Population genetic structure of the bank vole *Myodes glareolus* within its glacial refugium in peninsular Italy

Running title: Bank vole genetic structure in Italy

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Abstract

It is now well established that Southern European peninsulas have been major glacial refugia for temperate species during Pleistocene climatic oscillations. However, substantial environmental changes occurred also within these peninsulas throughout the Pleistocene, rising questions about the role and interplay of various of micro-evolutionary processes in shaping patterns of intraspecific diversity within these areas. Here, we investigate the patterns of genetic variation in the bank vole *Myodes glareolus* within the Italian peninsula. By using a panel of 13 microsatellite loci, we found more intra-specific variation than expected based on previous assessments. Indeed, both Bayesian and ordination-based clustering analyses of variation recovered five main geographic/genetic clusters along the peninsula, with three clusters geographically restricted to the southern portion of the peninsula. This clustering is supported by previous evidences of some morphological distinctiveness among these populations. This pattern can be explained by a refugia-within-refugia scenario, with the occurrence of multiple sub-refugia for the bank vole within the Italian, likely promoted by the major paleo-environmental changes which affected forested habitats within this area during the Pleistocene. Moreover, our results support a scenario whereby the high levels of intraspecific diversity observed within major Pleistocene refugia are better explained by dynamic micro-evolutionary processes occurred within these areas, rather than by long-term demographic stability of refugial population. Finally, the narrow and isolated distribution of some of the identified lineages, suggest the need for future assessments of their conservation and taxonomic status.

Introduction

Southern European peninsulas have provided an excellent research ground to investigate how past climate changes and topographic features influenced species' evolutionary histories (Hewitt, 2011). Plenty of studies in the last thirty years highlighted the role of these peninsulas as climatic refugia for temperate species during Pleistocene glacials (Bennet & Provan, 2008; Comes & Kadereit, 1998; Feliner, 2011; Hewitt, 1996; 2004; Schmitt, 2007; Weiss & Ferrand, 2007; Stewart, Lister, Barnes & Dalén, 2010). Due to the strong topographic complexity of these peninsulas, species underwent extreme population fragmentation within refugia (Gomez & Lunt, 2007; Hofreiter & Stewart, 2009). As a consequences, these areas have been found to be particularly rich of intraspecific genetic lineages – sometimes highly divergent from the closest relatives (e.g. Canestrelli, Cimmaruta, Costantini & Nascetti, 2006), often with narrow distribution (e.g. Bisconti et al., 2018) – detecting which is crucial to understanding species genetic structure and to defining evolutionary and management units for conservation planning (Avise, 2008; Frankham, 2010; Groves et al., 2017; Palsbøll, Berube & Allendorf, 2007). However, despite their disproportionate importance as both cradles and reservoir for Western Palearctic biodiversity, current knowledge of historical biogeographic patterns and processes within these areas is still far from satisfactory. For many taxa, the genetic structure is still little known, and the available knowledge if often flawed by limited sampling or limited number of genetic markers.

The bank vole *Myodes glareolus* (Schreber, 1780) is a small woodland-dwelling rodent, widespread throughout temperate and boreal forests of most of Europe (Amori, Contoli & Nappi, 2008a), which has been a key species in the study of the European fauna response to the Pleistocene climate changes. It has been one of the most convincing examples of a woodland species surviving glaciations within a cryptic northern refugium in Europe, i.e. a refugium located further north of the traditionally recognized refugia in the Southern European peninsulas (Bhagwat & Willis, 2008; Bilton, Mirol, Mascheretti, Fredga, Zima & Searle, 1998; Deffontaine et al., 2005; Filipi, Marková, Searle & Kotlík, 2015; Kotlík, Deffontaine, Mascheretti, Zima, Michaux, & Searle, 2006). However, bank vole populations survived Pleistocene glaciations also in southern refugia. There were identified distinct evolutionary lineages in either Balkan, Iberian and Italian peninsulas (Colangelo, Aloise, Franchini, Annesi & Amori, 2012; Deffontaine et al., 2005; Filipi et al., 2015). Within the Italian peninsula, four distinct evolutionary lineages have been characterized by mean of mitochondrial DNA variation: one widespread across Alps and northern Italy, one distributed mainly throughout

