Mamm Res (2016) 61:327–335
DOI 10.1007/s13364-016-0295-8

ORIGINAL PAPER

Pine marten vs. stone marten in agricultural lowlands: a landscape-scale, genetic survey

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Abstract We applied molecular analysis methods to faecal samples to determine both the overall level of occupancy for pine marten (*Martes martes*) and current stone marten (*Martes foina*) distribution in the western Po plain. Surveys were carried out in a 10 × 10-km grid, applying a hybrid sampling design. The specific identification of faecal samples was accomplished either by a polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) method or by amplifying and sequencing a 330-bp mtDNA fragment of the control region (D-loop). Identification success was 93.7 % by the PCR-RFLP and 71.7 % by DNA sequencing. Overall, we collected 47 pine marten records and 24 stone marten records. Thirty-six squares (81.8 %) were found to be positive for at least one marten species, the distribution range of the two species scarcely overlapping. The pine marten was shown to be widespread in lowland areas on the north bank of the River Po, which is probably acting as a barrier to its expansion. In this area, stone marten records were few, while it is was widespread on the south bank of the river. Pine marten expansion may have forced the stone marten to restrict itself to less suitable agricultural and urban areas. Nonetheless, we cannot exclude that stone marten range and/or numbers may being declining as a consequence of pine marten expansion. Six pine marten samples belonged to the Central-Northern European (CNE) phylogroup. The relatively high percentage of CNE martens is consistent with the hypothesis of an ongoing expansion of Alpine and trans-Alpine pine marten populations.

Keywords *Martes martes* · *Martes foina* · Range variation · PCR-RFLP · Phylogroups

Introduction

Species ranges can be highly dynamic, continuously changing in size and shifting in space as a consequence of variation in both abiotic and biotic conditions over time (Davis and Shaw 2001; Gaston 2003; Wisz et al. 2013). Research on species' distribution ranges has recently gained a renewed interest, due to the need to address large-scale, human-induced environmental changes (reviewed by Sexton et al. 2009). Several studies have focused on the expansion of invasive alien species (Andow et al. 1990; Hastings 1996; Von Holle and Simberloff 2005), which allows the investigation of spatio-temporal dynamics in the absence of historical complexities (Svenning et al. 2014); the impact of global climate change (Parmesan 2006; Moritz et al. 2008), which makes it urgent to assess the capability of species to track shifting environments (Cianfrani et al. 2011) and the effects on ecosystems of the

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Communicated by: Allan McDevitt

Electronic supplementary material The online version of this article (doi:10.1007/s13364-016-0295-8) contains supplementary material, which is available to authorized users.

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extinction of large carnivores (e.g. "mesopredator release"; Ritchie and Johnson 2009; Ripple et al. 2014).

Mesopredator spread has been related to the decline of several prey species (Palomares et al. 1995; Crooks and Soulé 1999; Galetti et al. 2009) and may facilitate the transmission of pathogens to domestic carnivores and humans (Whiteman et al. 2007). Nonetheless, the expansion of autochthonous mesopredators can also have positive effects, e.g. by reducing the number of alien species, as it is the case of American grey squirrel (*Sciurus carolinensis*) population crash following the recovery of pine marten in Ireland (Sheehy and Lawton 2014). Less attention has been devoted to the intra-guild effects of expanding medium-sized carnivores (Hersteinsson and Macdonald 1992; Tannerfeldt et al. 2002).

Long considered a "forest-specialist", in recent years, the pine marten *Martes martes* has been repeatedly reported to occur also in largely fragmented landscapes (reviewed by Virgós et al. 2012), suggesting a greater ecological flexibility than previously believed. In heterogeneous landscapes, individuals are confined to wood patches (Vergara et al. 2015), resulting in smaller home ranges compared to forested habitats (Mergey et al. 2011). Habitat fragmentation is also a major factor producing strong genetic differentiation between pine marten populations over short geographical distances (Ruiz-González et al. 2015).

