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Short communication

Preliminary study on microsatellite and mitochondrial DNA variation of the stone marten *Martes foina* in Bulgaria

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In Europe, distribution of many animal species has been severely restricted to small refugia during the last glacial period. The Balkan Peninsula including Bulgaria has been identified as one of the three main glacial refugia (Randi 2007). To know biogeographical history in Europe, molecualr phylogeographical studies about various European mammal species have been performed: for example, the least weasel *Mustela nivalis* by the mitochondrial DNA (mtDNA) control region and cytochrome *b* (Lebarbenchon et al. 2010), the ground squirrel *Spermophilus citellus* by cytochrome *b* (Kryštufek et al. 2009), the yellow-necked fieldmouse *Apodemus flavicollis* and the woodmouse *A. sylvaticus* by cytochrome *b* (Michaux et al. 2005), the brown bear *Ursus arctos* by mtDNA control region (Taberlet and Bouvet 1994; Taberlet et al. 1998) and the hedgehogs *Erinaceus europaeus* and *E. concolor* by cytochorome *b* (Santucci et al. 1998). These studies commonly show that distribution of European species was influenced by climate changes in the Quaternary glaciations, and they survived in different ways (Michaux et al. 2005). Studies on the genetic diversity of mammals in Bulgaria as part of the Balkan Peninsula provides further insights to understanding of biogeographical history from the last glacial maximum (LGM) to present in south Europe.

The stone marten *Martes foina* is a medium-sized carnivore of the family Mustelidae, and is distributed from Spain and Portugal in the west, through central and southern Europe, the Middle East, and central Asia, extending as far east as the Altai and Tien Shan Mountains and northwest China (Abramov et al. 2006). This species prefers more open areas than other martens (Sacchi and Meriggi 1995). Its habitat preferences vary in different parts of its range. *Martes foina* is typically found in deciduous forests, forest edges and open rocky hillsides. In some areas, however, *M. foina* also inhabits suburban and urban areas, with rare woods (Tikhonov et al. 2008). Because the Balkan Peninsula is one of the southern limit of distribution of *M. foina*, the analysis of its Balkan population would contribute to further understanding of refugia and phylogeography in Europe. Whereas there have been some phylogenetic studies about the family Mustelidae including *M. foina* (Marmi et al. 2003; Li et al. 2011) and a microsatellite variation study of *M. foina* in southern Portugal (Basto et al. 2010), no population genetic studies about this species in Bulgaria have been reported.

In the present study, to know the population structure and phylogeography of the Bulgarian population of *M. foina*, we studied its microsatellite polymorphisms and mtDNA control region variation, and then discuss the genetic diversity in the population.

Materials and methods

Samples and DNA extraction

Muscle tissue samples were obtained from 28 stone martens from 2010 to 2011 in Bulgaria. Sampling locations were Varna, Dolen Chiflik, Stara Zagora, Plovdiv, Sredna gora region, Pleven and Teteven (central and east part of Bulgaria) (Fig. 1). In addition, a sample of the pine marten *M. martes* was obtained near Sofia, Bulgaria in 2010. Total DNA was extracted from muscle tissues using DNeasy Blood & Tissue Kit (Qiagen).

Fragment analysis of microsatellite and data analysis

In the present study, 13 microsatellite loci (Mar02, Mar06, Mar08, Mar14, Mar15, Mar19, Mar21, Mar36,

