

358 and the Fu's Fs, value indicate a signal of population expansion. There is no clear evidence of recent

359 expansion in the Bayesian plot for the central north European sub lineage as the confidence interval

360 can reveal either an expansion either a decline.

361 REFUGIA AND POSTGLACIAL RECOLONIZATIONS

362 The phylogeographic analysis allowed the reconstruction of refugia for *M. avellanarius* although in

363 some cases more sampling and additional analysis is needed (e.g. Central-North European sub-lineage,

364 Balkan sub-lineage, Turkish specimen).

365 *Italian sub-lineage*. The high level of genetic diversity (Table 3) indicates that the Italian region was

366 one of the refugia for *Muscardinus avellanarius*. Furthermore, the mismatch analysis and the

367   neutrality tests suggest the existence of a stable population in the Italian region. During Quaternary

368   glaciations, this sub-lineage has been able to survive to the general cooling and to diversify genetically

369   in different parts of the peninsula. This lead to the appearance of three genetic groups corresponding to

370   the following regions: Central Italy, Sicily and Calabria. This separation is confirmed by the Fst values

371   which are very high and significant among the three groups (Table 4). These groups are probably the

372   result of geographic isolations in three different refugia, associated to the fragmentations of forests that

373   appeared during Quaternary cold stages (Magri *et al.*, 2006) .The long-term isolation of Sicily and

374   southern Calabria from the rest of Italy would be also due to marine-flooded graben in central

375   Calabria, which appeared for most of the Pleistocene, as attested by the presence of several endemic

376   plants (Pignatti, 1982) and animals in these regions (Malatesta 1985; Caloi, Malatesta, Palombo**,** 1989;

377   Santucci, Nascetti, Bullini, 1996; Canestrelli *et al.*, 2010). Our approximate time of divergence, with a

378   separation between the three groups around 1 Mya, seems to corroborate such biogeographic

379   scenarios. This result strongly suggests the possible existence of refugia within refugia in the Italian

380   peninsula as already observed for several other species (Michaux *et al.*,2003; Canestrelli *et al.*, 2006,

381   2007, 2008; Castiglia *et al.*,2007; Grill *et al.*,2009; Vega *et al.*,2010).

382   *Western sub-lineage*. The high endemism of the Italian sub-lineage strongly suggests that such

383   population did not contribute to the postglacial recolonization of Western Europe. This implies that *M.*

384   *avellanarius* living in France, Belgium and Switzerland and now grouping in the Western sub-lineage

385   had other refugia outside from those 'traditional'. There were areas situated outside the permafrost

386   during the maximum cooling (Sommer & Nadachowski, 2006) within the current range of the Western

387   sub-lineage, e.g. the area of the Dordogne in south-western France

388   *Balkan sub-lineage*. The high level of genetic diversity (Table 3) indicates the Balkan region was

389   another glacial refuge for *Muscardinus avellanarius*. The Balkan Peninsula is topographically the

390   most diverse landscape in Europe (Reed, Krystufek, Eastwood, 2004) and such variability could have

391   provided a suitable environment for altitudinal shifts in response to climatic change during glacial-

392   interglacial oscillations and also for small scale allopatric isolation (Krystufek *et al.*, 2007). This later

393   hypothesis could be confirmed by the high number of mutation steps within the Balkan sub-lineages in

394   the minimum spanning network (Fig 3). Recent studies suggest multiple glacial refugia in the Balkan

395   Peninsula for different species, such as *Dinaromys bogdanovi* (Krystufek *et al.,* 2007a), *Rana*

396   *(Pelophylax)* (Lymberakis *et al.*, 2007) and *Spermophilus citellus* (Krystufek *et al.*, 2009).

