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Original investigation

Hybridization between the European and Asian badgers (*Meles*, Carnivora) in the Volga-Kama region, revealed by analyses of maternally, paternally and biparentally inherited genes

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ABSTRACT

Two closely related species of *Meles* (Carnivora), the European badger (*M. meles*) and the Asian badger (*M. leucurus*), are distributed allopatrically in continental Eurasia but show a narrow contact zone around the Volga and Kama Rivers, Russia. We analyzed maternally (mitochondrial DNA), paternally (*SRY* gene and CAN-SINEs on the Y chromosome), and biparentally (*CFTR* gene and nine microsatellite loci) inherited genes for evidence of hybridization between the two species in the contact zone. Of 71 badgers examined, we identified 17 individuals as hybrids with mixed genotypes for the first time. Some hybrids appeared to have resulted from repeated backcrossing with the parental species. In addition, the hybridization was symmetric between the two species. Compared with previous palaeontological data, the hybridization between the two species could have resulted from secondary contact due to western expansion in distribution by the Asian badgers.

Introduction

Hybridization between different animal species in nature tends to be uncommon, inhibited by differences in chromosome number, morphology, mating behavior, and/or physiological requirements of the sperm or embryo. In addition, the first filial generation (F_1) is often sterile or exhibits reduced fertility (Dowling and Secor, 1997). However, recent molecular studies have indicated that hybridization is more frequent than previously believed in wild mammals. Study of interspecific hybridization is crucial both for reconstructing evolutionary processes and for conservation efforts. Several cases of hybridization in carnivoran species have been reported, e.g. between Neotropical cats (*Leopardus* spp.) in Brazil (Trigo et al., 2008, 2013); the red wolf (*Canis rufus*) and coyote (*C. latrans*) in the United States (Adams et al., 2007); the European mink (*Mustela lutreola*) and European polecat (*M. putorius*) in Europe (Cabria et al., 2011; Ternovsky and Ternovskaya, 1994; Tumanov and Abramov, 2002); and genet species (*Genetta* spp.) in Southern Africa (Gaubert et al., 2005). Hybridization and genetic introgression, which play an important role in the evolution and diversification of species, can best be studied through molecular analyses (Allendorf et al., 2001).

Eurasian badgers (*Meles*, Mustelidae, Carnivora, Mammalia) are distributed widely in the Palaearctic region from Ireland, through continental Eurasia, to Japan. Based on analyses of mitochondrial DNA (mtDNA) (Del Cerro et al., 2010; Marmi et al., 2006; Tashima et al., 2011a), nuclear DNA (Del Cerro et al., 2010) and morphometry (Abramov and Puzachenko, 2006, 2013), four species are currently recognized: the European badger, *M. meles* (Linnaeus, 1758), in Europe; the Southwest Asian badger, *M. canescens* (Blanford, 1875), in the Middle East and Caucasus; the Asian badger, *M. leucurus* (Hodgson, 1847), in continental Asia; and the Japanese badger, *M. anakuma* (Temminck, 1844), on the Japanese islands. *Meles meles* is distributed from Great Britain and Ireland to the west bank of the Volga River in Europe, whereas *M. leucurus* is distributed from the east bank of the Volga River to Far Eastern Russia, China and the Korean Peninsula. The presumed boundary between the two species is along the Volga and Kama Rivers in western Russia

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(Abramov et al., 2003; Abramov and Puzachenko, 2006; Kinoshita et al., 2017; Tashima et al., 2011a), where morphometric analyses (Abramov and Puzachenko, 2005, 2006; Baryshnikov et al., 2003) have shown that the distributional ranges overlap in a narrow contact zone. Gasilin and Kosintsev (2010) reported that *M. leucurus* has expanded its distribution during the Holocene, reaching the Volga region 100–200 years ago, and the current contact zone between the two species was thought to be recently formed.

Baryshnikov et al. (2003) indicated that the possibility of the hybridization between the *M. meles* and *M. leucurus* based on the morphological analysis using samples obtained from Zhiguli (contact zone between the two species, but the different site from Volga-Kama region). In addition, Abramov and Puzachenko (2007) reported the possibility of hybridization between another *Meles* species pair (*M. leucurus* and *M. canescens*) in a contact zone at the Tian Shan Mountain range in central Asia. It is important to reveal the hybridization of genus *Meles* accompanying the evolutionary processes. Thus, Tashima et al. (2011a) and Kinoshita et al. (2017) tried to obtain the genetic evidence of hybridization between *M. leucurus* and *M. meles* in the overlapping region of the distribution ranges (Volga-Kama region), but they found no evidence due to small sample sizes from Volga-Kama region and limited genetic markers. In order to clarify the possibility of hybridization between the two species, more detailed molecular analyses applying multiple genetic markers using larger number of samples were required. In the present study, the maternally inherited mtDNA control region, paternally inherited regions on the Y chromosome (the *SRY* gene, or sex-determing region on the Y chromosome, and CAN-SINEs, or short interspersed nuclear elements unique to genomes in order Carnivora), and biparentally inherited autosomal genes (*CFTR*, or cystic fibrosis transmembrane regulator, and nine microsatellite loci) were used as multiple genetic markers. Previous studies indicated that mtDNA control region and Y chromosomal genes showed clear genetic differences between *M. meles* and *M. leucurus* (Tashima et al., 2011a,b), and we used these markers for identifying the direction of hybridization. Since nucleotide sequences of *CFTR* can be easily determined by direct sequencing, it is also a useful genetic marker. Microsatellite markers were used to classify the different hybrid categories (F_1, F_2 or backcrosses). After presenting clear genetic evidence of hybridization between the two species, we discuss the history of this hybridization in the context of *Meles* phylogeography.

