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ORIGINAL PAPER

Genetic variability of central–western European pine marten (*Martes martes*) populations

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Abstract Recent studies highlighted the potential role of cryptic glacial refugia for temperate taxa in Europe beyond the Mediterranean peninsulas. To further investigate phylogeographic features of the European pine marten (*Martes martes*) in previously identified cryptic refugia located in central–western Europe, we analysed the hyper-variable diagnostic fragment of the mitochondrial control region in a total of 134 specimens, allowing for reliable comparisons with previous genetic studies of the species. We included samples from eight different European countries in central–western Europe (Belgium, France, Luxembourg and the Netherlands), in south–western Europe (Spain), in north–central Europe (Denmark) and in central Europe (Germany and Poland). The sequences collapsed in 17 haplotypes, which allowed us to

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determine the genetic composition of the pine marten populations throughout central–western Europe. Overall, our results showed that the population genetic variation, estimated by the standardised haplotype diversity, was high ($0.400 \leq Hs \leq 0.762$), and it was considerably higher in Germany (0.762) and the Netherlands (0.722) compared to the other countries. The nucleotide diversity was relatively low ($0.002 \leq \pi \leq 0.016$) even in Germany and the Netherlands (0.016 and 0.014, respectively), suggesting relatively small, long-term effective population sizes or severe bottlenecks. Out of the 17 haplotypes found in our study area, 13 were unique and limited to a single country: one in Denmark, one in Spain, four in Poland and seven in the Netherlands. The pairwise genetic distance ranged from 0.001 to 0.032 and did not show any evident correlation with the geographic distances between the populations. A genealogical relationship network was constructed, which provided evidence for a recent origin of many of the unique haplotypes. Approximately 82 % of the samples analysed in this study belonged to haplotypes grouped into a previously identified central–northern European phylogroup of the species. Our results support previous findings, indicating low contribution of southern refugial populations to the postglacial recolonization of central–western Europe and a predominant contribution of pine marten populations that survived the Last Glacial Maxima in cryptic northern refugia.

Keywords mtDNA · Haplotype diversity · Pine marten

Introduction

The Pleistocene ice age is thought to have had a major impact on the current flora and fauna in Europe (Avise 2000; Hewitt 1999, 2004) where extensive range dynamics and massive community rearrangement took place following climate transitions. The identification of refugia during the Last Glacial Maximum (LGM) (c. 19–26 kyr bp; Clark et al. 2009) has been a topic of active research for the last few decades (Hewitt 1999, 2004; Stewart et al. 2010). Recent evidence indicates that many temperate species have maintained populations at considerably higher latitudes than previously assumed (i.e. beyond the traditional Pleistocene refugia located at Mediterranean peninsulas). These simultaneously served as sources for the subsequent re-expansion of confined populations (Stewart and Lister 2001; Stewart et al. 2010; Bhagwat and Willis 2008; Provan and Bennett 2008; Schmitt and Varga 2012). Such locations were recently referred to as 'cryptic refugia' (Stewart and Lister 2001; Stewart et al. 2010), a term that nicely conveys the difficulties in inferring the past existence of these previously overlooked refugial populations. However, the existence and location of these refugia still remain uncertain for many species and little is known about the potential implications these refugia have in the intraspecific genetic variability patterns currently present in Europe (Stewart et al. 2010).

In this study, we focus on the European pine marten (*Martes martes*), a mammal species widely distributed throughout Europe and western Asia, from northern Portugal to western Siberia (Proulx et al. 2004; Kranz et al. 2008). It is a habitat specialist that is mainly confined to mature deciduous and coniferous forests (Pereboom et al. 2008). It has a limited dispersal ability compared to other mustelids (Bright 2000) and a slow reproduction rate (Zalewski and Jędrzejewski 2006), potentially rendering it vulnerable to habitat changes (Bright 2000).

The pine marten has a long history of hunting, resulting in a serious decline in the number of individuals and possible population bottlenecks (Proulx et al. 2004; Kranz et al. 2008). The species is either threatened or scarce in many countries where forest habitat loss and fragmentation are major threats (Kranz et al. 2008). Through this process, larger populations will get divided into smaller subpopulations, chances of inbreeding increase and a loss of genetic diversity due to drift will be the result.