In Italy, the pine marten and the closely related stone marten (*Martes foina*) occur sympatrically in mountainous areas, while in lowlands, only the latter has been reported (Prigioni et al. 2001; Genovesi and De Marinis 2003a, b; Sindaco and Carpegna 2010). The large plain crossed by the River Po is one of the most intensively cultivated and densely populated areas of Italy, where residual forest cover is <5 % (Falcucci et al. 2007). According to the analysis of available museum data (De Marinis and Lapini 1994; unpubl. data) and ca. 350 marten records collected between 1973 and 2007 (Balestrieri et al. 2010; Sindaco and Carpegna 2010; unpubl. data of Piedmont region Data Bank), the first record of the pine marten in the Po plain dates back to the end of 1988. In the following two decades, records of road-killed pine martens in the western section of this apparently unsuitable area (Piedmont region) grew exponentially (Balestrieri et al. 2010), with evidence of its stable occurrence in two small protected wooded areas (Remonti et al. 2012) and in riparian woodland on the banks of the River Ticino (Balestrieri et al. 2015a), a north-bank tributary of the River Po (Fig. 1). In the same period, in Piedmont, the stone marten showed an opposite trend (as assessed by comparing the number of records in 1991–1999 and 2000–2007), although being still considered widespread throughout the region (Sindaco and Carpegna 2010; unpubl. data of Piedmont region Data Bank). In contrast, except for a few recent records on the River Oglio (Lombardy region; Mantovani 2010), no evidence of pine marten presence is available for the central (Prigioni et al. 2001) and eastern (Bon et al. 1995; Mezzavilla F., pers comm. Oct. 2015) sections of the Po plain, where the stone marten is widespread. Although the more generalist and synanthropic stone marten should be more adapted to human-impacted, fragmented landscapes than the pine marten (Sacchi and Meriggi 1995; Rondinini and Boitani 2002; Santos and Santos-Reis 2010; Vergara et al. 2015), on the River Ticino, pine marten range increase seems to have been accompanied by a sharp restriction in stone marten range (Balestrieri et al. 2015a).

To determine both the overall level of occupancy for pine marten and current stone marten distribution in the western Po plain, we applied molecular methods on non-invasively collected faecal samples. DNA analysis was necessary because the faeces of the *Martes* species, while being relatively abundant and easy to collect, thus facilitating the extensive sampling of these elusive species, cannot be distinguished from each other visually and can also be confused with those of other carnivores (Davison et al. 2002). Faecal DNA-based genotyping has proven to be a cost-effective way to reliably verify and monitor these elusive species' presence (O'Reilly et al. 2008; Ruiz-González et al. 2013a). Consequently, we either applied a polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) method for mtDNA, which allows effective identification of the two marten species and reliable monitoring of their distribution patterns (Ruiz-González et al. 2008), or sequenced a mtDNA fragment of the control region (D-loop) that has been previously shown to be sufficiently informative to both allow the identification of *Martes* spp. and identify the main haplotypes and haplogroups described in previous studies (Davison et al. 2001; Pertoldi et al. 2008; Jordan et al. 2012; Ruiz-Gonzalez et al. 2013a).

The second method was applied following the unexpected discovery on the River Ticino of two haplotypes (i.e. Mm47 and Mm48) from the central-northern European (CNE) lineage (Ruiz-González et al. 2013b). By analysing an ~1600-bp long fragment of the mtDNA of 287 individuals sampled across the entire distribution range of the pine marten, Ruiz-González et al. (2013b) provided evidence for the occurrence in continental Europe of two main phylogroups: the CNE phylogroup and the Mediterranean (MED) phylogroup. As the latter was the only phylogroup recorded in the whole Mediterranean basin, the two CNE haplotypes found in NW Italy have been hypothesised to come from a recent colonisation event from Alpine or trans-Alpine CNE populations (Balestrieri et al. 2015a). MtDNA sequencing was then applied to get more detailed information about the distribution of main haplotypes and phylogroups in this contact zone between the Mediterranean area and continental Europe.

