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1 **Spatial mixing of mitochondrial lineages and greater genetic diversity in some invasive populations**

2 **of the American mink (*Neovison vison*) compared to native populations**

3

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15

16

17   **Abstract**

18

19   The genetic characteristics of introduced populations have a relevant impact on their ability to

20   establish and spread. The American mink (*Neovison vison*), native to North America, is an important

21   invasive species in the Iberian Peninsula. Here, we used mitochondrial DNA sequences data to investigate

22   the genetic diversity and phylogeographic structure of invasive vs. native populations of this species. We

23   also evaluated whether genetic diversity in invasive populations could be explained by the genetic

24   characteristics of the native sources from which they derived. Phylogenetic analysis revealed two major

25   lineages in the native range, which indicated a clear separation between western and eastern populations.

26   On the contrary, we found no evidence of genetic structure in the invasive range. This was probably the

27   result of the diverse origins of the released specimens and the rapid expansion and encounters of the

28   introduced populations. We detected spatial mixing of both North American lineages in several sampling

29   localities of the north central area of the Iberian Peninsula, giving rise to high levels of genetic diversity

30   in some areas compared to North American populations. This could potentially lead to higher fitness of

31   these individuals and thus increase the population viability and invasiveness of this species. These results

32   point to the need to better study the populations in which lineages mix and, if necessary, intensify control

33   efforts in them.

34

35   **Keywords:** American mink, mitochondrial genes, genetic diversity, native range, Iberian Peninsula.

1. Introduction

Identifying the biological attributes of successful invaders is among the most pressing questions yet to be answered (Sol et al. 2012). Although there is evidence that species differ in their invasion potential, controversy exits about the characteristics that differentiate successful and unsuccessful invaders (Blackburn et al. 2009). Other studies of introduced species show that genetics might play an integral role in the success of an invasion, and they suggest that this role is determined by the quality of the genetic variation introduced, rather than the total quantity of genetic variation (see Dlugosch et al. 2015). The genetic diversity of founding populations, along with potential increase in genetic diversity from subsequent introductions, may be important to the successful establishment and spread of introduced species (Suarez and Tsutsui 2008; Sol et al. 2012). Nevertheless, there is no consensus on whether invaders stemming from multiple native origins are more successful than those from single populations, or whether demographic bottlenecks may limit the invasion success of a species (Edelaar et al. 2015; Estoup 2016).

Overall, introduced populations lose genetic variation relative to their source populations (Dlugosch and Parker 2008). However, numerous introduction events and a greater number of source populations may buffer against such genetic losses (Kolbe et al. 2004; Dlugosch and Parker 2008; Uller and Leimu 2011). In fact, in some cases successful invaders show large increases in genetic diversity in the introduced range, apparently caused by mixing of populations from different source regions (Genton et al. 2005; Novak and Mack 1993). Therefore, it is crucial to obtain information on the phylogeographic structure and genetic diversity throughout the native and introduced ranges to understand the factors affecting genetic diversity during invasion (e.g. multiple introduction events or admixture levels among populations) and the interaction between genetic diversity and invasive potential (Edelaar et al. 2015).

The American mink (*Neovison vison*) is a successful invader with a native range restricted to North America and with invasive populations distributed across Europe, Asia and South America (Bonesi and Palazón 2007; Dunstone 1993). In Europe, this species was introduced for commercial fur farming during the 20th century. Populations established by escaped individuals are now naturalised in fresh water and coastal ecosystems (Bonesi and Palazón 2007), with detrimental consequences for the abundance of many native species of prey and competitors of conservation importance (Bonesi et al. 2004; Harrington et al. 2009; Macdonald and Harrington 2003; Melero et al. 2012a), including critically endangered species such as the European mink (*Mustela lutreola*) (Maran et al. 2016) and the Pyrenean desman (*Galemys pyrenaicus*) (Fernandes et al. 2008). Fur farms were established in many countries and harboured mink stocks from different parts of their native range. Because of the large scale and long-term nature of this farming, the number of escapees, which started to breed in the wild and became the founder individuals of feral populations, was very high (Bonesi and Palazón 2007). In Spain, mink farming started at the end of the 1950s and the number of farms reached a peak in the 1980s with about 400 farms concentrated mainly in the North West (Galicia) (Ruiz-Olmo et al. 1997). Today, feral mink populations are widely distributed across almost all of the north and centre of the Iberian Peninsula. During the last few decades, these populations experienced a rapid expansion and increased the number of nuclei in almost every

colonised area (Ruiz-Olmo et al. 1997; Santulli 2014). Although the number of mink farms in Spain was considerably reduced at the end of the 1980s, mink escapes from the last active farms are still ongoing (Bonesi and Palazón 2007).