Several studies already tried to get more insight into the phylogenetic history and genetic variability of the pine marten (see Schwartz et al. 2012 for a thorough review). Davison et al. (2001) analysed two separate mitochondrial DNA (mtDNA) fragments, control region and cytochrome *b* of DNA samples of current populations in Europe. They suggest that all populations in central and northern Europe descended from one single glacial *refugium*, but this study has overlooked part of the existing spatial variation (Schwartz et al. 2012). Based on fossil records found throughout Europe, Sommer and Bennecke (2004) and Sommer and Nadachowski (2006) concluded that pine martens were restricted to three refugia during the late glacial period: Iberia (Spain and Portugal), Italy and interestingly, a central European refugium, potentially located in the Carpathians/Moldova region. More recently, Ruiz-Gonzalez et al. (2013) analysed the final part of the cytochrome *b* gene, *tRNAPro*, *tRNAThr*, the control region (d-loop) and the initial part of *12S* ribosomal RNA (rRNA). They found that pine marten populations are split into three major assemblages: Mediterranean, central–northern European and Fennoscandian–Russian clades, showing a north–south pattern of spatial segregation, with some area of overlap and genetic admixture. This study highlighted a mixed pattern of recolonization of northern Europe from both Mediterranean and non-Mediterranean refugia and confirmed that most of Europe was colonized by the central–northern European phylogroup, which survived the last glaciations in northern cryptic refugia. This was previously suggested by palaeontological studies (Sommer and Benecke 2004; Sommer and Nadachowski 2006). In spite of the fact that previous studies have provided important insights

about the species' phylogeographic patterns, further research is needed in order to get more detailed information about genetic variability patterns of the species throughout its whole distributional range (Schwartz et al. 2012), with special emphasis on populations that potentially survived in cryptic northern glacial refugia in continental Europe (Ruiz-Gonzalez et al. 2013). When interpreting the reasons for the present genetic structure of the pine marten populations, we should also bear in mind that Davison et al. (2001) reported evidence for historic introgression of *M. martes* with the sable (*M. zibellina*). This pattern was further confirmed by Ruiz-Gonzalez et al. (2013) that identified a divergent phylogroup in Fennoscandia–Russia region, which included specimens from both *Martes* species.

Our main objectives are (1) to determine genetic variation of the populations still present in western and central Europe that potentially survived in cryptic refugia in Europe during the LGM and (2) to estimate the degree of genetic differentiation between these populations. To answer these questions, we analysed the hyper-variable fragment of the mitochondrial control region, which is a well-used tool in phylogenetic studies (Moritz et al. 1987; Larson et al. 2002). It is sufficiently informative to identify the main haplotypes and haplogroups described in previous pine marten studies (Davison et al. 2001; Pertoldi et al. 2008a; Jordan et al. 2012; Ruiz-Gonzalez et al. 2013) in order to provide new insights about the phylogeographic patterns and genetic variability of the species in previously suggested cryptic refugia in Europe (Ruiz-Gonzalez et al. 2013).

Material and methods

Study samples

All DNA samples were collected from traffic casualties and samples consisted of tongue tissue, muscle tissue, blood or hair. About 20 samples were either degraded or otherwise did not yield a reliable amplification product and had to be excluded from the analysis. Ultimately, a total of 134 individuals from eight European countries, mainly collected from central–western Europe, were successfully scored (see Table 1).

Molecular work

DNA was extracted using the QIAgen DNeasy tissue kit. A 172-bp mtDNA fragment of the hyper-variable control region was amplified using the primers LutCR1 (5′-CACCACCAAC ACCAAAGCT-3′) and LutCR2 (5′-CCTGAAGTAAGAAC CAGATG-3′) (Cassens et al. 2000). This DNA fragment was selected as it contains the main diagnostic mutations to reliably identify previously described haplotypes and/or haplogroups (Davison et al. 2001; Pertoldi et al. 2008a; Jordan et al. 2012; Ruiz-Gonzalez et al. 2013), thus allowing for comprehensive comparisons. Polymerase chain reaction (PCR) consisted of 10 μL reaction mix in total containing 1.0 μL template DNA, 1.0 μL 10x PCR buffer, 0.25 μL of 10 μM primer forward and reverse, 1.0 μL DNA polymerization mix, 2 mM deoxyribonucleotide triphosphate (dNTP), 0.1 μL Taq DNA polymerase (Roche) and 6.4 μL MilliQ (H_2O). Samples were heated to 94 °C for 3 min, followed by 30 cycles of 1 min at 94 °C, 1 min at 52 °C and ended with 1 min at 72 °C. Cycling was followed by 20 min at 72 °C. PCR products were purified with NucleoSpin® Extract II. Consecutively, the samples were sequenced in both forward and reverse direction using BigDye Terminator Cycle Sequencing Kits (Applied Biosystems). To 5 μL-PCR product, we added 8 μL MilliQ, 5 μL Sequence Reaction Buffer, 1 μL forward or reverse primer (5 μM) and 1 μL BigDye Termination mix (BDT). The samples were heated to 96 °C for 10 s, 50 °C for 5 s and 60 °C for 4 min. This was repeated for 25 cycles, after which samples were kept at 20 °C. The samples were precipitated following Sephadex protocol. PCR-labelled fragments were run on an ABI-PRISM 377 automated DNA sequencer (Applied Biosystems).