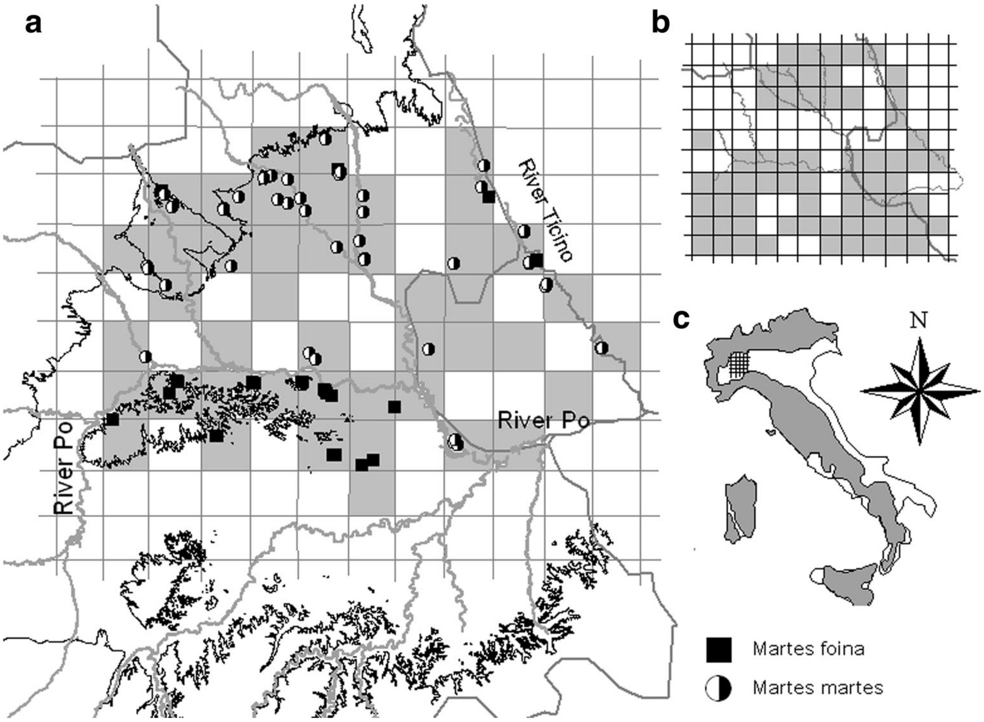

Fig. 1 Distribution of both the pine (MM) and stone marten (MF) in the western Po plain (**a**) as assessed by faecal mDNA analysis; the 300 m a.s.l. contour line, which marks the limit of the River Po plain, and sampling grid (10 × 10 km) are shown; sampled squares are in *grey*. The stone marten occurs in the whole peninsula; its past distribution in the study area (data collected in 1980–2010) is shown (**b**). The map of Italy (**c**) shows the location of the study area with respect to pine marten range (in *grey*)

Study area

The Po-Venetian alluvial plain is the largest in Italy (ca. 46,000 km^2). The pedogenetic and micro-morphological characteristics of the soils of the lower plain, crossed from west to east by the River Po (652 km in length), support high levels of agricultural productivity and are intensively managed for cattle husbandry and the production of rice, maize and wheat.

Since the second half of the nineteenth century, widespread urbanisation and industrialisation have led to a progressive depletion of soil resources, and built-up areas currently cover ca. 9 % of the area (Gherardi et al. 2009). About 70 % of residual forests are in the western and central plain (Camerano et al. 2010) and either consist of small, isolated fragments (mean patch size =4.5 ha; Lassini et al. 2007) scattered within the agricultural matrix or, as in most European lowlands (Coles et al. 1989), cover the banks of major rivers.