397   *Central-North European sub-lineage.* The star-like topology in the minimum spanning network, the

398   Fu's Fs value and the Strobeck's S index suggest a rapid expansion/colonization event for the Central-

399   North European sub-lineage. However, the refugium of this sub-lineage is unclear. All the analysis

400   (ML, MST, genetic distance analysis, Fst value) showed that the Balkan sub-lineage is highly

401   divergent from the Central-North European sub-lineage. These results tend to infer that the Balkan

402   sub-lineage did not contribute to the colonization of the Central-North Europe. As suggested for

403   several other species (Seddon *et al.,* 2002*;* Deffontaine *et al.*, 2005, Kotlik *et al.*, 2006), modern

404   populations of common dormouse from Central-North Europe could be derived from populations that

405   survived in the Carpathian region as it was covered with patches of mixed coniferous and deciduous

406   forests instead of a uniform steppe-like landscape (Willis *et al.,* 2000). Fossil data tend to suggest such

407   hypothesis. Indeed, numerous fossils records of *M. avellanarius* have been found during the Late

408   Vistulian (Pleistocene) period in the Deszczowa Cave in southern Poland (Nadachowski, 1989).

409   *Turkish sub-lineage*. The single Turkish specimen is also highly divergent as compared to the Central-

410   North European specimens. During the last glacial maximum, temperate forest remained in northern

411   Turkey in a narrow band along the southern shore of the Black Sea, with patchy extension to the

412   south-west Caucasus (Adams & Faure, 1997). Pollen records indicate that deciduous oak was present

413   in the southern, and particularly south-western, part of the Caucasus region from at least 12 000 years

414   BP (Huntley, 1990, 1992). It seems possible therefore that this area of Northern Turkey could be the

415   site of a glacial refugia (Seddon *et al.,* 2002). This hypothesis would need to be confirmed by a more

416   extensive sampling of this region.

417   TAXONOMIC AND CONSERVATION IMPLICATION

418   The genetic divergence between the Lineage 1 and the Lineage 2 (approximately 7.7%) falls within

419   the range of inter- and intraspecific cyt *b* distances observed in Mammals (Bradley & Baker, 2001),

420   more specifically in the Arvicolinae (Jaarola & Searle, 2002, 2004; Conroy & Cook, 2000; Haynes *et*

421   *al.,* 2003) and in the Glirinae (Bentz & Montgelard, 1999). Thus, the West European and Italian

422   populations could be described under the phylogenetic species concept (Cracraft, 1983) like a

423   subspecies or even an allo-species separated from a second subspecies formed by the Central-North

424   European and the Balkan populations. However such phylogenetic approach based on only one genetic

425   marker is not longer widely accepted (Avise & Ball, 1990). Additional data from genetically

426   independent loci is required before solid taxonomic conclusions can be made.

427   Corbet (1978) tentatively recognized five subspecies of *Muscardinus* based on the morphological

428   studies of Witte (1962) and Roesler & Witte (1969): *M. avellanarius avellanarius* (including *anglicus,*

429   *corilinum, muscardinus)* in Sweden*; M. a. kroecki* Niethammer & Bohmann, 1950 in Bulgaria*; M. a.*

430   *pulcher* Barrett-Hamilton, 1898 (including *niveus* and *speciosus*) in Italy and Sicily; *M. a. zeus*

431   Chaworth-Musters, 1932 in Greece; *M. a. trapezius* Miller, 1910 in Asia Minor. Namely, the

432   geographic variation has never been assessed throughout the species' range, and discontinuities in

433   morphological variation have not been demonstrated. A more recent view observed great

434   morphological homogeneity among the European populations which does not allow to distinct

435   different subspecies (Wilson & Reeder, 2005). According to our results, there is no congruence

436   between our genetic lineages and the 5 previously recognized subspecies. However, the present

437   genetic study would affirm that common dormouse could be composed of minimum 5 historically

438   isolated, independently evolving sets of populations. Even if taxonomic conclusions cannot still be

439   made those lineages could be regarded as independent units for conservation management purposes

440   (Hillis, Moritz, Mable, 1996; Kocher & Stepien, 1997). The high genetic divergence between all the

441 lineages, the ancient separation of the lineages reflects low mobility. The conservation of such

442 evolutionary significant units (ESU's) is regarded as an important goal preserving species (Moritz,

443 1994, 1999). Furthermore, the identification of cryptic refugia has important implications in current

444 and future periods of global climatic changes (Provan & Bennett, 2008).

445

446 ACKNOWLEDGEMENTS

453

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710   FIGURE LEGENDS

711   FIG.1. Geographical distribution of the *Muscardinus avellanarius* in Europe and proximate location of

712   the sampled population. The shaded zone corresponds to the distribution area of the species. Different

713   symbols refer to lineages in Fig 2, 3 and 4 and in the Tables 2, 3 and 4 (✱ = West European sub-

714   lineage, ◕ = Italian sub-lineage, ◆ = Turkish specimen, ▲ = Central North European sub-lineage, ■ =

715   Balkans sub-lineage).

716   FIG.2. Maximum likelihood tree for the 33 haplotypes of the common dormouse. Numbers indicated

717   on the branches correspond to bootstrap support obtained in the ML analysis (left) and Bayesian

718   probabilities (right). Haplotypes origins are indicated in Table 1.