Data analyses

Sequences were first checked by eye, edited with Chromas 1.5 (http://chromaspro.software.informer.com) and aligned with BioEdit 7.0.0 (Hall 1999). Identical haplotypes among the sequences were determined using the Collapse tool—Collapse ver. 1.2. (Posada 2004) (http://darwin.uvigo.es/software/modeltest.html). Intrapopulation standardised haplotype diversity (*Hs*) was calculated for every population (with the exception of Luxembourg and Spain, which showed only one and three samples, respectively) by the programme CONTRIB (Petit et al. 1998). Intrapopulation nucleotide diversity (π) and pairwise population genetic distances (using the K2P evolutionary model) were calculated by MEGA v. 5.0 (Tamura et al. 2011). To determine the genealogical relationships between the haplotypes found, we created a minimum spanning network by using TCS version 2.1 (Clement et al. 2000), in which a 98 % connection limit was used and gaps were treated as missing data. Furthermore, we plotted a pie chart showing the percentages of the different haplotypes found in the different populations and listing the presence of private haplotypes. We used BLAST software (Altschu et al. 1990) in order to determine the correspondence of each discovered haplotype with previously published sequences and the main phylogroups described by Ruiz-Gonzalez et al. (2013).

Table 1 Data on the number of individuals analysed (*n*), nucleotide diversity (π) within each country/population and standardised haplotype diversity (*Hs*). *Hs* values were calculated with the programme CONTRIB (Petit et al. 1998), and the minimum number of samples used was five, so the Luxembourg and Spanish populations could not be analysed (*NA* not applicable). The standard deviations (SD) of *Hs* and π are shown in parentheses. Haplotype number refers to that listed in the Electronic appendix 1

Population	*n*	π	*Hs*	Private haplotypes
Belgium	7	0.003 (0.003)	0.476 (0.171)	–
Denmark	22	0.005 (0.003)	0.541 (0.068)	Hap 11
France	5	0.002 (0.002)	0.400 (0.237)	–
Germany	7	0.016 (0.007)	0.762 (0.115)	–
Luxembourg	1	NA	NA	–
Spain	3	NA	NA	Hap 13
The Netherlands	74	0.014 (0.005)	0.722 (0.044)	Hap 04, Hap 05, Hap 06, Hap 07, Hap 08, Hap 09, Hap 10
Poland	15	0.006 (0.003)	0.648 (0.134)	Hap 14, Hap 15, Hap 16, Hap 17

Results

Haplotype, nucleotide diversity and unique haplotypes

In total, 17 haplotypes were identified with ten parsimony-informative sites (out of 14 total variable sites; see Electronic appendix 1) throughout the eight different populations. Some were only found once and were unique to specific populations, while others were common among several of them (see Electronic appendix 1). Out of the 17 haplotypes, 13 were found only in a single country (see Table 1). The geographic distribution and frequency of the 17 pine marten haplotypes are shown in Electronic appendix 1.

Haplotype 01 was most common and present in every population, with the exception of the Spanish population, but at different frequencies (see Fig. 1 and Electronic appendix 1). Haplotypes 01, 02, 03, 11, 12 and 16 were already registered in GenBank, whereas the others are new sequences closely related to previously identified haplotypes (pairwise identity>98.3 %; see Electronic appendix 1).

Overall, genetic variation estimated within countries was relatively high for haplotype diversity ($0.400 \leq Hs \leq 0.762$) and relatively low for nucleotide diversity ($0.002 \leq \pi \leq 0.016$). Particularly, *Hs* values were considerably higher in Germany (0.762) and the Netherlands (0.722) compared to the other countries. However, even for those populations (i.e. Germany and the Netherlands), π values were found to be relatively higher compared to the π values found for the other populations investigated (0.016 and 0.014, respectively; see Table 1). Because samples from Luxembourg and Spain were limited to one and three individuals respectively, they were removed from these analyses.