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northern and central Apennines, one restricted to the Gargano promontory (Apulia), and one found only in Calabria (Colangelo et al., 2012). This differentiation is supported also by some slight morphological distinctiveness (Amori et al., 2008a). Interestingly, the Calabrian clade showed strong and ancient (Early Pleistocene) genetic divergence from all other *M. glareolus* lineages, resulting as the basal clade of the entire bank vole phylogeny, whereas the Gargano clade does not cluster with any of the other lineages (Colangelo et al., 2012; Filipi et al., 2015). Nevertheless, all phylogenetic and phylogeographic inferences were based only on mitochondrial data, which have several limitations in inferring population genetic structure and patterns of gene flow among populations (Ballard & Whitlock, 2004). Indeed, despite Calabrian and Apulian populations resulted genetically differentiated from the other peninsular populations, and virtually geographically isolated by the rarefaction/fragmentation of species habitat, the lack of a multi-marker analysis of species genetic structure does not allow inferences on genetic isolation.

In this study, we further investigate the genetic structure of the bank vole in the Italian peninsula. We employ a set of thirteen microsatellite loci in order to complement previously published mitochondrial data (Colangelo et al., 2012), with the aim of better understanding the geographic structure of genetic variation and to shed more light on the bank vole evolutionary history. Moreover, considering the narrow ranges of the Calabrian and Apulian lineages and the ongoing reduction of the forest habitat (Scarascia-Mugnozza, Oswald, Piussi & Radoglou, 2000), a better understanding of the pattern of genetic isolation is mandatory to evaluate the need for conservation actions concerning southern bank vole populations.

Materials and methods

We collected 76 *Myodes glareolous* individuals from 15 localities spanning the Italian peninsula; collecting sites and sample sizes are given in Table 1 and Figure 1; voucher numbers are listed in Appendix I. Tissue samples were obtained from an auricle biopsy on live-trapped animals during field sessions, or from museum specimens (Museum of Comparative Anatomy G.B. Grassi of the University of Rome 'La Sapienza').

DNA extractions were performed by using the standard cetyltrimethylammonium bromide protocol (Doyle and Doyle, 1987). We analysed genetic variation at thirteen microsatellite loci: *Cg14E1, Cg15F7, Cg17A7, Cg3A8, Cg3F12, Cg4F9, Cg12A7, Cg13C12, Cg17E9, Cg10A11, Cg10H1, Cg13G2, Cg6A1* (Guivier et al., 2011; Rikalainen, Grapputo, Knott, Koskela & Mappes, 2008) following protocols published in Guivier et al. (2011). We chose a subset of

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available loci after excluding those that exhibited reaction inconsistency in over 30% of the samples analysed. The thirteen loci were assembled in three multiplex as described in Table 2. Forward primers were fluorescently labelled, and PCR products were electrophoresed by Macrogen Inc. on an ABI 3730xl genetic analyser (Applied Biosystems) with a 400-HD-size standard.

The microsatellite data were analysed using GeneMapper® 4.1. Micro-Checker 2.2.3 (Van Oosterhout, Hutchinson, Wills & Shipley, 2004) was used to test for null alleles and large-allele dropout influences. Allelic frequencies were computed by using GENETIX 4.05 (Belkhir, Borsa, Chikhi, Raufaste & Bonhomme, 1996), while FSTAT (Goudet, 1995) was used to test for deviations from the expected Hardy-Weinberg and linkage equilibria. Estimates of genetic diversity, based on the mean allelic richness and the mean observed and expected heterozygosity were computed by the DivRsity R package (Keenan, McGinnity, Cross, Crozier & Prodöhl, 2013), after excluding populations with $n < 4$; allelic richness was computed using the rarefaction method (Petit, Mousadik & Pons, 1998).