Material and methods

Samples and DNA extraction

Tissue samples from 66 Eurasian badger individuals were obtained from legal hunting and captures in Kirov Province (Russia), the sympatric zone between *M. meles* and *M. leucurus* (Fig. 1, Supplementary Table S1). In addition, Tashima et al. (2011a) had previously published sequences from the mtDNA and *SRY* genes from five individuals (MEL-KRV1–3, 5 and 6) obtained from the same region as the present study. Including these previous samples, our total number of samples was 71 (Supplementary Table S1).

Total DNA was extracted from muscle samples preserved in ethanol by using the DNeasy Blood and Tissue Kit (Qiagen), and from hair and skin samples (Kir-23, 67, 69, MEL-KRV5 and 6) by using the QIAamp DNA Investigator Kit (Qiagen), according to the manufacturer's protocols. The purified DNA was eluted with TE buffer and stored at 4 °C.

Amplification and sequencing of the mtDNA control region

The polymerase chain reaction (PCR) was used with primers UR1 (Taberlet and Bouvet, 1994) and ANK-R1 (Tashima et al., 2011a) to amplify a fragment of 542–572 base-pairs (bp) from the mtDNA control region. PCR amplifications were performed in 20 μl reaction volumes each containing 2 μl of 10× buffer, 1.6 μl of dNTP mixture, 0.1 μl of r*Taq* DNA polymerase (5 U/μl; Takara), 0.2 μl of each primer (25 pmol/μl each), 2 μl of DNA extract and 13.9 μl of distilled water. PCR conditions in a TP350 thermal cycler (Takara) were 94 °C for 1 min; 30 cycles of 94 °C for 1 min, 58.3 °C for 1 min and 72 °C for 1 min; and 72 °C for 4 min. PCR products were purified with the QIAquick PCR Purification Kit (Qiagen). Cycle sequencing conditions using the Big Dye Terminator v1.1 or v3.1 Cycle Sequencing Kit (Applied Biosystems) and an ABI3730 DNA Analyzer (Applied Biosystems) were 96 °C for 1 min and 25 cycles of 96 °C for 10 s, 50 °C for 5 s and 60 °C for 4 min.

Four samples (Kir-2, 16, 44 and 54) could not be directly sequenced, and amplicons were cloned before sequencing. To obtain PCR products for cloning, the PCR mixture contained 4.0 μl of 5× buffer (Takara), 1.6 μl of dNTP mixture, 0.3 μl each of phosphorylated primers UR1 and ANK-R1 (each 25 pmol/μl), 0.4 μl of bovine serum albumin, 0.4 μl of PrimeSTAR GXL DNA polymerase (Takara), 2 μl of DNA extract and 11 μl of distilled water. PCR conditions were 94 °C for 2 min; 30 cycles of 98 °C for 10 s, 60 °C for 1 min and 68 °C for 30 s; and 68 °C for 5 min. The PCR products were purified with the QIA quick PCR Purification Kit (Qiagen) according to the manufacturer's protocol and cloned into pBluescript II SK+ (Agilent Technologies). Positive clones containing target amplicons identified by blue/white selection were randomly picked and cultured overnight. Plasmid DNA was purified with the QIAprep Spin Miniprep Kit (Qiagen) and sequenced in both directions with M13 forward and reverse primers (Messing, 1983).

Amplification and sequencing of nuclear DNA sequences

PCR amplifications for the *SRY* gene and the CAN-SINEs in the final intron of the *ZFY* gene (zinc-finger protein on the Y chromosome) were performed as for the mtDNA control region. Primers MELSN-F2 and MELSN-R2 (Tashima et al., 2011b) were used to amplify the CAN-SINEs, and primers MSRY-F2 and MSRY-R2 (Yamada and Masuda, 2010) to amplify the *SRY* gene. Sequencing primers for the *SRY* gene were MELSR-f2 and MELSR-r2 (Kinoshita et al., 2017).

PCR amplifications of cystic fibrosis transmembrane regulator (*CFTR*) intron 25 were performed as for the mtDNA control region, with primers HCFTREX22D (Venta et al., 1996) and RMel-I22 (Del Cerro et al., 2010). For sequencing, we designed a new reverse intermediate primer, MelCFTR-r (5′-CACCTGTGTGGCTCAGTTG-3′), which gave clear sequencing chromatograms at the target position (arrows in Supplementary Figure S1).