719   FIG.3. A minimum spanning network constructed using the 33 haplotypes of mitochondrial Cytb gene

720   sequences. Geographic origins (Table 1) are noted. Numbers correspond to the mutational steps

721   observed between haplotypes, numbers in parentheses correspond to the number of animals presenting

722   this haplotype and the size of the circle is proportional to the numbers of haplotypes represented.

723   FIG.4. Maximum clade probability tree displayed from the Beast analysis. Numbers in bold indicate

724   the posterior mean estimates of divergence time. Node bars illustrate the width of the 95% Highest

725   Posterior Density (HPD).

726   FIG.5. Bayesian skyline plots of historical demography of *Muscardinus avellanarius*. Time is shown

727   in million years to present day (= 0). Demographic trends for the Central-North European, Italian and

728   Western European sub-lineages as well as central Italian group are shown. Solid lines and shaded

729   areas represent the median estimates and the 95% HPD.

730   FIG.6. Mismatch distribution of cyt *b* sequences (704 pb) for the Central North European, the Western

731   European, the Italian sub-lineages and the central Italian group.

732   TABLES

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733   TABLE 1. – Map references, geographic locations, corresponding sub lineages, sample symbols,

734   collectors and GenBank accession numbers of *M. avellanarius* haplotypes used in this study.

735   Accession numbers with an asterisk were downloaded from the GenBank database.

	Geographic origin		Sub lineages	Total numbers of animals	Samples symbols	Haplotypes	Genbank accession number
Hazel dormouse *(Muscardinus avellanarius)*							
	Macedonia	Mt. Galičica	Balkans	5	Ma1	Hap01, 04, 05	xxxx
		Popova Šapka	Balkans	1	Ma2	Hap05	xxxx
		Mt. Pelister	Balkans	1	Ma3	Hap05	xxxx
	Serbia	Mt. Cer	Balkans	1	Ser1	Hap05	xxxx
	Slovenia	Mt Krim	Balkans	2	Slo1	Hap03, 05	xxxx
		Pogorelec Mt. Kocevski Rog	Balkans	1	Slo2	Hap02	xxxx
	Lithuania	Šakiai district	Central North	12	Lit1	Hap06, 07,08, 09	xxxx
	Latvia		Central North	3	Let1	Hap11, 12,13	xxxx
	unknown		Central North	1	?	Hap 16	xxxx
	Poland		Central North	2	Po1	Hap10, 15	xxxx
	Germany		Central North	3	Ge1	Hap06, 14,18	xxxx
	Turkey	Mt.Ulu dag	Turkish	1	Tur1	Hap17	xxxx
	Switzerland	Canton de Vaud	West European	6	Swi1	Hap19	xxxx
		(Bentz & Montgelard, 1999)	West European	1	Swi2	Hap22	AJ225117*
	France	Normandie	West European	2	Fr1	Hap20	xxxx
	Belgium	Mechelen	West European	6	Be1	Hap21	xxxx
	Italy	Tevere Farfa(Lazio)	Italian	1	It10	Hap23	xxxx
		Castel di Guido (Lazio)	Italian	10	It7	Hap24, 27	xxxx
		Arcinazzo Romano (Lazio)	Italian	1	It8	Hap24	xxxx
		Viterbo (Lazio)	Italian	4	It11	Hap24, 26, 28	xxxx
		Filettino (Lazio)	Italian	1	It9	Hap25	xxxx
		Castelporziano (Lazio)	Italian	9	It6	Hap24, 27,28	xxxx
		Perugia (Umbria)	Italian	2	It12	Hap24	xxxx
		Calabria	Italian	1	It3	Hap29	xxxx
		Cosenza (Calabria)	Italian	1	It4	Hap29	xxxx
		Catena Costiera (Calabria)	Italian	1	It5	Hap29	xxxx
		High Madonia (Sicily)	Italian	3	It2	Hap30, 32, 33	xxxx
		Low Madonia (Sicily)	Italian	2	It1	Hap31, 32	xxxx
Outgroups							
Garden dormouse *(Eliomys quercinus)*							
	(Bentz & Montgelard, 1999)			1			AJ225030*
	(Bentz & Montgelard, 1999)			1			FM16427*
				1			xxxx