Genetic differentiation and genealogical relationship

The genetic distance (calculated through K2P evolutionary model) between populations ranged from 0.001 to 0.032 (Table 2). Based on the TCS network (Fig. 2), the haplotype genealogical relationship net constructed showed evidence that many unique haplotypes might be of recent origin (i.e. only one or two base substitutions).

Several haplotypes found in this study were already found in the previous studies by Pertoldi et al. (2008a), Davison et al. (2001) and Ruiz-Gonzalez et al. (2013) (see Electronic appendix 1). The most comprehensive study in terms of geographic range and sampling by Ruiz-Gonzalez et al. (2013) found that pine marten populations in Europe are subdivided into three major assemblages [i.e. Mediterranean (MED), central–northern European (CNE) and Fennoscandian–Russian (FNR) phylogroups]. Using BLAST, we identified that Hap 1–10 and Hap 14–17 were identical or nearly identical (pairwise identity> 98.3 %) to previously identified haplotypes of the CNE phylogroup. Hap 11–13 were equal or closely related (pairwise identity>99.4 %) to the Mediterranean phylogroup haplotypes. Eighty-two point eighty-four percent (113 out of 134) of the analysed samples belonged to the CNE phylogroup and were found in all the populations sampled from central–western Europe, suggesting that this haplogroup is predominant in this area. In contrast, only 17.16 % (23 out of 134) of the samples came from the MED phylogroup. None of the samples retrieved from the studied populations was grouped within the FNR phylogroup, which included haplotypes from both pine marten and sable *M. zibellina* specimens, originated due to ancient introgression (Davison et al. 2001; Schwartz et al. 2012; Ruiz-Gonzalez et al. 2013).

Discussion

Population structure of pine martens in central–western Europe

The haplotype diversity found in our study was relatively high ($0.400 \leq Hs \leq 0.762$). Previous studies on different mammal