Sequence analyses

The nucleotide sequences we obtained were validated and aligned by using MEGA ver. 6 (Tamura et al., 2013). Phylogenetic trees for the mtDNA control region and *SRY* gene were constructed by the maximum likelihood and neighbor-joining methods (Saitou and Nei, 1987) using MEGA. Kimura's two-parameter model (Kimura, 1980) was selected as the best-fit substitution model for the maximum likelihood trees of mtDNA control region and *SRY* gene, respectively. In addition to the haplotype sequences we obtained, the phylogenetic analyses included sequences for control region and *SRY* haplotypes from across the distributional ranges *M. meles* and *M. leucurus*, taken from DNA databases (Supplementary Table S2). Bootstrap values were calculated with 1000

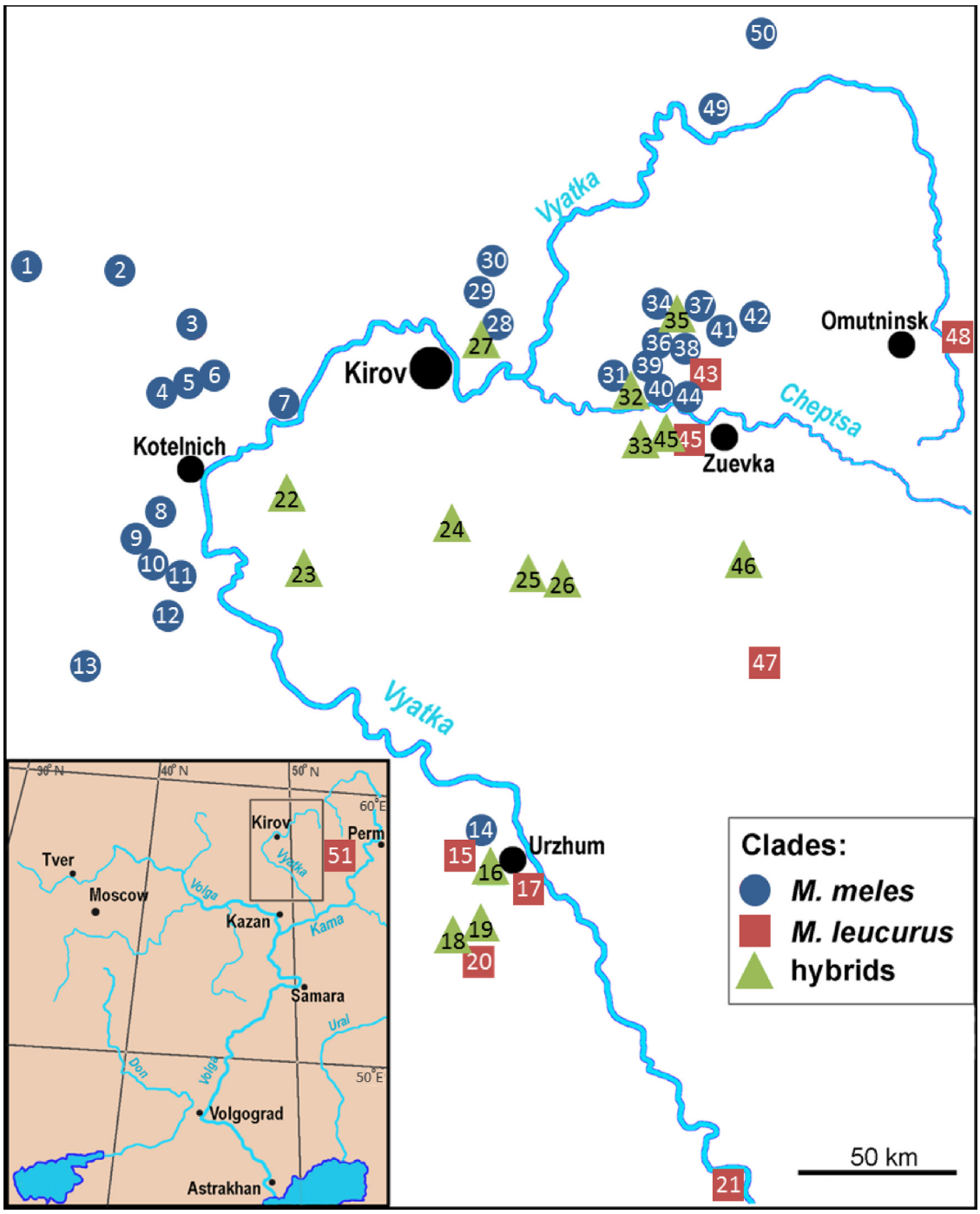

Fig. 1. Sampling locations for European badgers (*Meles meles*) and Asian badgers (*M. leucurus*) examined in the present study. Numbers within the symbols correspond to 'No. on the map' in Supplementary Table S1.

replications for neighbor-joining and maximum likelihood methods. The mtDNA control region of the hog badger (*Arctonyx collaris*; AJ563704; Marmi et al., 2006), and the *SRY* genes of the Japanese marten (*Martes melampus*; AB491589; Yamada and Masuda, 2010) and the sable (*M. zibellina*; AB491590; Yamada and Masuda, 2010) were included as outgroup taxa.