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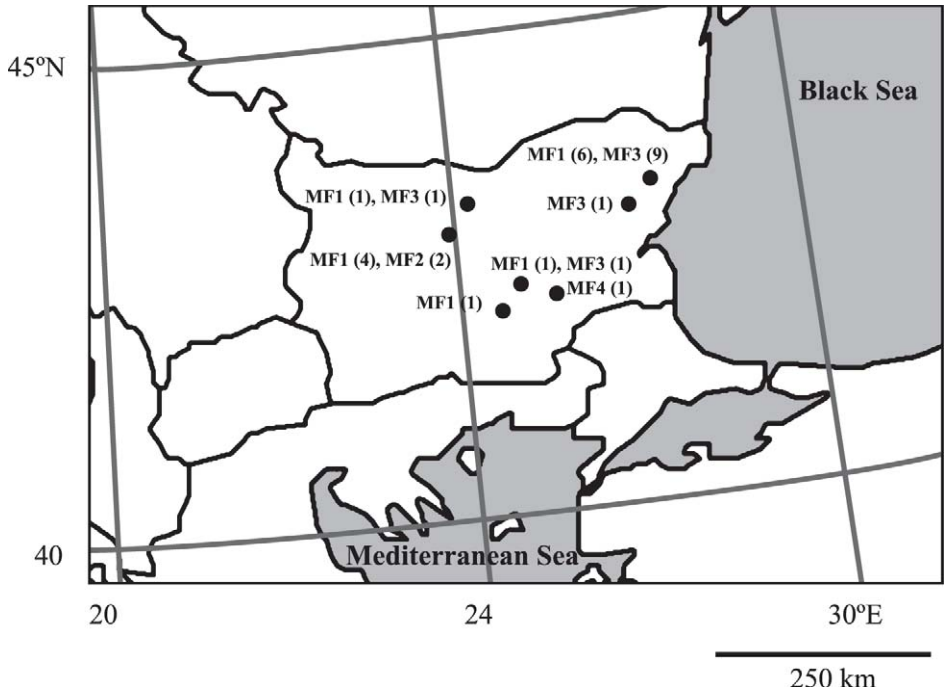

Fig. 1. Sampling locations (dots) of *M. foina* in Bulgaria. MtDNA haplotypes with animal numbers in parentheses are indicated near the locations.

Mar43, Mar53, Mar56, Mar58 and Mar64), which were developed from *M. martes* genome by Natali et al. (2010), were used. The 5' end of each forward primer was fluorescently labeled with FAM, NED, VIC or PET. Polymerase chain reaction (PCR) was performed in 10 µl of the reaction mixture, which consisted of 0.05 µl of r*Taq* DNA polymerase (5 units/µl, Takara), 1.0 µl of 10 × reaction buffer, 0.8 µl of dNTP, 0.1 µl of each primer (25 pmol/µl), 2.0 µl of DNA extracts and 5.95 µl of the distilled water. After denaturing at 94ºC for 5 min, the following reaction was repeated 35 times using a thermal cycler Takara TP-600: denaturing 94°C for 40 sec; annealing at 50–55°C for 40 sec; extension at 72°C for 1 min; and then reaction completion at 72°C for 5 min. Each PCR product was electrophoresed using a DNA sequencer ABI3170, and then molecular sizes were measured with Gene Mapper 4.0 (ABI) to determine alleles and genotypes.

The PCR products were then sequenced to test whether they included microsatellite regions. A total volume of cycle PCR reaction mixture was 10 µl consisted of 0.4 µl of premix (BigDye Terminator Sequence Kit ver.1: ABI), 2.0 µl of diluent 5 × buffer, 1.6 µl of primer (1 pmol/µl), 1.0 µl of DNA product and 5.0 µl of distilled water. Cycle PCR amplification was performed using a thermal cycler Takara TP-600 with the following program: 30 cycles of denaturing at 94°C for 10 sec, annealing at 50°C for 5 sec and extension at 60°C for 4 min. Cycle PCR products were purified with isopropanol precipitation for electrophoresis in a DNA sequencer ABI3170.

STRUCTURE 2.3.3 (Pritchard et al. 2000; Falush et al. 2003) was used for estimation of the population structure. The log-likelihood ratio [LnP(D)] was calculated to estimate the number of clusters (*K*). Observed (*Ho*) and expected (*He*) heterozygosities were calculated using ALEQUIN 3.11 (Excoffier et al. 2005). Departures from Hardy-Weinberg equilibrium and linkage equilibrium were examined for each microsatellite locus using ALEQUIN 3.11. Evaluation of recent population size decline was tested using BOTTLENECK 1.2 (Cornuet and Luikart 1997).