		1			xxxx
Asian Garden dormouse (*Eliomys melanurus*)					
		1			xxxx
		1			xxxx
Edible dormouse (*Glis glis*)					
(Hurner *et. al.*, 2010)		1			FM16065*

736

737 TABLE 2. - Cytochrome *b* Net Genetic Distance (NGD) between lineages

			Balkans	CentralNorthEurope	Western Europe		
						Central Italy	Sicily
		Sample size (n)	NGD	NGD	NGD	NGD	NGD
All		**84**	/	/	/	/	/
Lineage 2		**33**	/	/	/	/	/
Balkans		11	/	/	/	/	/
Turkey		1	4%	3,30%	/	/	/
Central North Europe		21	2,60%	/	/	/	/
Lineage 1		**51**	/	/	/	/	/
Western Europe		15	8%	7,70%	/	/	/
Italy		36	/	/	/	/	/
	central Italy	28	8,60%	8,60%	3,20%	/	/
	Sicily	5	8,10%	8,10%	4%	1,20%	/
	Calabria	3	8,40%	8,50%	3,40%	0,80%	0,70%

738

739 TABLE 3. – Summary of haplotypes (Hd) and nucleotide diversity (Pi) and their standard deviation

740 observed within the main genetic groups of the common dormouse.

		Sample size (n)	Pi (± SD)	Hd (± SD)
All		**84**	0,05992 ±0,00193	0,921 ±0,015
Lineage 2		**33**	0,0211 ±0,00262	0,841 ± 0,049
Balkans		11	0,00668 ±0,00294	0,491 ±0,175
Turkey		1	/	/
Central North Europe		21	0,00337 ±0,00707	0,786 ±0,096
Lineage 1		**51**	0,0225 ±0.00	0,844 ±0,00124
Western Europe		15	0,00168 ±0,00013	0,705 ±0,074
Italy		36	0,006 ± 0,00125	0,742 ± 0,064
	central Italy	28	0,00151 ±0,00024	0,566 ±0,079
	Sicily	5	0,002 ±0,00081	0,7 ±0,218
	Calabria	3	0,00095 ±0,00045	0,667 ±0,314

741

742 TABLE 4. - Pairwise Fst between lineages. * = (p < 0.5)

|		Sample size (n)	Balkans Fst	CentralNorthEurope Fst	Western Europe Fst	Central Italy Fst	Sicily Fst
All		**84**	/	/	/	/	/
Lineage 2		**33**	/	/	/	/	/
Balkans		11	/	/	/	/	/
Turkey		1	/	/	/	/	/
Central North Europe		21	0.88234 *	/	/	/	/
Lineage 1		**51**	/	/	/	/	/
Western Europe		15	0.95963 *	0.97283 *	/	/	/
Italy		36	0.93593 *	0.94808 *	0.88739 *	/	/
	central Italy	28	0.97113 *	0.97843 *	0.96398 *	/	/
	Sicily	5	0.94403 *	0.96967 *	0.96726 *	0.90824 *	/
	Calabria	3	0.94175 *	0.97091 *	0.96487 *	0.87564 *	0.84239 *

743

744 TABLE 5. - Results of neutrality tests (Tajima's D , Fu's Fs, R2) and mismatch analyses for the three

745 sub lineages and the Central Italian group (n>15). *= (p <0.05)

|		Sample size (n)	Tajima's D	Fu's Fs	R2	Strobeck's S	Raggedness index, Rg	Mismatch distribution, SSD
All		84	/	/	/	/	/	/
Lineage 2		33	/	/	/	/	/	/
Balkans		11	/	/	/	/	/	/
Turkey		1	/	/	/	/	/	/
Central North Europe		**21**	-0.507	-6.318*	0.107	1*	0.09014	0.03325
Lineage 1		51	/	/	/	/	/	/
Western Europe		**15**	0.833	0.034	0.193	0.751	0.10204	0.02245
Italy		**36**	0.218	0.634	0.285	0.509	0.09384	0.04341
	central Italy	**28**	-0.484	-0.552	0.105	0.827	0.240	0.05832
	Sicily	5	/	/	/	/	/	/
	Calabria	3	/	/	/	/	/	/