Microsatellite genotyping

We used nine previously reported pairs of PCR primers for microsatellite loci identified in the European badger (*Mel*101, *Mel*102, *Mel*104–110) (Carpenter et al., 2003). Carpenter et al. (2003) found no linkage disequilibria for any locus combination. Each forward primer was labeled with a fluorescent dye (6-FAM, PET, VIC or NED; Applied Biosystems). PCR amplifications were performed in 5.5 μl reaction volumes each containing 2.5 μl of 2×Multiplex Mix (Qiagen), 0.5 μl of primer mixture (0.5 pmol/μl each primer), 1 μl of DNA extract and 1.5 μl of distilled water. PCR conditions in a TP350 thermal cycler (Takara) were 95 °C for 15 min; 25 cycles of 94 °C for 30 s, 58 °C for 90 s and 72 °C for 1 min; and 60 °C for 30 min. One μl of PCR product was added to 10 μl of HiDi Formamide with the size standard GeneScan 500 LIZ (Thermo

Fisher Scientific). PCR fragments were separated by size with an ABI3730 DNA Analyzer (Applied Biosystems), and microsatellite genotypes were determined by using Peak Scanner 2.0 software (Applied Biosystems).

Microsatellite analyses

Number of alleles (N_A) and observed (H_O) and expected (H_E) heterozygosities were calculated for each microsatellite locus by using Arlequin 3.5.1.2 (Excoffier and Lischer, 2010). Deviation of the microsatellite loci from Hardy-Weinberg equilibrium (HWE) was tested using Fisher's method (Freeman and Halton, 1951).

We used two different Bayesian clustering methods, STRUCTURE and NEWHYBRIDS, to estimate proportions of admixture from microsatellite data. STRUCTURE version 2.3.4 (Pritchard et al., 2000; Falush et al., 2003, 2007) was used to infer assignment probabilities (q) to either the *M. meles* or *M. leucurus* species group. Values of q were estimated by the number of populations from one to five (K = 1–5) and running 1,000,000 Markov chain Monte Carlo (MCMC) iterations, after a burn-in of 100,000 iterations, under the admixture ancestry and correlated allele frequency models without prior genetic information. Ten runs were carried out for each value of K, and we adopted the values of K based on the rate of change in the log probability of data between successive K values (delta K) (Supplementary Table S3). Figure was constructed based on the run with the highest likelihood values.

NEWHYBRIDS version 1.1 beta (Anderson and Thompson, 2002) estimates Bayesian posterior probabilities (Q) generated by the MCMC method for identifying hybrid individuals generated during n = 2 or n = 3 generations of potential inbreeding. Six genotype frequency classes were defined: pure *M. meles*, pure *M. leucurus*, F_1 hybrids, F_2 hybrids, F_1 hybrid backcrosses to *M. meles* (BxMm) and F_1 hybrid backcrosses to *M. leucurus* (BxMl). Simulations were run using a burn-in period of 100,000 sweeps followed by 1,000,000 MCMC iterations.

Patterns of genetic differentiation were visualized with a factorial correspondence analysis (FCA) implemented in GENETIX version 4.02 (Belkhir et al., 2000).

Detection of hybrids

Del Cerro et al. (2010) reported a polymorphic site in the *CFTR* gene between *M. leucurus* and *M. meles*, with the former showing an A nucleotide at position seven in *CTFR* (accession nos. GU247586–GU247590), and the latter a T nucleotide (GU247574–GU247578). Individuals resulting from hybridization events were detectable as showing a double sequencing peak in this position (Supplementary Figure S1).

Potential hybrids were identified by STRUCTURE (q < 0.90) and NEWHYBRIDS (Q <0.90) scores, and by lack of congruence in the mtDNA and Y chromosomal gene phylogenies. For example, because Kir-29 possesed *meles*-type microsatellites (q = 0.948 and Q = 0.968) and *leucurus*-type mtDNA, we judged this individual as a hybrid, and removed it from the non-hybrid populations.

Results

MtDNA control region

From 71 badgers collected in the contact zone, we identified 13 haplotypes for the mtDNA control region (542–544 bp) (Supplementary Table S1); haplotype KL-1 from individual Kir-44 had a 30 bp insertion, and so the total lengh of its mtDNA control region was 572 bp. We constructed a phylogenetic tree using the 13 haplotypes we detected and sequences obtained from the DNA Data Bank of Japan (DDBJ) (Fig. 2A). Of the 13 haplotypes, four (KL-1, E-1, E-3 and E-4, marked by asterisks in Fig. 2A) were distributed among 17 individuals and grouped within a *leucurus* clade. Nine haplotypes (KM-1–6, FN1, W-1 and W-4) were distributed among 54 individuals and grouped within in a *meles* clade. Haplotype KL-1 in the *leucurus* clade, and haplotypes KM-1–6 in the *meles* clade were novel haplotypes detected in our study (accession nos. LC333397–LC333403).

Table 1
Number of individuals used each genotype of *CFTR* gene.