Determination of mitochondrial DNA sequence and data analysis

The 5'-portion of mtDNA control region (523 base-pairs, bp) was amplified using two PCR primers, UR-1 (Taberlet and Bouvet 1994) and MZCRP-R1 (Inoue et al. 2010). Polymerase chain reaction (PCR) was carried out in 20 µl of the reaction mixture: 0.1 µl of r*Taq* DNA polymerase (5 units/µl, Takara), 2.0 µl of 10 × reaction buffer, 1.6 µl of dNTP, 0.2 µl of each primer (25 pmol/µl), 1.0 µl of DNA extracts and 11.9 µl of the distilled water. After denaturing at 94°C for 3 min, 40

Table 1. Genetic diversity of 13 microsatellite loci in all individuals

Locus	Number of samples	Number of alleles	Molecular size of alleles (bp)	Heterozigosity	
				Ho	*He*
Mar02	28	2	140–142	0.250	0.399
Mar06	28	4	202–208	0.143	0.171
Mar08	28	6	141–151	0.500	0.709*
Mar14	28	4	214–220	0.571	0.595
Mar15	27	7	175–191	0.815	0.794
Mar19	28	3	198–204	0.428	0.434
Mar21	28	4	149–159	0.214	0.346*
Mar36	28	6	220–232	0.750	0.742
Mar43	28	7	139–163	0.786	0.818
Mar53	28	5	231–239	0.643	0.705
Mar56	28	6	199–209	0.643	0.684
Mar58	28	4	232–240	0.714	0.697
Mar64	28	2	159–163	0.036	0.036
Mean		4.6		0.499	0.549

*Significant departure from Hardy-Weinberg equilibrium ($P < 0.05$).

cycles were performed using a thermal cycler Takara TP-600 with the following program: denaturing 94°C for 1 min; annealing at 55°C for 1 min; extension at 72°C for 1 min; and then reaction completion at 72°C for 10 min. To check PCR amplification, 5 μl for each PCR product was electrophoresed on a 2% agarose gel, stained by ethidium bromide and visualized under an ultraviolet illuminator. The remaining 15 μl of each PCR product was purified using QIAquick PCR Purification Kit (Qiagen). Purified PCR products were subjected to cycle PCR and subsequent sequencing in the method same as mentioned above.

The haplotype diversity (h) and nucleotide diversity (π) were calculated by ARLEQUIN 3.11. A parsimony network among mtDNA haplotypes obtained in the resent study were made using TCS 1.21 (Clement et al. 2000). A neighbor-joining tree among *M. foina* haplotypes cited from the DNA database in addition to obtained haplotypes was constructed by MEGA 5 (Tamura et al. 2011). Tajima-Nei model for genetic distance and bootstrap values obtained from 500 replications were used in MEGA 5. The *M. foina* mtDNA sequences for comparison were cited as follows: accession no. AF336973 (Davison et al. 2001) from Europe; AB601564 (Hosoda et al. 2011) from Germany; EF200701 and EF200702 (Ruiz-González et al. 2008) from northern Iberian Peninsula; and HM106325 (Li et al. 2011) from China. As an outgroup, the homologous mtDNA sequence of *M. martes* (AB705460) determined in the present study was used.

Results and discussion

Microsatellite diversity

We found that PCR products from all 13 microsatellite loci included microsatellite sequences; therefore, genotypes of the 13 microsatellite loci were determined for 28 individuals from Bulgaria. Mean *Ho* and *He* in all individuals were 0.499 (0.036–0.815) and 0.549 (0.036–0.818), respectively (Table 1). We found two to seven alleles on each locus, and the mean number of alleles was 4.6 (Table 1). Disequilibrium for all individuals was statistically significant at seven pairs of loci (Mar08/Mar56, Mar14/Mar56, Mar19/Mar36, Mar21/Mar53, Mar21/Mar56, Mar36/Mar53 and Mar43/Mar64). In addition, departure from Hardy-Weinberg equilibrium ($P < 0.05$) was detected at two loci (Mar08 and Mar21) for all individuals (Table 1).