In Europe, there is genetic information on feral American mink populations from several countries. Previous studies in Spain using microsatellite data determined that American mink populations from disjunct geographical areas appeared differentiated and not yet connected by gene flow (Lecis et al. 2008). In Scotland, Zalewski et al. (2009) and Fraser et al. (2013) found that landscape features restricted gene flow within this species. Other studies in Poland found that multiple introductions determine the genetic structure of American mink populations (Zalewski et al. 2010) and that these populations exhibit high mitochondrial genetic diversity (Zalewski et al. 2011). A study in France using microsatellite data suggested recent admixture between populations that had recently encountered each other (Bifolchi et al. 2010). Similarly, in Sweden it was found, also using microsatellite data, that genetic diversity decreased over six consecutive years of mink culling as part of a population control programme (Zalewski et al. 2016).

Despite the importance of understanding the genetic diversity within the native populations for investigating the evolutionary dynamics of invasions, no phylogeographic study has been performed so far in the North American native range of the American mink. Therefore, many genetic studies performed thus far are difficult to interpret. We used mitochondrial DNA (mtDNA) sequence data from populations in the native range to elucidate whether invasive populations in the Iberian Peninsula have single or multiple origins from the North American sources. We aimed to determine the phylogeographic structure of native and invasive populations, and to evaluate whether mitochondrial genetic diversity observed in established invasive populations can be explained by genetic characteristics of native populations. We examined whether the genetic variability of the Iberian population represented only a small part of that in its native range, as expected for many invasive species or, due to the introduction history and particular origin of these populations, this genetic variability was larger. Overall, the study aims to provide recommendations for mink control in the Iberian Peninsula with respect to determining key control areas.

2. Materials and methods

2.1 Sample collection

We used hair and tissue samples from invasive populations provided by different Autonomous Communities of Spain. These samples were collected during population control operations conducted by the local administrations between 2007 and 2016 as part of eradication programs and were conserved in tubes containing absolute ethanol (ESM Appendix: Table S1). A total of 105 samples from Catalonia (16), La Rioja (15), Galicia (12), Basque Country (20), Navarra (11), Castellon (7) and Castilla-Leon (24) were included in the study.

117   Moreover, tissue samples of 21 specimens from North America (ESM Appendix: Table S2) were

118   obtained from different biological collections: University of Alaska Museum (12), Museum of

119   Southwester Biology (5) and Cornell University Museum of Vertebrates (4).

120

121   **2.2 DNA extraction, PCR and sequencing of mitochondrial DNA**

122

123   We extracted DNA using the QIAGEN DNeasy Blood and Tissue Kit, following the manufacturer's

124   instructions, in a final elution volume of 100 μl. These extractions were carried out in a separated UV-

125   irradiated area with dedicated equipment. Samples were incubated in a lysis buffer with proteinase K at

126   56°C overnight to ensure maximum tissue lysis.

127

128   For each sample, we amplified a 573 bp fragment of the 5′ distal part of the mitochondrial DNA

129   control region (D-loop). For this purpose, we designed novel primers using complete mitochondrial

130   sequences of several mustelids: Neovison_tRNA_pro (5'-ATC AGC ACC CAA AGC TGA CAT TCT

131   A-3') and Neovison_Dloop_556r (5'-TGT GTA TGT CCT GTG ACC ATT GAC T-3'). PCR reactions

132   were set up in a dedicated PCR clean room that is physically separated from post-PCR working areas and

133   regularly decontaminated by UV-irradiation. PCR reactions were performed in a final volume of 25 μl,

134   containing 2–4 μl of genomic DNA, 1 μM of each primer, 0.2 mM dNTPs, 0.75 units of Promega GoTaq

135   DNA polymerase and 17.5 μM of bovine serum albumin under the following conditions: an initial

136   denaturation of 2 min at 95°C, followed by 35 cycles of denaturation (30 s at 95°C), annealing (30 s at

137   54°C) and extension (30 s at 72°C). PCR products were visualised by electrophoresis in a 1% agarose

138   SYBR-Safe (Invitrogen) stained gel.

139

140   PCR products were purified using ExoSAP-It (Affymetrix), and both strands were sequenced using

141   the original PCR primers at Macrogen Europe (Amsterdam, The Netherlands). Sequences were inspected,

142   trimmed and assembled using Geneious Pro (Biomatters Ltd.). All sequences obtained in this study were

143   deposited in European Nucleotide Archive/GenBank under accession numbers LT854061-LT854186.