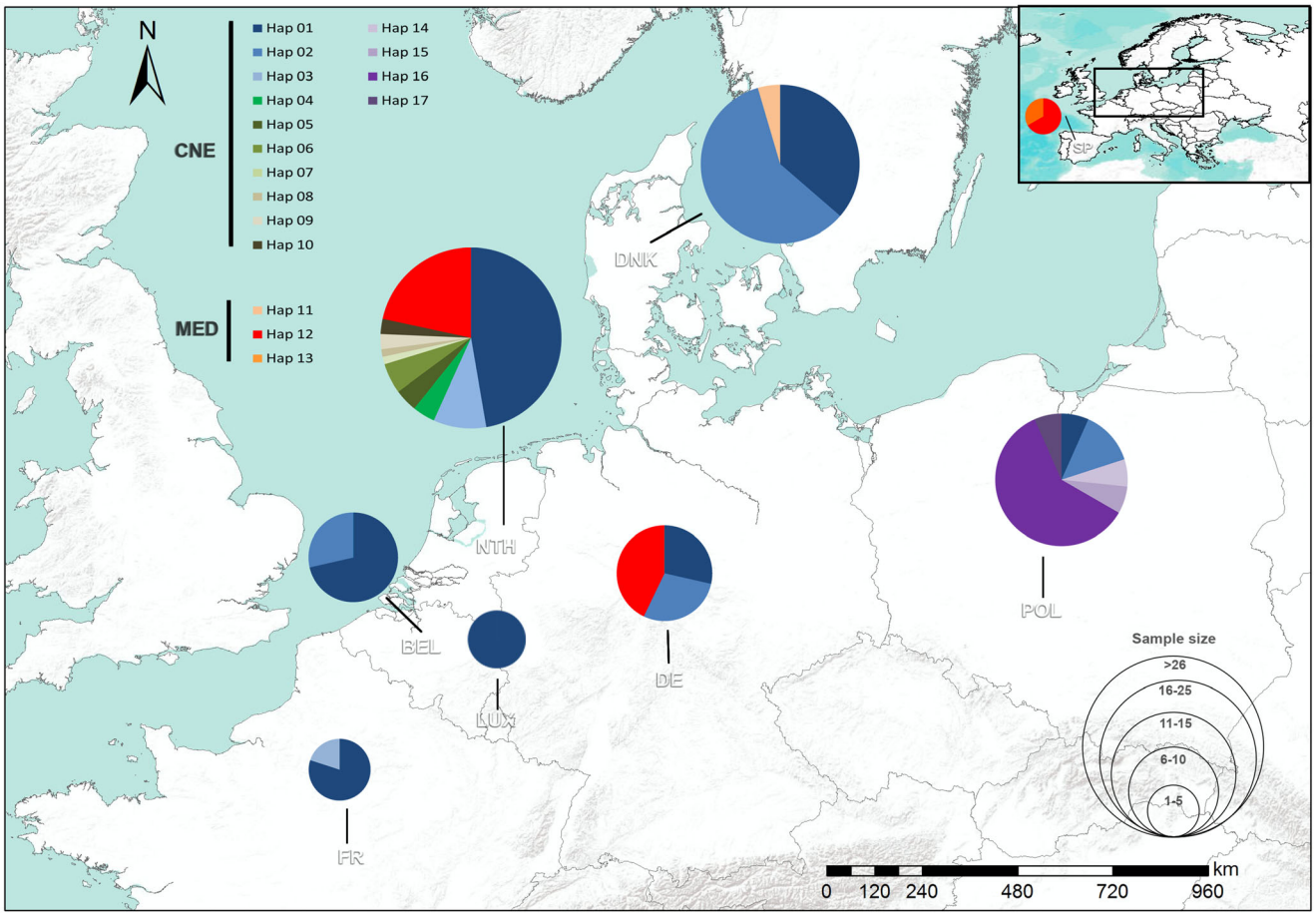

Fig. 1 Geographic distribution and frequency of the 17 different haplotypes found in the hyper-variable control region fragment of the mitochondrial DNA (172 bp) from the 134 samples of martens *Martes martes* analysed throughout Europe are shown in *pie charts*. *Hap 1–10* and *Hap 14–17* corresponded to the central–northern European phylogroup (*CNE*) while *Hap 11–13* corresponded to the Mediterranean phylogroup (*MED*) identified by Ruiz-Gonzalez et al. (2013). For details on haplotype information, see Electronic appendix 1, and for private haplotype within each country, see Table 1

species that have populations exhibiting bottlenecks found *Hs* values between 0.4 and 0.5 (Bickham et al. 1996; Hoelzel 1997; Eizirik et al. 1998; Huchon et al. 1999; Pope et al. 2000; Randi et al. 2000). This suggests that the pine marten populations in western Europe could not yet be genetically exhausted. However, the relatively low level of nucleotide diversity found ($0.002 \leq \pi \leq 0.016$), despite the relatively high level of haplotype diversity, suggests a long-term, low effective population size and/or population bottlenecks. Given the quite high heterogeneity of the haplotype and nucleotide diversity found among countries, we suggest that the pine marten populations across Europe have experienced different demographic events.

Table 2 Pairwise genetic distance between countries/populations calculated through K2P evolutionary model. Standard deviations are shown in parentheses

	The Netherlands	Spain	Germany	Denmark	France	Luxembourg	Belgium
The Netherlands	–						
Spain	0.024 (0.010)	–					
Germany	0.016 (0.006)	0.017 (0.007)	–				
Denmark	0.013 (0.005)	0.029 (0.012)	0.014 (0.006)	–			
France	0.010 (0.003)	0.027 (0.012)	0.013 (0.005)	0.005 (0.004)	–		
Luxembourg	0.009 (0.003)	0.026 (0.012)	0.012 (0.005)	0.004 (0.003)	0.001 (0.001)	–	
Belgium	0.010 (0.003)	0.028 (0.012)	0.013 (0.006)	0.004 (0.003)	0.003 (0.002)	0.002 (0.002)	–
Poland	0.015 (0.005)	0.032 (0.013)	0.017 (0.007)	0.010 (0.005)	0.007 (0.005)	0.006 (0.004)	0.007 (0.005)