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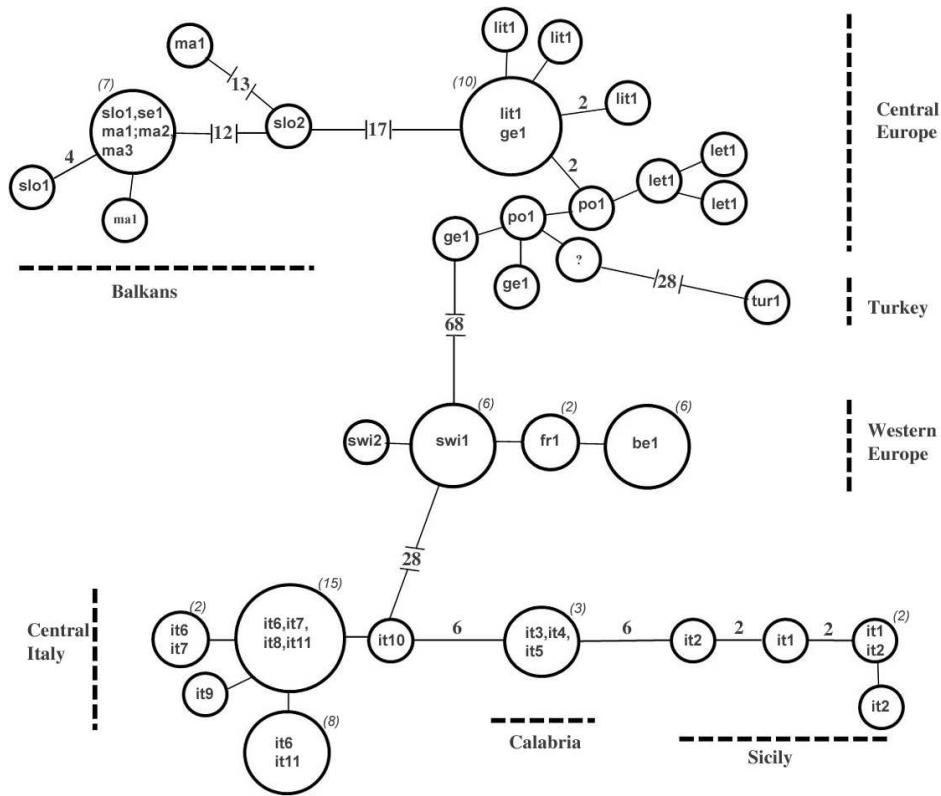58
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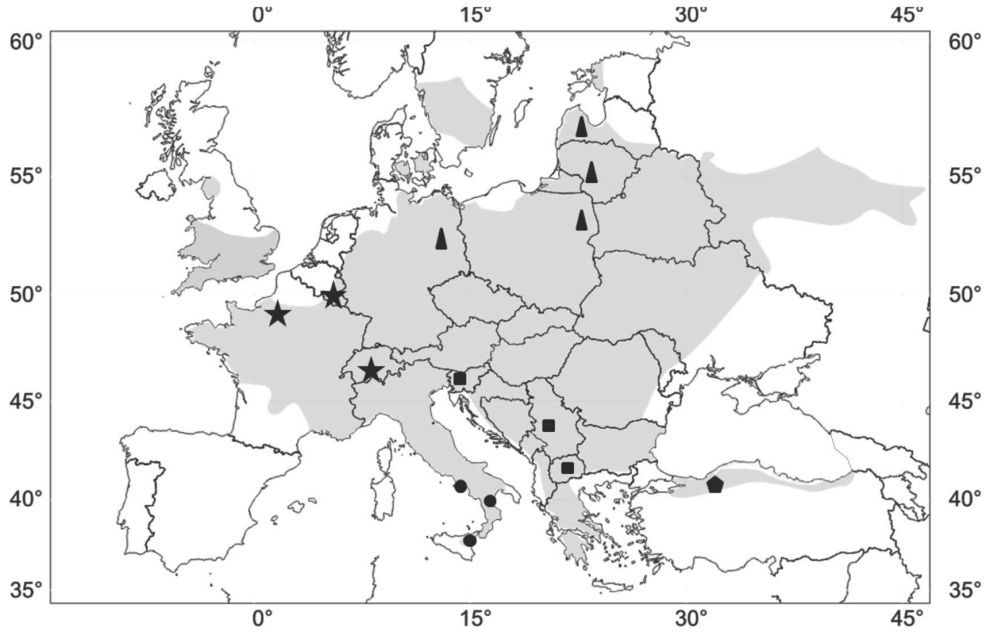
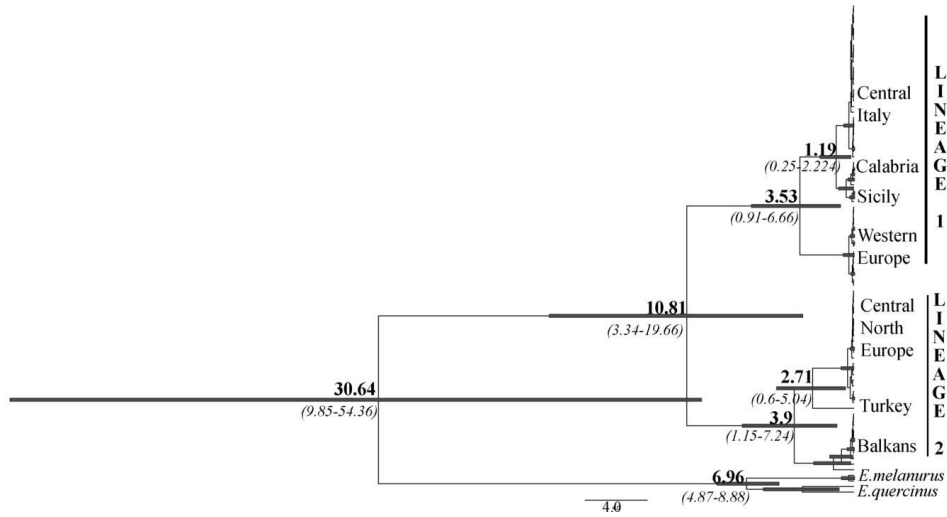Scale: 1:39576053 at Latitude 0°