Based on previous pine marten records (see Balestrieri et al. 2010), we focused on the western sector of the Po plain (< 300 m above sea level, Piedmont region), covering an area of ca. 7000 km^2 (Fig. 1). The Eastern limit of the study area was marked by the valley of the River Ticino, including the largest and best-conserved riparian forests of the Po-Venetian plain. In Piedmont, the mean annual discharge of the River Po ranges between 43 and 121 m^3/s (sampling station of Turin, period 1995–2013; ARPA 2015).

Climate was sub-continental temperate, with mean yearly temperature of 12.0 °C and mean yearly rainfall of 1000 mm.

Methods

To assess the overall level of occupancy for both marten species in the western Po plain, surveys were carried out in a 10 × 10-km grid, superimposed on the kilometric grid of digitised, 1:10,000 Regional Technical Maps. We did not assume that occupancy and detection probabilities were constant across space (MacKenzie et al. 2003; Royle and Nichols 2003), as in Mediterranean agricultural landscapes, the occurrence of both the pine marten (Pereboom et al. 2008; Balestrieri et al. 2015a) and stone marten (Virgós and Garcia 2002; Mortelliti and Boitani 2008) depends on forest availability, i.e. size and degree of connectivity of residual wood patches. As in fragmented habitats carnivores concentrate in the remnant forest patches (Barrull et al. 2014; Šálek et al. 2014), where they are more often detected than into the surrounding agricultural matrix (Santos et al. 2016), faecal samples were searched for along linear transects (mean length of each transect ± SE = 2.6 ± 0.17 km) placed along wood/field margins, paths and country roads as to cover available wood patches and their surrounding open habitats.

Because faeces can be washed away by rain, surveys were not carried out in rainy periods, being delayed by at least 1 week after heavy rain.

To determine how many surveys should be conducted per square, we followed the recommendations by Balestrieri et al. (2015a), who suggested that genetic survey protocols must involve multiple visits (between 1 and 3.9 per sampling unit, depending on marking intensity). We then applied a “hybrid” sampling design, which, for large study areas, represents a good compromise between the robustness of standard designs (all sites surveyed K times) and cost efficiency (Mackenzie and Royle 2005). About 60 % of sampling squares were surveyed only once and the others up to a maximum of four times, depending on the number (N) of "marten-like faeces found per kilometre of transect during each survey ($N > 1$ and $N < 0.5$, respectively; Balestrieri et al. 2015a). Repeated surveys of a same square were conducted as multiple discrete visits (i.e. a minimum of two transects per square surveyed on up to four different days) and/or multiple transects within a single visit (up to four transects in a single day, all or partially re-surveyed on one to three different days; Mackenzie and Royle 2005).

Sampling was conducted between January 2013 and December 2014. Hypothesising that river corridors may facilitate and drive pine marten colonisation of the interior of the plain (Balestrieri et al. 2015a), surveys were mainly conducted from north to south (i.e. squares at approx. The same latitude were sampled in the same sub-period), so as to follow the course of the major tributaries of the River Po (Fig. 1).

A portion (ca. 1 cm) of each faeces suspected of belonging to a *Martes* spp. was picked up using sticks, stored in autoclaved tubes containing ethanol 96 % and frozen at −20 °C until processed (Ruiz-González et al. 2008). All samples were georeferenced and projected onto a GIS (Arcview, ESRI).

DNA was isolated using the QIAamp DNA Stool Mini Kit (Qiagen) according to the manufacturer's instructions. The specific identification of faecal samples was accomplished either by a polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) method ($N = 35$), according to Ruiz-González et al. (2008), or by amplifying and sequencing a 330-bp mtDNA fragment of the control region (D-loop) ($N = 46$).