144

145   **2.3 Phylogenetic and genetic diversity analyses**

146

147   Consensus sequences were aligned using MAFFT version 7.130 (Katoh et al. 2002) with default

148   settings. Due to the presence of several gaps and ambiguous positions in a very specific region of the

149   alignment, we manually removed positions 92 to 110, thus leaving 554 bp in the alignment.

150

151   A maximum likelihood phylogenetic tree was calculated with RAxML version 8.0 (Stamatakis 2014)

152   using a general time reversible substitution model and rate heterogeneity modelled with a gamma

153   distribution plus a proportion of invariable sites, as recommended by the program. From this tree, a

154   haplotype genealogy was generated using Haploviewer 1.0 (Salzburger et al. 2011).

155

156   The nucleotide diversity (π) was estimated using the R package PEGAS (Population and Evolutionary

157 Genetics Analysis System, Paradis 2010). To analyse the variation in nucleotide diversity across the
158 Iberian Peninsula, we used a visualization method that does not require the pre-definition of populations.
159 The nucleotide diversity was estimated at each sampling location by using all samples present within 30
160 km of the location. This allowed the estimation of genetic diversity from a sufficient number of samples
161 at each point, yet the resolution was sufficient to distinguish regional differences in genetic diversity.
162 QGIS (http://qgis.osgeo.org) was used to plot the nucleotide diversity in the map. In the case of the North
163 American native range, it was not possible to apply this analysis due to the limited number of samples.
164 Therefore, we selected three areas with a sufficient number of samples: North West (British Columbia,
165 six samples), South East (Texas, three samples) and East (New York, four samples), in order to estimate
166 the nucleotide diversity in these specific areas.
167
168 **3. Results**
169
170 **3.1 Mitochondrial phylogeographic analysis**
171
172 A total of 16 different haplotypes of the mitochondrial control region were detected in 126 American
173 mink, 11 of which were recorded in North America and 7 in the Iberian Peninsula (ESM Appendix: Fig.
174 S1). The maximum likelihood phylogenetic tree showed two large clades found throughout western and
175 eastern North America, respectively (Fig. 1). The haplotype genealogy reconstructed from the maximum
176 likelihood tree revealed the same two large groups (Fig. 2a). As indicated in this genealogy, two
177 mutations unambiguously differentiate both clades. The map of samples clearly shows the geographical
178 separation of the two clades in North America (Fig. 2b). In the Iberian Peninsula, we found more samples
179 belonging to the western than to the eastern clade of North America (68 vs. 37 samples, respectively).
180 However, samples belonging to both clades were detected together in four regions of the north central
181 area of the Iberian Peninsula: Basque Country, La Rioja, Navarra and Avila (South of Castilla-Leon) (Fig.
182 2c).
183
184 In addition, the phylogeographic structure was also different at a finer scale in the native and invasive
185 ranges. While there was evidence of a geographical pattern for the individuals collected from the native
186 range, with individual haplotypes concentrated in a single locality or close localities (Fig. 1), many
187 haplotypes were widely dispersed in the Iberian Peninsula (Fig. 1, Fig. S1), showing that haplotypes are
188 not informative with regards to the geographical origin of any sample.
189
190 **3.2 Mitochondrial genetic diversity**
191
192 The global genetic diversity of the American mink estimated with the control region was relatively
193 low for all samples, with a value of nucleotide diversity of $\pi = 0.68\%$, whereas the nucleotide diversity
194 for North America and the Iberian Peninsula was 0.78% and 0.64%, respectively (haplotype diversity was
195 0.81, 0.92 and 0.76, respectively). However, the nucleotide diversity varied considerably when different
196 localities and populations were considered separately. Among the three areas of the native range in which