In order to investigate the extent of ordination in microsatellite data attributable to population genetic structure, without using previous information on the origin of each individual, a Discriminant Analysis of Principal Components (DAPC, Jombart et al., 2010) was performed using Adegenet R package (Jombart et al., 2008). DAPC optimizes variation between clusters and minimizes variation within them, and it is free of assumptions such as Hardy-Weinberg and linkage equilibria (Jombart, Devillard & Balloux, 2010). At first data are transformed using a Principal Component Analysis (PCA), and then clusters are identified using discriminant analysis. The number of clusters (K) was identified by the *find.clusters* function using the "K-means" algorithm, and the Bayesian information criterion (BIC) was used to choose the most relevant K values for population structure. The discriminant analysis was then performed using the optimal number of principal components identified by a spline interpolation of the a-scores (i.e. the difference between the proportion of successful reassignment of the analysis and the values obtained using random groups).

The population genetic structure across the study area was also investigated using the Bayesian clustering algorithm implemented in TESS 2.3.1 and the geographical location of individuals as prior information (Chen, Durand, Forbes & François, 2007; Francois & Durand 2010). The analysis was performed by modelling admixture using a conditional autoregressive model (CAR). Preliminary analyses were carried out to assess model performance, with 20 000 steps (the first 5 000 were discarded as burn-in) and 10 replicates for each K value (i.e. the number of clusters) between 2 and 10. The final analysis contained 100 replicates for each K

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144   value, with K = 2–10; each run consisted of 80 000 steps, with the first 30 000 discarded as

145   burn-in. The spatial interaction parameter was initially kept at the default value (0.6), and the

146   updating option was activated. The model that best fitted the data was selected using the

147   deviance information criterion (DIC). DIC values were averaged over the 100 replicates for

148   each K value, and the most probable K value was selected as the one at which the average DIC

149   reached a plateau. For the selected K value, the estimated admixture proportions of the 10 runs

150   with the lowest DIC were averaged using CLUMPP 1.1.2 (Jakobsson & Rosemberg, 2007).

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Results

153   The final dataset consisted of a multi-locus genotype for 76 individuals at thirteen

154   microsatellite loci, with 9.8% of missing data. Micro-Checker detected the possible occurrence

155   of null alleles at locus *Cg14E1* in population 13 and at locus *Cg15F7* in population 11. Except

156   for these two populations, no significant deviation from the Hardy-Weinberg and linkage

157   equilibria was found after the Bonferroni correction was applied. Allelic richness and mean

158   expected heterozygosity estimates for each population are shown in Table 1. Population 13

159   (Sila Massif, central Calabria) and population 4 (Foresta della Lama, Tuscan-Emilian

160   Apennines) showed the highest values of genetic diversity, whereas the lowest values of

161   heterozygosity and allelic richness were observed in population 12 (Gargano, N Apulia).

162   DAPC identified K = 5 as the best clustering option, being the one with the lowest BIC value.

163   The optimization of the spline interpolation of the a-scores suggests to use only the first 11

164   principal components (accounting for 54,4% of the total variance) as the more informative ones

165   for the discriminant analysis. The inspection of the scatterplot resulting by the DAPC analysis

166   (Fig. 2) clearly identified five main genetic clusters, including individuals from: i) the Western

167   Alps (pops. 1-2), ii) the Northern and Central Apennines (pops. 3-11), iii) the Gargano

168   Promontory (pop. 12), iv) the Sila Plateau and Catena Costiera massif (central Calabria, pops.

169   13-14), and v) the Aspromonte Massif (southern Calabria, pop. 15).

170   The Bayesian clustering analyses carried out with TESS revealed a clear geographic

171   structuring of genetic variation, consistent with results of the DAPC analysis. The plots of DIC

172   values versus K values reached a plateau at K = 5 and only a minor decrease in the DIC values

173   was observed at higher K values. The spatial distribution of the five clusters had a clear

174   geographical structure: one is widespread in the Alps and, a lower frequency, in the Northern

175   Apennines; one is found from Northern to Central Apennines; one is restricted to the Gargano

176   Promontory region; one ranges from the Sila Plateau to the Catena Costiera, and one from the

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Catena Costiera to the Aspromonte Massif. Bar-plots showing the individual admixture proportions and pie-charts showing the average proportion of each cluster within each sampled population are given in Figure 3. Large genetic admixture is observed in individuals from Catena Costiera (pop. 14), as well as in those from northern (pop. 3-5) and central Apennines (pop. 6-11).