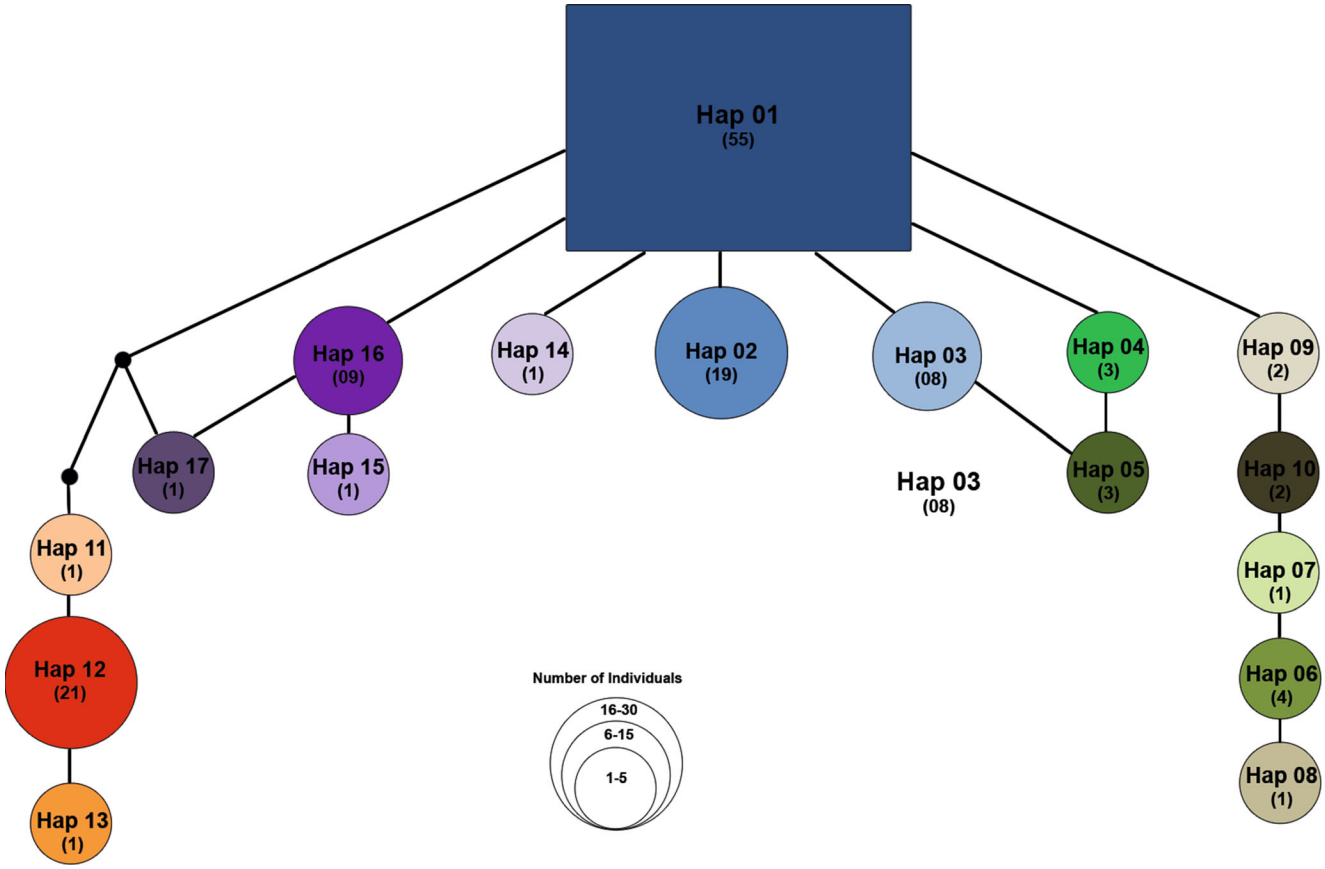

Fig. 2 Haplotype network obtained by TCS v. 1.21 showing the genealogical relationships between the 17 mitochondrial haplotypes found in this study (a total of 134 specimens; 172 bp from a fragment of the mitochondrial control region). The *square* indicates that haplotype with the biggest outgroup weight, which could be considered as the putative ancestral haplotype. The *circles* represent the other haplotypes found in this study and are proportional to the number of individuals shared (*number in parenthesis*). Each *line* represents one nucleotide substitution and the *black dots* indicate missing or not sampled haplotype. Haplotype number refers to that listed in the Electronic appendix 1

When we focus on the populations individually, we found that the Dutch populations had a relatively high genetic variation, which could be due to the fact that the Netherlands (with a mixing of different lineages) is considered a genetic melt pot or suture zone (Taberlet et al. 1998). However, it might also partially be explained by the much larger sample size for the country. Indeed, the Dutch and the German populations are the only populations with representative haplotypes of the two main pine marten phylogroups identified in Europe (i.e. MED and CNE phylogroups; Ruiz-Gonzalez et al. 2013).