6

197 π was calculated separately, the population of British Columbia showed the highest nucleotide diversity
198 (0.53%) whereas New York (0.12%) and Texas (0%) registered lower values. In agreement with the
199 spatial mixing of clades in the central areas of the Iberian Peninsula, the map derived from the genetic
200 diversity values of the samples around each locality clearly showed maximum levels of genetic diversity
201 in these regions (Fig. 3). When values of several sampling localities were averaged, the Basque
202 population concentrated the highest nucleotide diversity (0.61%), followed by Avila (0.50%) and La
203 Rioja (0.25%). From these areas, genetic diversity decreased towards the remainder of the mink
204 distribution, reaching values of 0% in some areas.
205
206 **4. Discussion**
207
208   Our results show that the establishment of the invasive American mink population in the Iberian
209 Peninsula is the product of the introduction of individuals from the two main genetic lineages found in
210 western and eastern North America. In the invasive range, these lineages registered a complete lack of
211 phylogeographic structure. This was probably the result of the additive effect of multiple continuous
212 introductions, and the rapid expansion and encounters of the introduced populations. Overall, genetic
213 diversity was lower in the introduced than in the native populations, although in some introduced
214 populations diversity was higher to any one native population, at least with respect to the reduced native
215 area analysed so far. These results provide evidence for the potential of introduced populations to
216 maintain or increase the genetic diversity.
217
218 **4.1 Phylogeographic patterns**
219
220   Phylogenetic analysis revealed two large native phylogeographic lineages, which indicated a clear
221 separation between the western and eastern populations in North America. Therefore, the relationship
222 between the mtDNA haplotypes and the location from which they were sampled provides evidence of
223 phylogeographic structure among populations of American mink in their native range. Unfortunately, the
224 lack of information about sequence data of mtDNA of the American mink in North America did not allow
225 our results to be compared with those of similar studies. Other studies of plant and animal species from
226 North America have illustrated a complex phylogeographic history of the biota of this region (Shafer et
227 al. 2010; Soltis et al. 2006). The data currently available suggest that vicariance, dispersal and the
228 existence of refugia have worked in concert to produce the species' distribution and genetic divergence
229 patterns of the region (Brunsfeld et al. 2001). Future research focused on filling the gaps by sampling at
230 locations between the two clades seen in this work is required to achieve a deeper understanding of the
231 evolutionary history of the American mink in its native range.
232
233   The pattern of spatial structure recorded in North America allowed for a more precise identification of
234 the origin of invasive populations in the Iberian Peninsula. Our analysis revealed that there were
235 introductions from both western and eastern regions in North America. Additionally, the distribution of
236 the invasive haplotypes in the Iberian Peninsula suggests that at least the north central area of the Iberian

7

237   Peninsula (populations of the Basque Country, La Rioja, Navarra and Avila) experienced multiple

238   introductions from both regions of North America, while the western and eastern Iberian populations

239   (Galicia, Catalonia and Castellon) originated from introductions from western North America, contrary to

240   what was previously believed (Palazón et al. 1997). This situation most likely occurred as a result of the

241   multiple origins of mink from different farms and the fast range expansion of the occupied area since

242   their introduction in the 1960s-1970s. It is also likely that farms received founder individuals from

243   different source populations in the native range.

244

245   The occurrence of the two main mitochondrial lineages in the north central area of the Iberian

246   Peninsula suggests that the intermixing of individuals of both lineages may potentially take place.

247   However, it will be necessary to perform analysis with multiple genomic markers in the future to

248   determine whether individuals belonging to these two lineages effectively interbreed and, if so, to

249   estimate admixture levels in these populations (Pritchard et al. 2000). It is also important to note that, due

250   to the limited information derived from the mitochondrial DNA, which only reflects the maternal lineage,

251   the existence of some admixture levels in other Iberian populations cannot be discarded until genomic

252   analyses are performed. In fact, a previous study in the Iberian Peninsula based on microsatellites showed

253   that the highest admixture levels between populations were found in the Basque Country population,

254   which in principle is consistent with our results (Lecis et al. 2008). However, since no native specimens

255   were available in that study, it was not possible to determine how the identified populations were related

256   to the source populations. This confirms that it is crucial to obtain samples from the native range to shed

257   light on the relationship between invasive and native populations and to identify potential source

258   populations.