Discussion

The geographic structure of microsatellite variation supports the existence of five main genetic clusters within *Myodes glareolus* in the Italian peninsula, in contrast to the four lineages identified by the mitochondrial DNA. Indeed, previous studies identified a genetic lineage from Europe widespread in northern Italy, a distinct genetic lineage in central Italy, a slightly differentiated lineage restricted to the Apulian region, and a highly differentiated lineage restricted to the Calabrian region (Colangelo et al., 2012; Filipi et al., 2015). Our data support for the independent evolution of these lineages, and identified further sub-structuring within the Calabrian region. We found evidence for genetic differentiation between populations from Aspromonte and Sila, and for admixture between these two groups within the Catena Costiera. Genetic differentiation is also corroborated by some morphological distinctiveness between these populations, which has led to the description of distinct subspecies: *Myodes glareolus curcio* (von Lehmann, 1961) for the Sila Massif and *Myodes glareolus hallucalis* (Thomas, 1906) for the Aspromonte Massif (Amori et al., 2008a; Viro & Niethammer, 1982). Moreover, our results suggest strong genetic isolation between southern populations and the other Apennine populations and claim for considering the Calabrian and Gargano lineages as independent evolutionary and conservation units, deserving special attention in conservation planning.

Strong genetic differentiation and high intra-specific variation of Calabrian populations is a fairly common pattern in both animal and plant species (Bisconti et al., 2018; Canestrelli et al., 2006; Canestrelli, Cimmaruta & Nascetti, 2008; Canestrelli, Aloise, Cecchetti & Nascetti 2010; Canestrelli, Sacco & Nascetti, 2012; Chiocchio, Bisconti, Zampiglia, Nascetti & Canestrelli, 2017; Hewitt, 2011; Vega, Amori, Aloise, Cellini, Loy & Searle, 2010). The Calabrian region is a well-known glacial refugium for temperate species in peninsular Italy, and provides one of the best examples of highly sub-structured refugia, a scenario known as refugia-within-refugia (Gomez & Lunt, 2007). Indeed, for most of the temperate species studied to date in this area, the Calabrian region provided suitable albeit fragmented habitats through most of Pleistocene,

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allowing long-term survival of relict populations (Bisconti et al., 2018; Senczuk, Colangelo, De Simone, Aloise & Castiglia, 2017). Accordingly, the Early-Middle Pleistocene origin of Calabrian bank vole populations was suggested by both molecular dating and fossil evidence (Colangelo et al., 2012; Sala & Masini 2007). Moreover, palynological data support the expansion of Alpine forests in Calabria during the Early-Middle Pleistocene transition, as a consequence of particularly humid glacial cycles (Capraro et al., 2005; Palombo, Raia & Giovinazzo, 2005). The southward expansion of forest habitats promoted southward colonization of several forest and woodland-dwelling species, and might have promoted the establishment of the bank vole populations in Calabria, which probably remained trapped after the following shrinking of woodlands. The almost complete absence of admixture between the Calabrian cluster and those located more to the north, strongly suggests an ancient isolation of Calabrian populations.

On the other hand, the sub-structuring of the bank vole populations within the Calabrian region appears of more recent origin and it is likely related to the high palaeogeographic instability of this region. According to palaeogeographic reconstructions, the repeated glacio-eusthatic sea level oscillations of the Pleistocene caused repeated marine floods in the lowlands, turning the main mountain massifs into paleo-islands (Bonfiglio et al., 2002; Caloi, Malatesta & Palombo, 1989; Cucci, 2004; Ghisetti, 1979, 1981; Tansi, Muto, Critelli & Iovine, 2007; Tortorici, Monaco, Tansi & Cocina, 1995). The repeated insularization of Sila and Aspromonte massifs heavily affected population structure in most of the terrestrial fauna inhabiting these areas (Canestrelli et al., 2006, 2008, 2010, 2012). This historical process could also have triggered genetic (and morphological) differentiation in the bank vole populations, although caution should be adopted in the present case, due to the lack of molecular dating analyses.