The average *He* of Bulgarian *M. foina* (0.549) was lower than that reported from other mustelids such as Portuguese *M. foina* (*He*: 0.334–0.817, mean *He*: 0.596) (Basto et al. 2010), Canadian *M. pennanti* (*He*: 0.562–0.683, mean *He*: 0.623) (Kyle et al. 2001) and North American *Gulo gulo* (*He*: 0.420–0.684, mean *He*: 0.63) (Kyle and Strobeck 2001). By contrast, the average *He* of Bulgarian *M. foina* was higher than that of Scandinavian *G. gulo* (*He*: 0.269–0.376, mean *He*: 0.369) (Walker et al. 2001). In addition, the average *He* of Bulgarian *M. foina* was lower than that of *M. martes* in Tuscany, Italy (*He*: 0.400–0.856, mean *He*: 0.698) (Natali et al. 2010), although analyzed microsatellite loci

were the same as those in the present study. These comparisons indicated that genetic diversity of Bulgarian *M. foina* obtained in the present study was as much as or lower than that of other mustelids.

Population structure based on microsatellite genotypes

STRUCTURE analysis showed that the most optimal number of cluster was one (K = 1). Each geographical subpopulation (Eastern subpopulation including Varna and Dolen Chiflik; and Western subpopulation including Stara Zagora, Plovdiv, Sredona gora, Pleven and Teteven) did not form any single cluster, indicating that no significant genetic differentiation between the two populations was found. Because the Balkan Peninsula has been identified as a main glacial refuge area during the Pleistocene in Europe (Randi 2007), the distribution of *M. foina* could have expanded rapidly after the LGM. Zachos et al. (2009) performed STRUCTURE analysis on microsatellite data for population genetic study of the golden jackal *Canis aureus* in Serbia, the Balkan Peninsula, and reported that the maximum probability was found at two clusters (K = 2). But only 31.7–42.5% of the jackals were correctly assigned to their sampling sites. In addition, there was very little differentiation among Serbian *C. aureus*, and the genetic variability estimated by allelic richness and observed and expected heterozygosities was very low (Zachos et al. 2009).

To evaluate recent population size decline from allele data frequencies in Bulgarian *M. foina*, two bottleneck tests were performed in the present study. The first test showed significant heterozygosity excess under some models, but the second test indicated normal L-shaped distribution of allele frequencies. The results indicated that the Bulgarian population of *M. foina* did not experienced bottleneck events recently.

mtDNA diversity

Three nucleotide sites in the 5'-portion of the mtDNA control region (523 bp) were variable among 28 individuals of *M. foina* (Table 2). Based on the substitutions, four haplotypes (MF1–MF4) were identified: haplotype MF1 was most frequent in Bulgaria (46.4%, 13/28 individuals) and possessed one substitution with the other three haplotypes (MF2, MF3 and MF4) (Fig. 2). Haplotype diversity (h) and nucleotide diversity (π) were 0.62 and 0.001371, respectively (Table 2). Inoue et al. (2010) analyzed homologous mtDNA sequences (535–537 bp) in the sable *Martes zibellina*, and reported that h and π of the Hokkaido Island population were 0.73 and 0.00242, respectively. Meanwhile, h and π of the Russia population were 1.00 and 0.00874, respectively. Thus, the genetic diversity of Bulgarian *M. foina* was lower than that of *M. zibellina*. Zachos et al. (2009) analyzed mtDNA control region (392 bp) of Serbian *C. aureus*, and reported that their haplotypes were monomorphic. Such a loss of genetic diversity could have resulted from the recent expansion in the Balkans and indicates a strong founder effect (Zachos et al. 2009). The low genetic diversity in Bulgarian *M. foina* obtained in the present study could be ascribed to the similar biogeographic history to *C. aureus*.