	Seqencing peak at nucleotide position 7	Number of individuals
All		66
Meles meles	homozygous of T	49
M. leucurus	homozygous of A	12
Hybrid	heterozygous of T and A	5

SRY and CAN-SINEs

We identified two *SRY* haplotypes distributed among 27 male badgers. One was identical to previously reported haplotype Mw1 (1052 bp; accession no. AB539136; Tashima et al., 2011a) and occurred in six individuals, and the other, Me5 (1058 bp; accession no. LC333404), was novel and occurred in 21 individuals (Supplementary Table S1). In the phylogenetic tree of *SRY* haplotypes (Fig. 2B), Mw1 from *Meles meles* formed a sister group to haplotype Mc1 from *M. canescens*, while Me5 formed a clade with four other *M. leucurus* haplotypes taken from the DDBJ database.

Two CAN-SINE haplotypes were distributed among the 27 male badgers, and these were identical to previously reported haplotypes SNE1 (AB551119) or SNW1 (AB551120) (Tashima et al., 2011b). The resultant phylogenetic tree based on CAN-SINEs (figure not shown) was identical to that in the previous study (Tashima et al., 2011b).

CFTR gene

We analyzed partial sequences of the *CFTR* gene (160 bp), which is biparentally inherited, for evidence of hybridization between the two species. *Meles leucurus* individuals were homozygous for an A nucleotide in position 7 from the 5′ end, whereas *M. meles* individuals were homozygous for a T nucleotide in this position (Supplementary Figure S1). We detected five heterozygous individuals that showed a mixed sequencing peak at this position, indicative of hybridization events. Amplification was not successful for five of 71 badger specimens (Kir-23, 30, 38, 67 and 69). Among 66 badgers, 12 were homozygous A/A (*leucurus* type), 49 were homozygous T/T (*meles* type), and five badgers were heterozygous A /T (hybrids) (Table 1).

Microsatellite analysis

We did not obtain any signals for the microsatellite loci from six of 71 badger specimens (Kir-23, 30, 38, 60, 67 and 69). All nine microsatellite loci were polymorphic in *M. leucurus* and all but one (*Mel* 109) were polymorphic in *M. meles* (Table 2). Microsatellite analyses were conducted after exclusion of the potential hybrids, thus the number of all individuals was 65 (containing hybrids), including 42 pure *M. meles* and six pure *M. leucurus*. The number of alleles ranged from one to ten in *M. meles* and three to five in *M. leucurus*; observed heterozygosity (*Ho*) ranged from 0.167 to 0.786 in *M. meles* and 0.333 to 1.000 in *M. leucurus*, and expected heterozygosity (H_E) ranged from 0.155 to 0.827 in *M. meles* and 0.439 to 0.864 in *M. leucurus* (Table 2). In addition, each microsatellite locus did not show a significant deviation from HWE.