By the first method, two primers—Mm_L1 (5′- CCC AAA GCT GAC ATT CTA AC −3′) and Mm_H1 (5′ - ATG GGC CCG GAG CGA GAA GAG GTA CAC-3′)—amplify the mtDNA from *Martes martes* and *Martes foina* and four *Mustela* species. The simultaneous digestion of amplified mtDNA by two restriction enzymes (RsaI and HaeIII) generates different restriction patterns for each mustelid species (i.e. DNA fragments differing in both number and length), allowing the unambiguous identification of faecal samples (see Ruiz-González et al. 2008 for further details).

By the second method, a 330-bp mtDNA fragment of the control region (D-loop) was amplified using the primers MFABDL228 (5′-AGA CTC AAG GAA GAA GCA ATA GCC-3′) and CLUDH350 (5′-GGG CCT GAA GTA AGA ACC AGA TGC C-3′) (Randi et al. 2000). This DNA fragment was selected as it contains the main diagnostic mutations which reliably identify both the samples at species level and previously described pine marten haplotypes and/or haplogroups (Davison et al. 2001; Pertoldi et al. 2008; Rozhnov et al. 2010; Jordan et al. 2012; Ruiz-González et al. 2013a), thus allowing for comprehensive comparisons. The standard polymerase chain reaction (PCR) amplifications were conducted in 15-μL reactions containing 3 μL of diluted (1:10) template DNA, 3.2 pmol of each primer, 1.75 μM dNTP, 1.33 μM MgCl2, 1.56 μL of Gold STAR 10× buffer, and 0.6 U Taq DNA polymerase, using the following cycling conditions: an initial denaturing step at 94 °C for 5 min, 42 cycles of denaturing at 94 °C for 50 s, annealing at 58.5 °C for 45 s, and extending at 72 °C for 90 s, with a final extending step of 72 °C for 10 min. The PCR products were purified using EXO-SAP IT (USB, Cleveland, OH, USA) and sequenced using the BigDye Terminator Kit V1.1 (Applied Biosystems, Foster City, CA, USA) in an ABI PRISM Model 3130 Genetic Analyser (Applied Biosystems). Electropherograms were visually inspected and edited using SEQSCAPE 2.5 (Applied Biosystems), and nucleotide sequences were further aligned and edited in BIOEDIT 5.0.9 (Hall 1999). Identical haplotypes were matched using DNASP v.5 (Librado and Rozas 2009). We then used BLAST software (Altschul et al. 1990) to determine (1) species' identity and (2) the correspondence of each discovered haplotype with already published haplotypes in GenBank and the pine marten phylogroups described by Ruiz-González et al. (2013b) (Table S1).

Additionally, we included unequivocal species records from road-killed individuals collected in 2013–2014.

Results

We surveyed 44 100-km^2 squares, for a total of 180.9 km of transects, collecting 107 faecal samples. To maximise the cost effectiveness of genetic analyses, a subsample of 81 samples was selected (discarding apparently "old" and dubious surplus samples) and analysed. Identification success was 93.7 % by the PCR-RFLP method (24 *Martes martes* and 9 *Martes foina* out of 35 samples) and 71.7 % by DNA sequencing (33 out of 46). By means of DNA sequencing, 21 samples were identified as *Martes martes*, 8 as *Martes foina*, 3 as *Vulpes vulpes*, and 1 as *Mustela putorius*. Overall, by the application of both molecular methods, 45 samples were assigned to the pine marten and 17 to the stone marten. Additionally, two pine and seven stone marten records from road kills were included. Thirty-six squares (81.8 %) were found to be positive for at least one marten species (Fig. 1a). All squares surveyed more than once were found to be positive (Table 1).