The low level of genetic variability found for the Danish population is in accordance to a previous study conducted by Pertoldi et al. (2008a), which shows approximately the same result with Hs=0.560 and π=0.003. Next to mtDNA analysis, Pertoldi et al. (2008b) conducted also a microsatellite analysis, which shows a strong decrease in effective population size over time. The results of the present study are therefore supporting the hypothesis that the Danish population has gone through a population bottleneck.

Due to the low sample sizes, the other populations included in the analyses such as those found in Spain, Belgium, Germany, Luxembourg, Poland and France make the comparisons of the level of genetic variability with other populations difficult as the estimates of genetic variability might not be very representative. However, despite the low sample size, a relatively high Hs and π were found for the German and Polish populations, whereas there is relatively low genetic variation for the Belgian and French population. A larger sample size would be necessary to make some realistic conclusions. Noteworthy, of the five haplotypes found in Poland, four were private, indicating that the Polish population must in some way be separated from the other populations that have been investigated. This isolation is also reflected by the relatively high pairwise genetic distance between the Polish population and all the other populations.

Differentiation and gene flow between pine marten populations: phylogeographic implications

No evidence for endemic clades or lineage groups was found. This may be due to the somewhat small fragment of mtDNA used (172 bp) that was not sufficiently informative to resolve

intraspecific phylogenetic patterns. However, the correspondence of each discovered haplotype with previously outlined phylogroups by Ruiz-Gonzalez et al. (2013) allowed inferring detailed phylogeographic patterns in the study area. Ruiz-Gonzalez et al. (2013) found a strict phylogeographic pattern throughout the species range, with the presence of three major phylogroups—MED, CNE and FNR, showing a global pattern of spatial segregation and a south–north replacement with some area of overlap and genetic admixture. The distribution of the phylogroups is subdivided into three main geographic regions in Europe: southern Europe (MED) (i.e. the Mediterranean peninsulas), central and northern Europe (CNE), and Fennoscandia–Russia (FNR) region. Our results are in accordance with the results reported in Ruiz-Gonzalez et al. (2013), showing a low proportion of pine martens from the Mediterranean lineage in continental Europe (Ruiz-Gonzalez et al. 2013, 17.6 %; This study, 14.6 %). These results confirm that the Mediterranean phylogroup, which survived the LGM in southern European refugia, did not significantly contribute to the postglacial recolonization of most of the Palaearctic range of the species (Sommer and Benecke 2004; Sommer and Nadachowski 2006; Ruiz-Gonzalez et al. 2013). According to this data, we exclusively identified haplotypes linked to the MED group in the Spanish population and in a few individuals from central–western Europe. As previously suggested by comprehensive phylogeographic and paleontological studies (Sommer and Benecke 2004; Sommer and Nadachowski 2006; Ruiz-Gonzalez et al. 2013), our results support that most of Europe was colonized by the central–northern European phylogroup, which survived the last glaciations in northern cryptic refugia, probably located in the Carpathians (Sommer and Benecke 2004; Sommer and Nadachowski 2006; Ruiz-Gonzalez et al. 2013). Our data is in agreement with this hypothesis, indicating a clear predominance of the CNE lineage in continental Europe (i.e. >80 % of the analysed samples). Thus, this study further confirms previously outlined phylogeographic patterns for the pine marten in Europe (Ruiz-Gonzalez et al. 2013), suggesting that this species did not only respond to the last glaciation by simply shifting their distributions to the Mediterranean region but also survived at higher latitudes previously considered inhospitable (i.e. central Europe). As previously reported in several temperate species (e.g. Deffontaine et al. 2005; Kotlik et al. 2006; Valdiosera et al. 2007; McDevitt et al. 2012), this work provides further insights into the cryptic northern glacial refugia in Europe (Bhagwat and Willis, 2008; Provan and Bennett 2008; Stewart et al. 2010). Additionally, the absence of haplotypes related to the FNR phylogroup in central–western Europe confirms that the introgression processes between closely related martens (i.e. pine marten and sable) were exclusively confined to the Fennoscandian and Russian regions (Ruiz-Gonzalez et al. 2013). Furthermore, we confirmed the absence of introgressed haplotypes with the American marten that up to now has been exclusively identified in England (Davison et al. 2001; Kyle et al. 2003; Jordan et al. 2012).

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