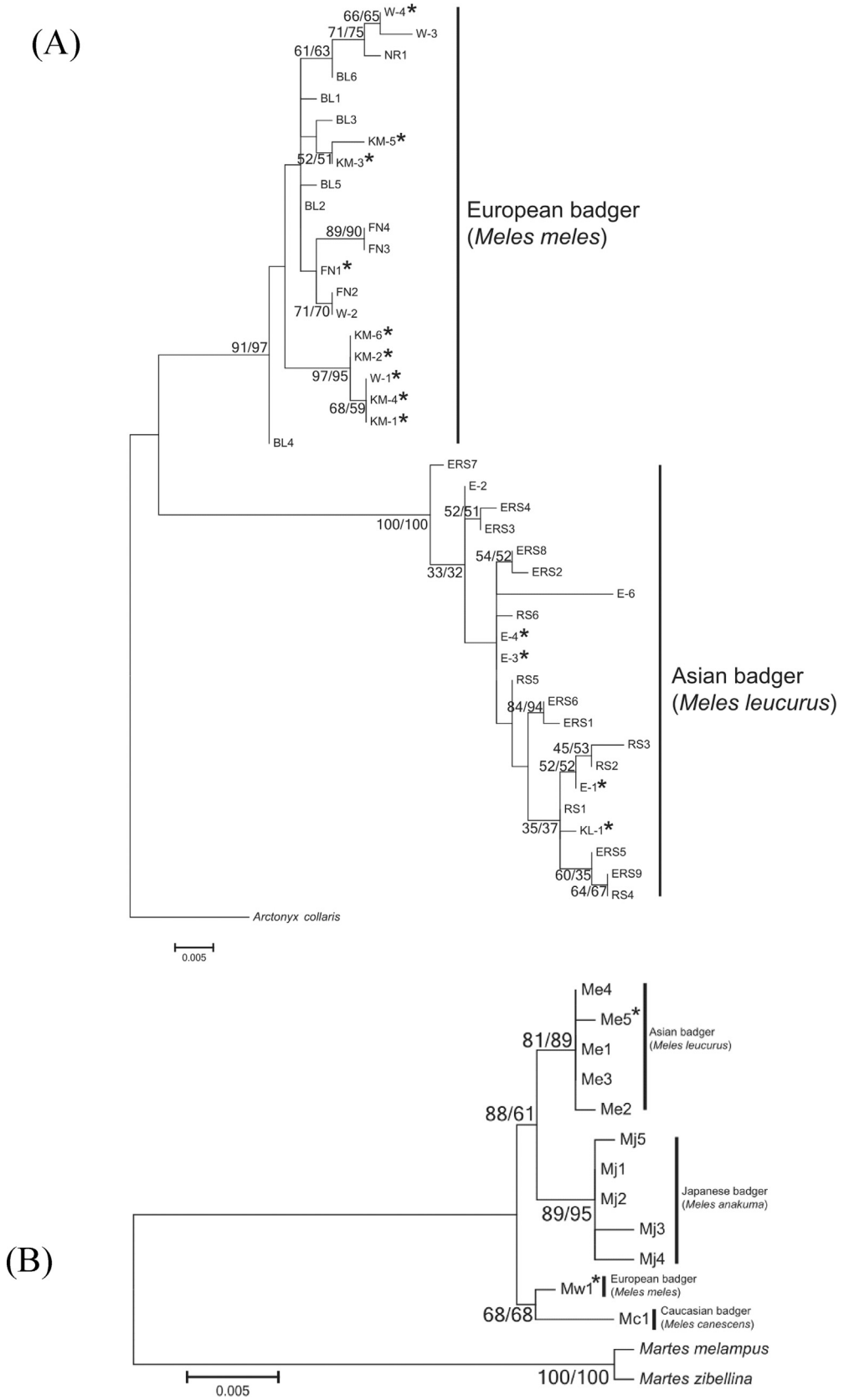

Fig. 2. Phylogenetic trees of mtDNA control region haplotypes (A) and *SRY* gene haplotypes (B) using two reconstruction methods (maximum likelihood / neighbor-joining). Numbers near internal branches are bootstrap values (>30%) derived from 1000 replications for maximum likelihood / neighbor-joining methods. The scale below the tree shows Kimura's two–parameter distance. Asterisks indicate haplotypes found in the present study. Haplotypes without asterisks were taken from the DDBJ database (Supplementary Tabele S2). Homologous sequences of *Arctonyx collaris* (A) and *Martes melampus* and *Martes zibellina* (B) were used as outgroups.

Table 2
Summary of the genetic variation for nine microsatellite loci in the badgers *Meles meles* and *M. leucurus*.

		All (n = 65)	*M. meles* (n = 42)				*M. leucurus* (n = 6)			
Locus	allele range	N_A[a]	N_A[a]	H_O[b]	H_E[c]	*P*-value[d]	N_A[a]	H_O[b]	H_E[c]	*P*-value[d]
*Mel*101	99-119	7	2	0.167	0.155	1.000	5	1.000	0.864	0.797
*Mel*102	175-192	9	5	0.595	0.687	0.436	4	0.500	0.682	0.132
*Mel*104	302-341	14	7	0.786	0.793	0.870	3	0.833	0.621	0.636
*Mel*105	121-142	9	6	0.786	0.827	0.366	4	0.667	0.561	1.000
*Mel*106	196-208	7	5	0.690	0.687	0.323	3	0.333	0.439	0.275
*Mel*107	256-292	12	10	0.786	0.798	0.575	4	0.833	0.742	1.000
*Mel*108	302-315	6	4	0.667	0.632	0.401	3	0.500	0.439	1.000
*Mel*109	98-109	5	1	–	–	–	5	0.667	0.727	0.501
*Mel*110	308-328	7	5	0.429	0.492	0.102	3	0.500	0.682	0.305

[a] Number of alleles.
[b] Observed heterozygosity.
[c] Expected heterozygosity.
[d] HWE *P*-values ($P < 0.005$) were estimated using Fisher's method (Freeman and Halton, 1951).