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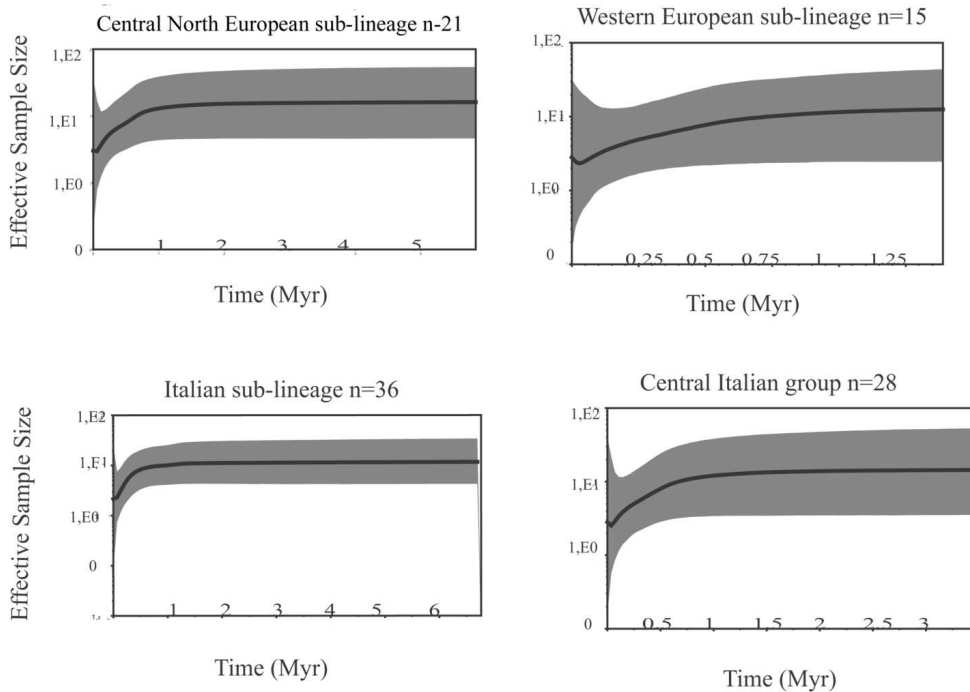43
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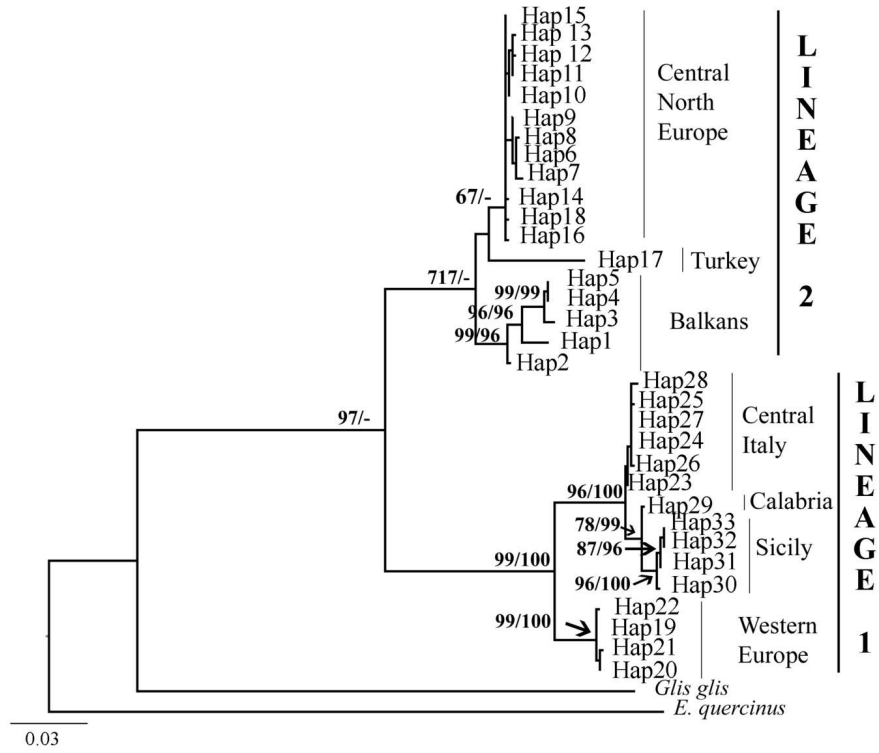
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