The relatively high levels of genetic diversity and genetic admixture observed in these populations could be explained by a more recent secondary contact between the two gene pools. Under this scenario, the high level of genetic variation observed within the Calabrian Pleistocene refugia would be better explained by dynamic microevolutionary processes, which involve cycles of allopatric divergence and secondary contact, rather than by a prolonged demographic stability (Canestrelli et al., 2010).

Conversely, the population from the Gargano Promontory - described as a distinct subspecies *Myodes glareolus garganicus* (Hagen, 1958; see Amori et al., 2008a) - showed strong genetic divergence and low genetic diversity, probably as a consequence of a historical isolation and small population size, which favoured genetic erosion by drift. However, a role for the strong

243   anthropogenic impact on the bank vole's habitats, which affected this region during the last

244   decades (Parise & Pascali, 2003; Ladisa, Todorovic, & Liuzzi, 2010), cannot be excluded.

245   Populations from Alps and from north-central Apennine belong to two different genetic

246   clusters. According to previous studies (Deffontaine at al., 2005, Colangelo et al., 2012, Filipi

247   et al., 2015), the genetic cluster widespread in the Alps originated through a post-glacial

248   expansion from a western European refugium. On the contrary, the north-central Apennine

249   cluster is likely derived from an ancient Mediterranean lineage, originally widespread from

250   Balkans up to the Italian and Iberian peninsulas, and more recently isolated in an Apennine

251   refugium. The substantial levels of admixture between these two genetic clusters is consistent

252   with that showed by mtDNA data (Colangelo et al., 2012) and suggests good habitat

253   connectivity and high levels of gene flow throughout Central Apennine and Alps, at least after

254   the last glacial phase. Therefore, the genetic structure of bank vole populations in the Italian

255   peninsula, as inferred by both microsatellites and mitochondrial DNA markers, is consistent

256   with a scenario of independent evolution of multiple genetic lineages within distinct glacial

257   refugia. Interestingly, the postglacial range expansion of the lineages from Northern and Central

258   Italy was more extensive than that showed by the southern lineages, which appear still confined

259   to their refugial areas. However, we suggest caution in considering the geographic and genetic

260   isolation of Calabrian and Apulian populations. The paucity of observations from

261   geographically intermediate populations, as well as the lack of morphological and genetic data

262   does not allow to trace neither geographic nor genetic boundaries among these lineages. Further

263   research should be focused on the intermediate areas, in order to ascertain the presence of bank

264   vole populations and to estimate their genetic structure.

265   Our results have implications for the management of the bank vole populations in Southern

266   Italy. Due to its widespread distribution throughout most of Europe, *Myodes glareolus* is

267   currently categorized as *Least Concern* by the IUCN red list of threatened species, both at the

268   global (Amori et al., 2008b) and national (Rondinini, Battistoni, Peronace, & Teofli, 2013)

269   level. Nevertheless, we identified at least three unique evolutionarily significant units (Moritz,

270   1994) in Southern Italy, with narrow and endemic ranges. Assessments of their demographic

271   consistence, as well as of the current threats to their populations have to be planned in the near

272   future, in order to better integrate these endemic bank vole lineages into the regional strategy

273   of biodiversity conservation.

274   Concluding, this study highlights the importance of investigating species genetic structure

275   with a multi-marker approach, in order to find hidden diversity and fine-scale genetic

276   structuring also in supposedly well-known species. The analysis of bank vole genetic structure

in the Italian peninsula revealed more biological diversity than expected (see Colangelo et al., 2012), suggesting the need for thorough research also in other apparently well-known taxa. Finally, the evolutionary history of *Myodes glareolus* provides further evidence supporting the hypothesis that Pleistocene refugia were not so stable as previously thought, and that dynamic micro-evolutionary processes, triggered by the paleoclimatic and palaeogeographic instability of these areas, better explain the high levels of intraspecific diversity they harbour.