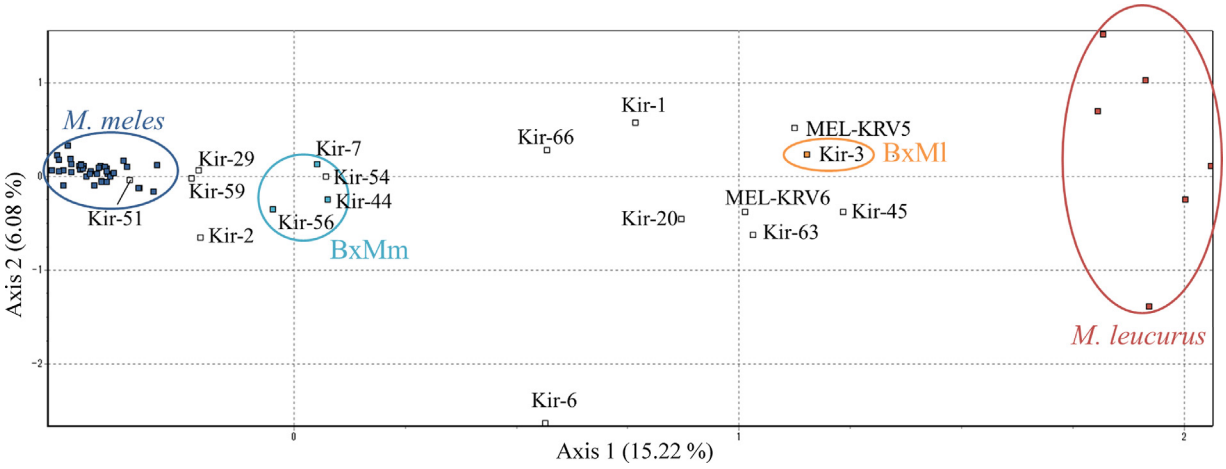

Fig. 3. Factorial correspondence analysis (FCA) based on nine microsatellite loci, showing strong separation between *M. meles* (blue) and *M. leucurus* (red). Progeny from F_1 hybrids backcrossed to *M. meles* (BxMm) (light blue) and F_1 hybrids backcrossed to *M. leucurus* (BxMl) (orange) show intermediate positions. White squares indicate individuals not correctly assigned to genotype class based on the NEWHYBRIDS analysis ($Q < 0.90$) (Fig. 4), or mtDNA or Y chromosomal alleles (Table 3) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

The results of the factorial correspondence analysis (FCA; Fig. 3) clearly show separate clustering between *M. meles* and *M. leucurus*. This analysis clearly indicated clustering of the two species (non-hybrids) and the hybrids.

Detection of hybridization

In all, we determined 42 individuals to be non-hybrid *M. meles* and six individuals to be non-hybrid *M. leucurus*. Two Bayesian analyses (Fig. 4) also revealed the clustering of individuals between *M. meles* and *M. leucurus*, and hybrid individuals. The number of populations was identified to be two ($K = 2$) based on delta *K* value (Supplementary Table S3). The STRUCTURE analysis at population number $K = 2$ showed 45 individuals as pure *M. meles* ($q > 0.90$), 13 individuals as pure *M. leucurus* ($q > 0.90$) and seven individuals as an ancestral admixture ($q < 0.90$). The NEWHYBRIDS analysis indicated 44 individuals as pure *M. meles*, six as pure *M. leucurus*, three (Kir-56, 44 and 7) as backcrosses with *M. meles* (BxMm), and one (Kir-3) as a backcross with *M. leucurus* (Bxml), at *Q* thresholds above 0.90. The remaining eleven individuals were not correctly assigned to any genotype frequency class because of low *Q* (0.538–0.887) (Table 3). We did not estimate the frequencies of F_2 and F_1 hybrids. Among the 65 individuals analysed, 17 individuals were identified as hybrids based on the genotyping of microsatellite, mtDNA and Y chromosomal sequences (Table 3).

Discussion

This study obtained the first genetic evidence for hybridization between *M. leucurus* and *M. meles* in the Volga-Kama region, where the two species' distribution ranges overlap, based on maternally (mtDNA control region), paternally (Y chromosomal DNA) and biparentally (*CFTR* gene and nine microsatellite loci) inherited genes. Among 71 badger specimens from this contact zone, 17 individuals showed features of ancestral admixture (Table 3). Only four among these 17 individuals (Kir-3, 7, 44 and 56) were clearly assigned to genotype frequency classes with *Q* thresholds above 0.90; the other 13 individuals were not clearly assigned to any genotype frequency class based on the three types of genetic data. For example, microsatellite data indicated Kir-51 and 29 to be pure *M. meles*, but the mtDNA phylogeny included them in the *M. leucurus* clade.

Our results show that at least some hybrids between *M. meles* and *M. leucurus* are viable and fertile, and that repeated backcrossing with the parental species occurs. For instance, Kir-18 was heterozygous for *M. meles* and *M. leucurus CFTR* alleles, but the microsatellite data indicated pure *M. meles* (score $q = 0.994$ and $Q = 0.9985$). We consider this individual to have resulted from repeated backcrossing with *M. meles* after hybridization occurred between its ancestors, leading to genetic introgression in the Volga-Kama region.