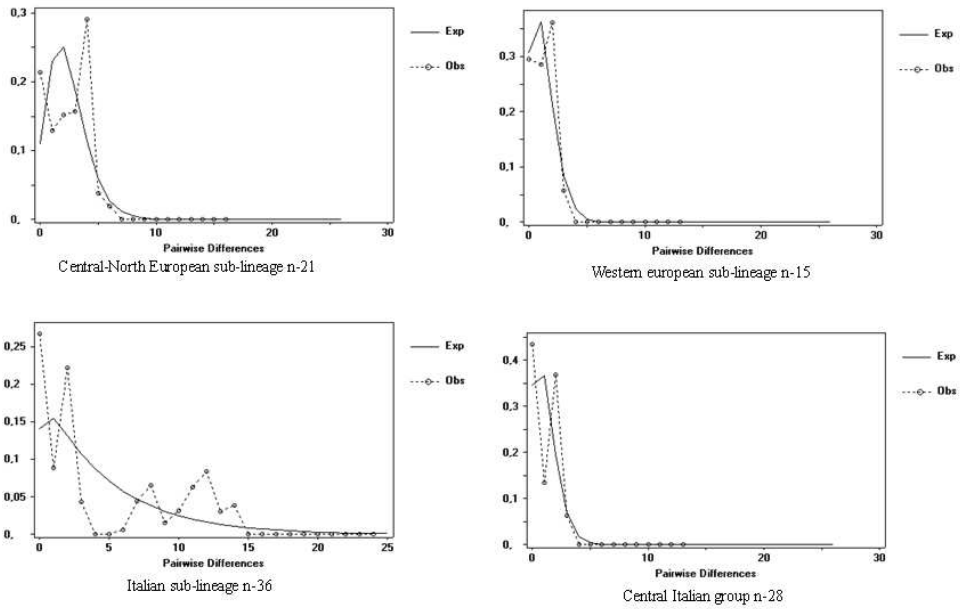54
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Central-North European sub-lineage n-21

Western european sub-lineage n-15

Italian sub-lineage n-36

Central Italian group n-28