Acknowledgements

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Figure legends

Figure 1 Geographical localization of the 15 populations of *Myodes glareolus* sampled and analysed. Localities are numbered as in Table 1; dashed lines delimits the main geographic regions named in the text. The map was drawn using the software Canvas 11 (ACD Systems of America, Inc.). Photo: *Myodes glareolus* (from Rudmer Zwerver via Photodune.net).

Figure 2 Discriminant Analysis of Principal Components (DAPC). (a) Scatterplot resulting from the DAPC performed on *Myodes glareolus* genotypes from the Italian peninsula, using K=5 as clustering option; axes represent the first two principle components; the box shows the relative contribution of the eigenvalues to the discriminant analysis; clusters are named following their geographic distribution. (b) Optimal number of informative principal components, suggested by the optimization of the spline interpolation of the a-scores. (c) line chart showing the BIC values versus the number of genetic clusters (K) ranging from 1 to 15.

Figure 3 Genetic structure of Italian populations of *Myodes glareolus* at 13 microsatellite loci estimated using TESS. (**a**) The bar plot shows the admixture proportions of each individual for the five genetic clusters recovered. (**b**) The pie diagrams on the maps show the frequency distributions of each cluster among the populations; populations with only one individual were grouped with the nearest population as follow: (1,2), (3,4,5), (6,7), (8,9,10,11); the line chart shows the mean values of the DIC statistics (averaged over 100 runs) for the number of genetic clusters (K) ranging from 2 to 10. The map was drawn using the software Canvas 11 (ACD Systems of America, Inc.).

17

Tables

Table 1 Sample number, collecting locality, sample size (analyzed specimens), geographic coordinates, allelic richness (Ar), and expected heterozigosity (He).

Sample	Locality	N	Latitude	Longitude	Ar	He
1	Gressoney	1	45.76902	7.82552	-	-
2	Aosta	11	45.73644	7.31298	2.302	0.594
3	Cantagallo	1	44.03176	11.05226		-
4	Foresta della Lama	10	43.81710	11.81270	2.869	0.718
5	Passignano sul Trasimeno	1	43.21675	12.13462	-	-
6	Tolfa	1	42.14959	11.93806	-	-
7	Settebagni	1	42.13705	12.53497	-	-
8	Civitella del Tronto	1	42.79336	13.67409	-	-
9	Montereale	3	42.49670	13.19747	2.564	0.564
10	L'Aquila	4	42.35686	13.38911	2.435	0.555
11	Majella Massif	6	42.14480	14.07530	2.584	0.605
12	Gargano Promontory	11	41.75540	16.01180	1.757	0.417
13	Sila Plateau	14	39.37211	16.57618	2.687	0.724
14	Catena Costiera	3	39.27578	16.09198	2.687	0.584
15	Aspromonte Massif	8	38.15910	15.92060	2.357	0.599
		76				

Table 2 Marker name, repeat motif, and colour dye of the thirteen microsatellite loci assembled in three multiplex.

Multiplex	Marker	Repeat	Dye
A	Cg4F9	$(CA)_{20}$	FAM
	Cg14E1	$(CT)_{20}$	FAM
	Cg17A7	$(ATGT)_9$	HEX
	Cg15F7	$(CT)_{20}$	HEX
	Cg3F12	$(GT)_{16}$	TAMRA
	Cg3A8	$(GT)_{21}$	TAMRA
B	Cg12A7	$(GA)_{21}$	FAM
	Cg17E9	$(GTAT)_9$	FAM
	Cg13C12	$(CT)_{21}$	HEX
C	Cg13G2	$(GT)_{14}$	FAM
	Cg10H1	$(GACA)_7$	FAM
	Cg10A11	$(GT)_{15}$	HEX
	Cg6A1	$(CAT)_{18}$	HEX

18