259

260   **4.2 Genetic diversity in the native versus invasive range**

261

262   Based on mitochondrial DNA control region sequence data, our results provide evidence that the

263   introduced American mink populations in the Iberian Peninsula have overall less genetic diversity than

264   native mink populations in North America. These results are in agreement a priori with the "genetic

265   paradox" (Allendorf and Lundquist 2003; Estoup et al. 2016), which predicts that low genetic variability

266   can be expected in invasive species because of the loss of rare alleles, as a result of the founder effect and

267   population bottlenecks (Allendorf and Lundquist 2003; Grapputo et al. 2005). Nevertheless, when

268   analysed at the population level within each range, nucleotide diversity in the population of the Basque

269   Country and La Rioja in the Iberian Peninsula was found to be greater than that recorded for North

270   American populations. The pattern registered here is consistent with other studies (e.g. Edelaar et al.

271   2015; Genton et al. 2005; Roman and Darling 2007), providing another example of an invasive species

272   retaining genetic diversity. Similarly, previous studies have demonstrated that the genetic diversity of

273   invasive populations in their new range may be increased through the admixture of lineages from multiple

274   native populations (Keller and Taylor 2010; Kolbe et al. 2008). As already indicated, the pattern observed

275   in the Iberian Peninsula could be the result of multiple introductions of mink deriving from different local

276   lineages of North America, their rapid expansion, and the encounter of lineages in the north central area

of the Iberian Peninsula that may potentially hybridise. However, we cannot discard that cross-breeding of the different North American subspecies or populations took place within farms (Belliveau et al. 1999; Dunstone 1993).

High values of genetic diversity have also been registered in some mink population of Poland (Zalewski et al. 2011). In this case, mean π for all feral mink analysed was 0.94%, with values for different populations ranging between 0 and 1.41%. Although the mitochondrial control region fragment used in that work was different to ours and a direct comparison cannot be performed, the values found in some populations in Poland were much higher than in others. It is therefore likely that the same processes suggested for the Iberian Peninsula (i.e. multiple introductions, rapid expansion and encounters of individuals of different lineages) can be applied to other areas with invasive populations of American mink.

4.3 Implications for management

This study provides an understanding of the genetic structure and diversity of the populations of the American mink in the Iberian Peninsula, which can be used to identify key control areas. Thus, one way to improve the effectiveness of management programmes of an invasive species such as the American mink is by incorporating molecular-based information into these programs (Hampton et al. 2004). In this regard, the current spatial mixing of individuals from two different native lineages in the north central area of the Iberian Peninsula (Basque Country, La Rioja, Navarra and Avila populations) and the potential interbreeding between such lineages that may take place could translate, among other outcomes, into a higher fitness in these individuals (e.g. Vandewoestijne et al. 2008). In fact, previous studies have found a positive correlation between genetic diversity and colonization success as measured by the reproduction rate (e.g. Crawford and Whitney 2010). Interestingly, Melero et al. (2012b) found that mink of the Basque Country were bigger in size than mink of the rest of the Iberian Peninsula. According to Melero et al. (2012b), this phenotypic variation could be due to a mixing of individuals from different origins, among other possibilities. If the link between spatial mixing of mitochondrial lineages, genome admixture and higher fitness is demonstrated for some populations, then higher efforts to control and eradicate the American mink should be undertaken in such populations in order to impede or minimise their expansion.

Current controls of the American mink in the Iberian Peninsula focus on reducing their effects on ecosystems and biodiversity (i.e., areas with local populations of threatened species, wetlands where they can cause high mortalities in waterfowl and protected natural areas, etc.). In this regard, it is important to consider that the areas of the Iberian Peninsula with presence of both American mink lineages coincide with critical areas for the conservation of two of the most threatened mammals of the Iberian Peninsula, the European mink and the Pyrenean desman. The American mink compete successfully for habitat and prey with the European mink (Sidorovich et al. 1999; Sidorovich and MacDonald 2001), whose population of the North of Spain and South West of France is highly isolated from the rest of the

distribution (Michaux et al. 2005). On the other hand, the populations of Pyrenean desman of the Central System have experienced a strong reduction in the last few years and are threatened with extinction. Although the reasons for this decline are not well known, predation by the American mink may have contributed to it (Fernandes et al. 2008). Thus, the presence of the two native lineages of the American mink in the areas with these threatened species should be taken into account in future conservation plans due the putative higher expansive potential of the American mink in them.