The distributional range of the two species scarcely overlapped, both species occurring in only four squares, two at foot hills and two in the valley of the River Ticino. Overall, pine marten dominated the north of the River Po, while the stone marten was the only *Martes* species occurring south of

Table 1 Distribution of the 44 100-km^2 large squares sampled for the occurrence of pine and stone martens in the western Po plain (*N* negative, *P* positive) in relation to the number of surveys per square

N of surveys	*N* of squares	*Martes* spp. presence	Percent
1	8	N	18.2
	21	P	47.7
2	11	P	25.0
3	3	P	6.8
4	1	P	2.3
Total	*44*		100.0

the river (Fig. 1a). North of the River Po, the stone marten was not found in 11 out of 13 surveyed squares where it has been previously recorded (Fig. 1b). In general, few samples were found in the central part of the study area, which was intensively cultivated for rice.

In total, three different pine marten haplotypes and six stone marten haplotypes were identified (Table S1 in Electronic Supplementary Material). Five *Martes foina* haplotypes were novel (Mf_It_1–5; GenBank accession nos.: KX649915–KX649919), while Mf_It_6 had been previously identified in Bulgarian samples (Table S1). All the three pine marten haplotypes retrieved in this study had already been found in previous studies (see Table S1). Using BLAST, haplotype Mm_It_1 was found to be identical (pairwise identity = 100 %) to previously identified haplotypes of the CNE phylogroup, while Mm_It_2 and Mm_It_3 belong to the Mediterranean phylogroup.

Fifteen out of the 21 pine marten samples (71.5 %) assigned to the pine marten through DNA sequencing belonged to the Mediterranean phylogroup (MED), while six samples (28.5 %) were from the central-northern European phylogroup (CNE). Pine martens from the CNE occurred throughout the northern sector of the study area (Fig. 2).

Discussion

Considering the overall high species identification success, faecal DNA analysis confirmed to be an effective method for landscape-scale sampling of sympatric martens (Ruiz-González et al. 2008, 2013a; O'Reilly et al. 2008).

Based on sampling efficacy, i.e. the relatively high probability of marten detection and the 100 % positivity of sites which were surveyed repeatedly, pine marten can be considered to have colonised all the northern section of the study area, at least wherever wood patches still occur.

The current pine marten distribution in the western Po plain suggests that expansion has mainly followed the main watercourses flowing southwards from the Alps, with the River Po, which crosses the study area from the south-west to the east, acting as a natural barrier. Considering that the inner part of the plain has been reached by the pine marten only recently (Balestrieri et al. 2010), the crossing of this barrier may occur in the near future as south of the river, there is large availability of areas suitable for the pine marten (Balestrieri et al. 2016).

Spatial tracking of the environmental niche in response to changing environmental conditions has been often invoked as an explanation for range variation (Davis and Shaw 2001; Svenning et al. 2008). Variations in either climate (Sexton et al. 2009) or land use (Brooks et al. 2002; Cousins et al. 2015) are unanimously considered major determinants of species' range limits.

As a consequence of gradual global warming, in the northern hemisphere, the ranges of temperate species are shifting northwards or upwards in elevation (Parmesan and Yohe 2003). In this context, pine marten expansion in arable lands would follow a counter-intuitive direction.

Woodlands are key features for pine marten (Virgós et al. 2012); accordingly, in lowland, riparian areas, its abundance has been related to the size and degree of fragmentation of wood patches (Balestrieri et al. 2015a). There is no evidence that in recent decades, forest cover has increased in the western Po plain, favouring pine marten colonisation. In contrast, the analysis of land cover change between 1960 and 2000 at country scale showed that both intensive agriculture and, particularly urbanisation have gradually increased in lowland areas of the whole peninsula (Falcucci et al. 2007). An opposite pattern was recorded for European mountain areas, where following widespread abandonment of low-intensity farming and livestock rearing, forest cover has increased (MacDonald et al. 2000), with a positive effect on forest-dwelling species, such as roe deer *Capreolus capreolus* (Vernesi et al. 2002; Jepsen and Topping 2004). Alpine pine marten populations may have taken advantage of this increase in forest cover (ca. 50 % between the 1960s and 2000; Falcucci et al. 2007), recolonising mountain districts from which pine martens had disappeared in the twentieth century (Balestrieri et al. 2016) and expanding in less suitable lowland areas. Similarly, the recent expansion of the pine marten in Ireland and Britain has been related to increased rates of afforestation (O'Mahony et al. 2012; Croose et al. 2013).