Table 2. Genetic diversity of mtDNA haplotypes in all individuals

Haplotype	Number of samples	Variable sites			Accession no.*
		75	158	257	
MF1	13	G	A	G	AB705456
MF2	2	A	·	·	AB705457
MF3	12	·	·	A	AB705458
MF4	1	·	G	·	AB705459
Haplotype diversity (h)	0.62 (0.05 *SD*)				
Nucleotide diversity (π)	0.001371 (0.001178 *SD*)				

*Sequences will appear in DDBJ/Genebank/EMBL data based with the accession numbers.

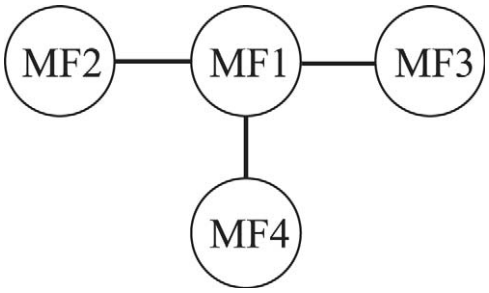

Fig. 2. Parsimony network of mtDNA control region haplotypes for *Martes foina* in Bulgaria. Haplotypes MF1–MF4 in circles are haplotype names. A bar between two circles indicates one base substitution.

Phylogenetic relationships among mtDNA haplotypes

A neighbor-joining tree among haplotypes (Fig. 3) shows that four haplotypes, which were obtained in the present study, were newly identified among European *M. foina*. These haplotypes were clustered to a common group and were closely related to haplotypes reported in Germany (Hosoda et al. 2011) and Europe (Davison et al. 2001: no information on sampling location is available), and two haplotypes of northern Iberian Peninsula (Ruiz-González et al. 2008) formed another single clade. The results suggest that there are at least two mtDNA lin-

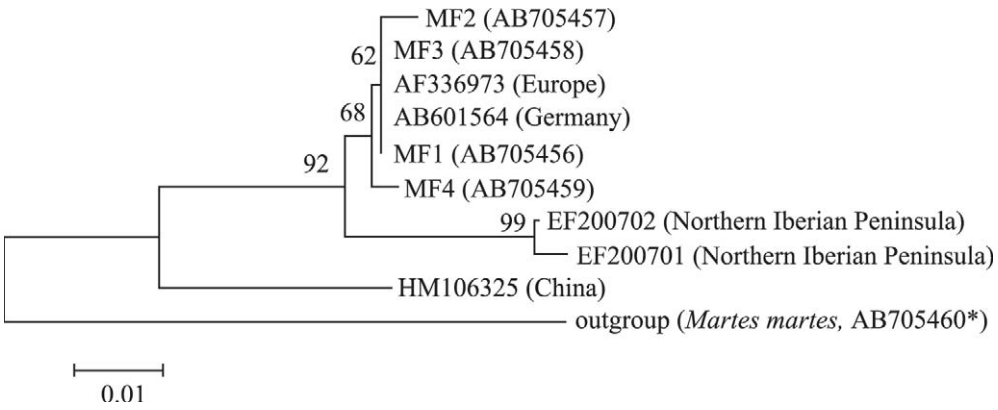

Fig. 3. A neighbor-joining tree constructed for *Martes foina* mtDNA control region haplotypes (251 bp). The homologous sequence of *M. martes* was used as an outgroup. Numbers (%) near internal branches show bootstrap values (>50%) derived from 500 replications. Haplotypes (MF1–MF4) obtained in the present study were compared with previously reported haplotypes of German and European *M. foina* (no information on precise sampling locations is available). These haplotypes from Europe were clustered into a common group, whereas Northern Iberian haplotypes formed another cluster. A haplotype of China is more distantly located. Accession numbers appearing in DDBJ/Genbank/EMBL databases are shown with mtDNA haplotypes. A bar below the tree indicates Tajima-Nei distances.

eages in European populations of *M. foina*, and that the Bulgarian population is included in one of them. Similar to this, the mtDNA lineage of the Balkan *U. arctos* is different from that of the Iberian among European *U. arctos* (Taberlet and Bouvet 1994). Further genetic information on mtDNA as well as microsatellites of *M. foina* around Bulgaria provides insights into better understanding of biogeographical history in the Balkan Peninsula.

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