The results of this work are in line with previous studies suggesting that mink control should also be directed to minimise the contact between genetically differentiated populations (Bifolchi et al. 2010; Fraser et al. 2013; Lecis et al. 2008; Robertson and Gemmell 2004), and so counteract a possible increase in mink fecundity and dispersal (Melero et al. 2015; Oliver et al. 2016). The present study has shown how molecular-based information obtained from the invasive range, together with that from the native range, was critical to characterise the sources from which the Iberian populations of the American mink originated. Further studies are now necessary to obtain more detailed information, particularly in areas where admixture of lineages may potentially occur, to help prioritise efforts to control and manage this invasive species.

5. Acknowledgements

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509   **Fig. 1** Maximum-likelihood phylogenetic tree derived from the partial mitochondrial control region
510   sequences for 126 American mink, with eastern and western clades indicated. The number of individuals
511   per locality is shown in brackets. Sequences from the native range are underlined. The scale indicates
512   number of substitutions per position.
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515   **Fig. 2** Phylogeographic analysis of the mitochondrial control region sequences of *Neovison vison*. (A)
516   Haplotype genealogy based on the maximum-likelihood tree. Circle sizes are proportional to the
517   haplotype frequency and small filled circles are inferred haplotypes not present in the sample. (B, C) Map
518   of native (B) and invasive (C) ranges showing the 126 samples of *N. vison* used in this study. Red and
519   blue colours represent the eastern and western clades, respectively. Grey areas represent the original
520   distribution of *N. vison* downloaded from the IUCN Red List of Threatened Species website (Reid et al.
521   2016) (B) and the occupancy area of mink in the Peninsula (MAGRAMA, 2013) (C). AL: Alaska, BC:
522   British Columbia, MT: Montana, WY: Wyoming, TX: Texas, NY: New York, GA: Galicia, CL: Castilla-
523   Leon, AV: Avila, LR: La Rioja, BC: Basque Country, NAV: Navarra, CAS: Castellon, CAT: Catalonia