The relatively high percentage of CNE martens found in the western Po plain, which currently represents a *unicum* in southern Europe (Ruiz-González et al. 2013b), is consistent with the hypothesis of an on-going expansion of Alpine and trans-Alpine pine marten populations.

The negligible occurrence of stone marten records north of the River Po agreed with the trend reported for the valley of the River Ticino, where pine marten expansion has coincided with a contraction of stone marten range (Balestrieri et al. 2015a). In contrast, on the south bank of the river, the stone marten is still widespread, as demonstrated by genetically identified faecal samples (this study) and available road kill

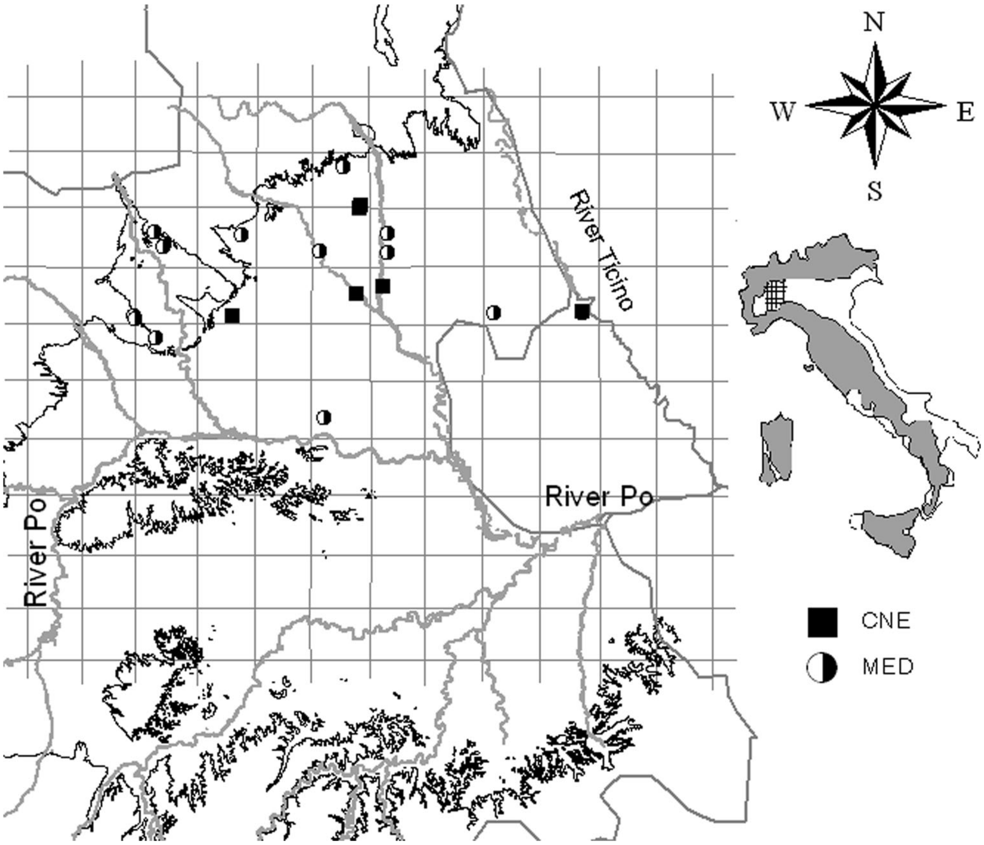

Fig. 2 Distribution of pine marten samples belonging to the Mediterranean (MED) and central-northern European (CNE) phylogroups; the 300 m a.s.l. contour line, which marks the limit of the River Po plain, and sampling grid (10 × 10 km) are shown; the map of Italy shows the study area and pine marten distribution in *grey*, while the stone marten occurs in the whole peninsula

records (Sindaco and Carpegna 2010). Interestingly, and in spite of small sample size and surveyed area, we identified a high number of closely related stone marten haplotypes. These findings will require additional analysis encompassing a longer DNA fragment and a European-scale survey (currently underway).