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526   **Fig. 3** Map plotting genetic diversity (π) in different sampling localities of *Neovison vison* in the Iberian
527   Peninsula. Colours of the sample points indicate the nucleotide diversity according to the provided scale.
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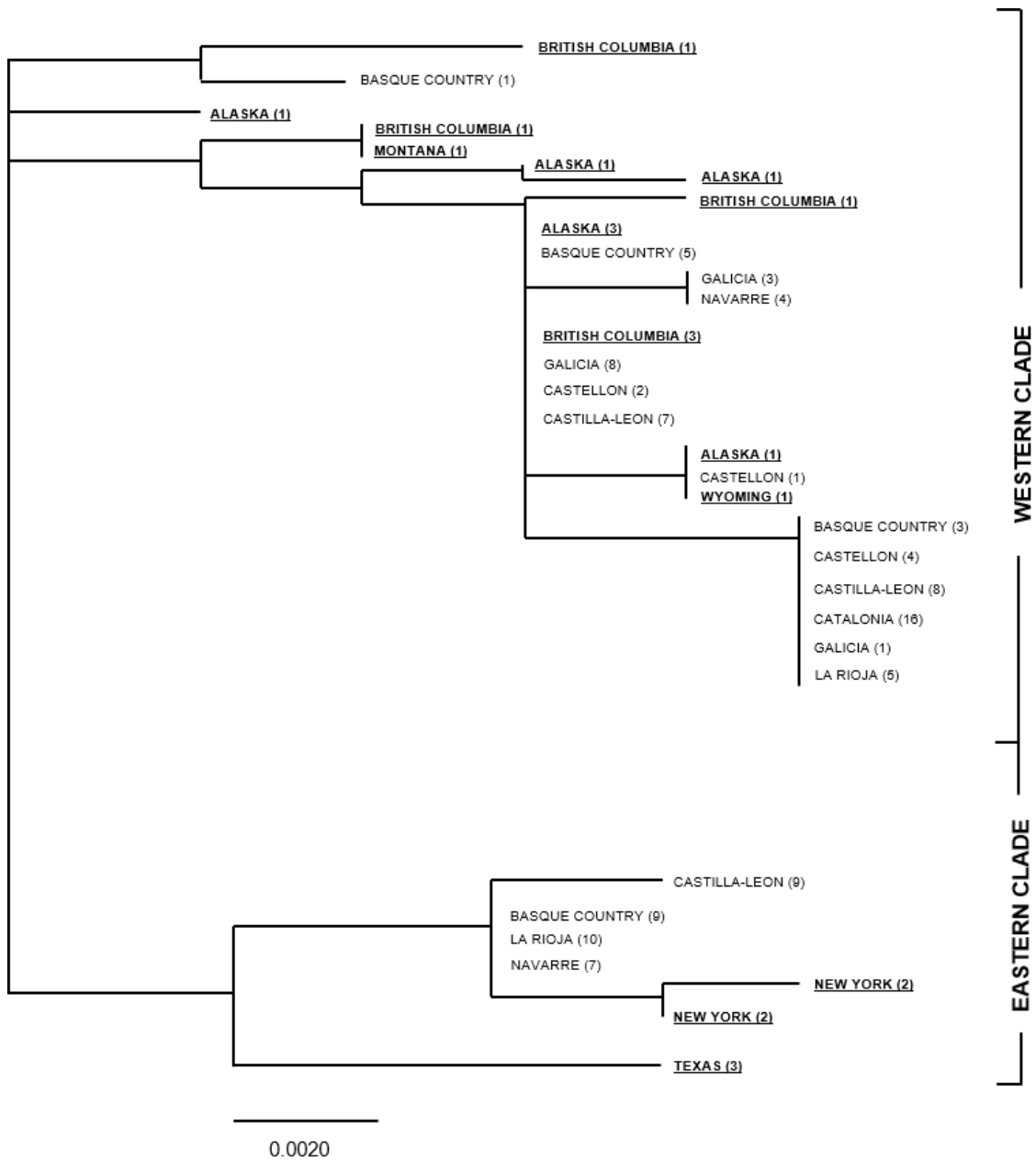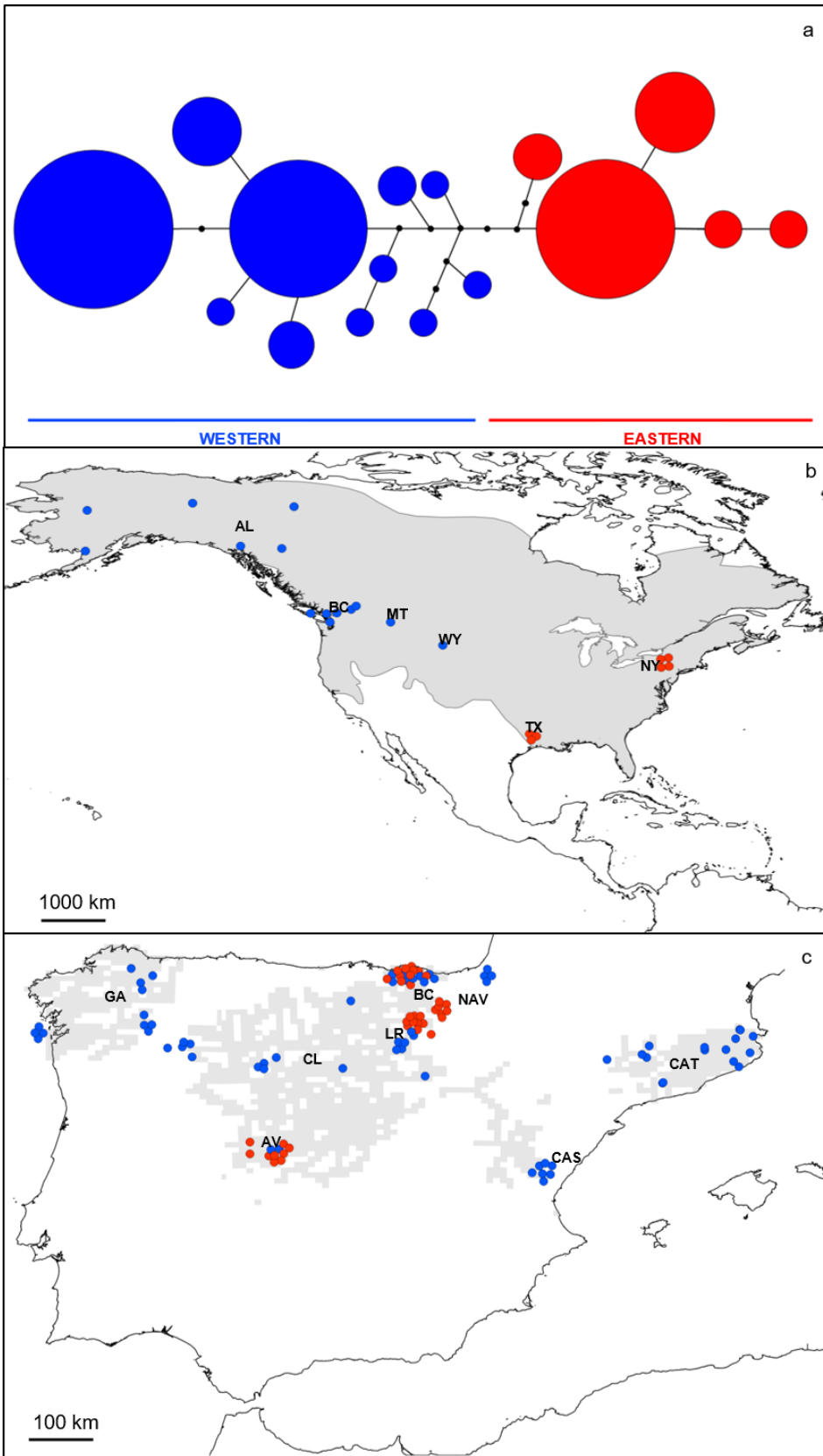532
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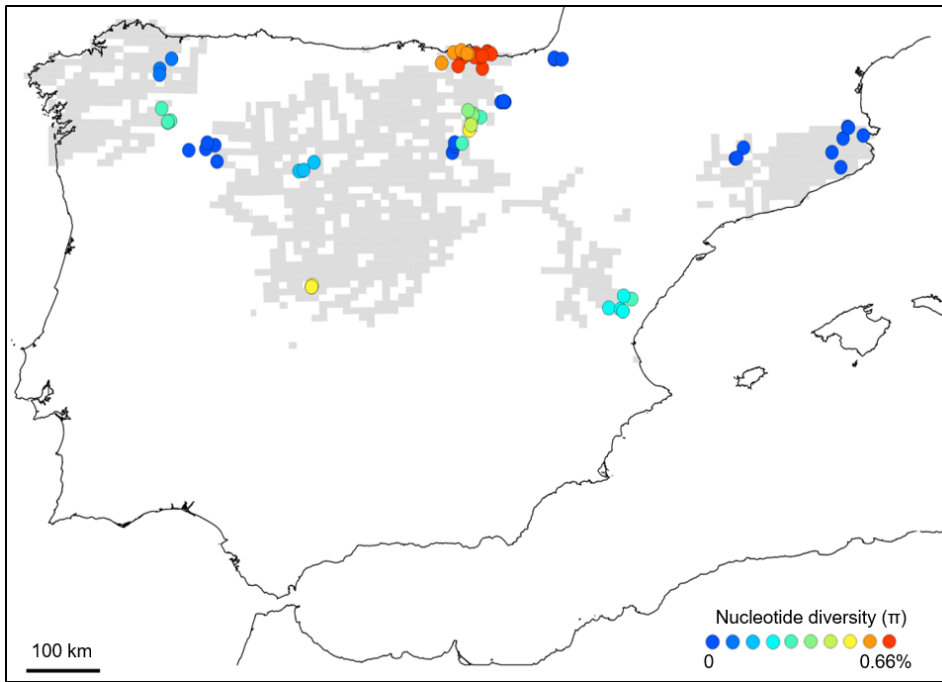540
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BRITISH COLUMBIA (1)
BASQUE COUNTRY (1)
ALASKA (1)
BRITISH COLUMBIA (1)
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BRITISH COLUMBIA (1)
ALASKA (3)
BASQUE COUNTRY (5)
GALICIA (3)
NAVARRE (4)
BRITISH COLUMBIA (3)
GALICIA (8)
CASTELLON (2)
CASTILLA-LEON (7)
ALASKA (1)
CASTELLON (1)
WYOMING (1)
BASQUE COUNTRY (3)
CASTELLON (4)
CASTILLA-LEON (8)
CATALONIA (16)
GALICIA (1)
LA RIOJA (5)
CASTILLA-LEON (9)
BASQUE COUNTRY (9)
LA RIOJA (10)
NAVARRE (7)
NEW YORK (2)
NEW YORK (2)
TEXAS (3)

WESTERN CLADE

EASTERN CLADE

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562   Fig. 2
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Fig. 3