Within Mustelidae, there are several sympatric species with similar ecological requirements that have evolved mechanisms to coexist (e.g. *Mustela frenata–Mustela erminea*, St-Pierre et al. 2006; *Mustela erminea–Mustela nivalis*, Aunapuu and Oksanen 2003; *Lutra lutra–Mustela vison*, Bonesi et al. 2004). Pine and stone martens are sympatric across a large part of continental Europe (Proulx et al. 2004) and have also been reported to be syntopic (Pilot et al. 2007; Ruiz-González et al. 2008). In fact, these two martens are quite similar in size, morphology and feeding habits (Marchesi et al. 1989; Balestrieri et al. 2011, 2013) and, when syntopic, their trophic niches can overlap extensively (Posluszny et al. 2007). There is still little information about the factors that may facilitate their coexistence, although some degree of spatial segregation (i.e. differential habitat use) has been reported to potentially reduce interspecific competition (Vergara et al. 2015; Wereszczuk and Zalewski 2015). In sympatry, the pine marten predominates in woodland, while the stone marten mainly occurs in rocky areas and suburban areas (Virgós et al. 2012). This shift in habitat use by the stone marten has been associated to interspecific interactions with the more competitive pine marten (Delibes 1983). Accordingly, in the western Po plain, pine marten expansion may have forced the stone marten to restrict itself to less suitable agricultural and urban areas, where food diversity is expected to be lower—and human disturbance higher—than in residual wood patches (Benton et al. 2003; Balestrieri et al. 2015b).

It is still not clear why pine martens should be able to force stone martens to use suboptimal habitats (Larroque et al. 2015). Remonti et al. (2012) compared the diets of martens and red fox *Vulpes vulpes* in northern Italy, reporting that the food niche of each marten species highly overlapped with that of the fox. They concluded that the entry of the pine marten could have altered the existing ecological relationship within the carnivore guild to the detriment of the stone marten, the diet of which overlapped to a larger extent that of the red fox (Remonti et al. 2012). Recently, it has been proposed that the lower ability of the stone marten to avoid interference competition at community-level may play a major role in determining its exclusion from forested areas by the pine marten (Balestrieri 2016). Whatever the mechanism determining the lower competitive ability of the stone marten, as sampling was focused on residual forest patch segregation of space use, may explain the small number of stone marten records found north of the River Po.

Nonetheless, we cannot exclude a priori that stone marten population is declining, as the current distribution patterns of the two species and the negative trend of stone marten road kill records (Sindaco and Carpegna 2010) suggest that the pine marten may being actually replacing the stone marten in the lowlands of NW Italy. As population declines are often dictated by complex combinations of environmental and biotic factors (Sinclair and Byrom 2006), particularly for same-sized Mustelidae (Powell and Zielinski 1983), further studies on niche partitioning among species belonging to the same guild are needed to predict short- and long-term outcomes of their interactions.

Acknowledgments This study has been partially funded by the Basque Government through the Research group on “Systematics, Biogeography and Population Dynamics” (Ref. IT317-10; GIC10/76; IT575/13), the SAIOTEK research programme (Ref: S-PE11UN028). Ruiz-González holds a post doc fellowship awarded by the Dept. of Education Universities and Research of the Basque Government (Ref. DKR-2012-64). A. Balestrieri was supported by a Ph.D. fellowship awarded by the Dept. of Biosciences of the University of Milan. Lesley C. Wright kindly revised the English language. The comments and suggestions of two anonymous referees helped to greatly improve the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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