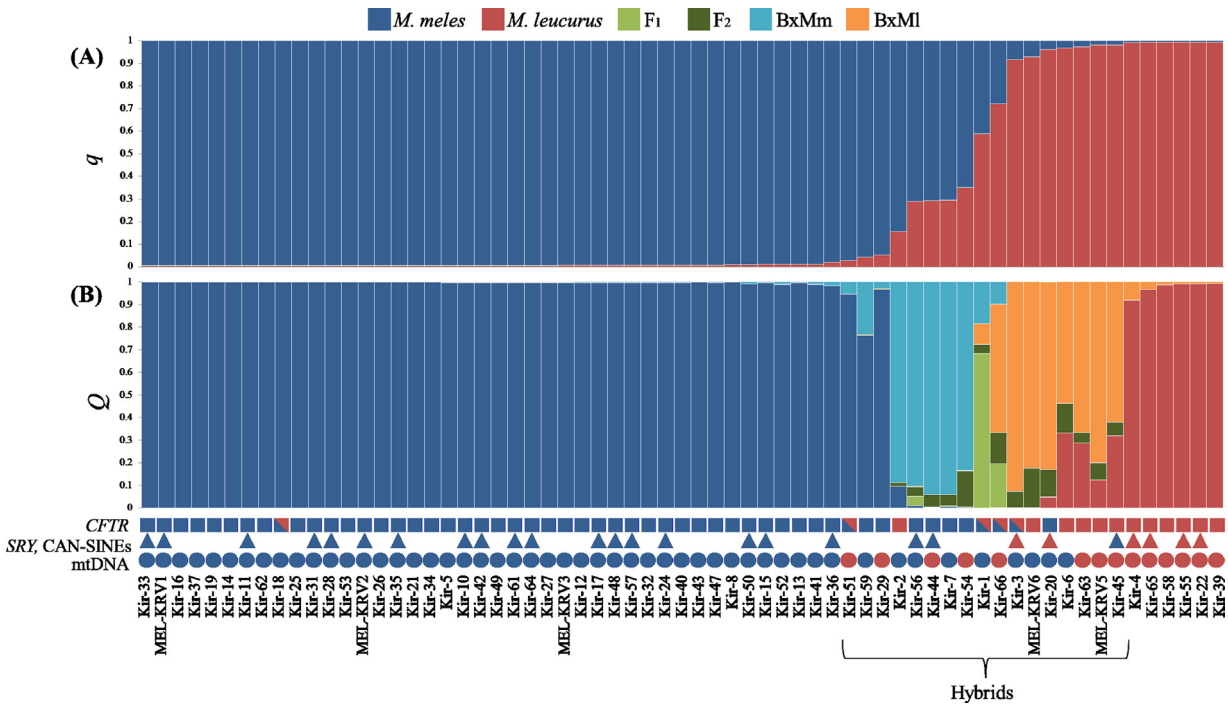

Fig. 4. Results of Bayesian clustering analyses based on nine microsatellite loci. (A) STRUCTURE analysis, with posterior probability *q* for $K = 2$. (B) NEWHYBRIDS analysis, with posterior probability Q estimated for each of the following genotype frequency classes: *M. meles*, *M. leucurus*, F_1 hybrids (F_1), F_2 hybrids (F_2), F_1 hybrid backcross to *M. meles* (BxMm) and F_1 hybrid backcross to *M. leucurus* (BxMl). The color key at the top defines parental and hybrid genotypes, with non-hybrid *Meles meles* genotypes in blue and non-hybrid *M. leucurus* genotypes in red. Individuals are labeled at the bottom. Symbols indicate the genotype indicated by the mtDNA allele (circles), the Y-chromosomal *SRY* and CAN-SINE alleles (triangles) and the nuclear *CFTR* allele (squares); individuals heterozygotic for *M. meles* and *M. leucurus CFTR* alleles are indicated by red/blue squares (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Table 3
Samples detected as hybrids from microsatellite, mtDNA and Y chromosomal data.

					NEWHYBRIDS (*Q*)[b]						STRUCTURE (*q*)[c]	
Sample ID	Sex[a]	mtDNA	*SRY*, CAN-SINEs	*CFTR*	*M. meles*	*M. leucurus*	F_1	F_2	BxMm	BxMl	*M. meles*	*M. leucurus*
Kir-1	F	*M. meles*	–	heterozygote	0.000	0.000	0.684	0.039	0.185	0.092	0.411	0.589
Kir-2	F	*M. meles*	–	*M. leucurus*	0.096	0.000	0.001	0.017	0.887	0.000	0.843	0.157
Kir-3	M	*M. meles*	*M. leucurus*	heterozygote	0.000	0.000	0.001	0.072	0.000	0.928	0.084	0.916
Kir-6	F	*M. meles*	–	*M. leucurus*	0.000	0.332	0.000	0.130	0.000	0.538	0.031	0.969
Kir-7	F	*M. meles*	–	*M. meles*	0.006	0.000	0.003	0.049	0.941	*0.000*	0.705	0.295
Kir-20	M	*M. meles*	*M. leucurus*	*M. meles*	0.000	0.048	0.001	0.120	0.000	0.830	0.038	0.962
Kir-29	F	*M. leucurus*	–	*M. meles*	0.968	0.000	0.000	0.003	0.029	0.000	0.948	0.052
Kir-44	M	*M. leucurus*	*M. meles*	*M. meles*	0.000	0.000	0.002	0.056	0.942	*0.000*	0.706	0.294
Kir-45	M	*M. leucurus*	*M. meles*	*M. leucurus*	0.000	0.320	0.000	0.060	0.000	0.620	0.018	0.982
Kir-51	F	*M. leucurus*	–	heterozygote	0.946	0.000	0.000	0.001	0.054	0.000	0.972	0.028
Kir-54	F	*M. leucurus*	–	*M. meles*	0.003	0.000	0.000	0.160	0.834	*0.002*	0.649	0.351
Kir-56	M	*M. meles*	*M. meles*	*M. meles*	0.009	0.000	0.043	0.041	0.904	*0.003*	0.711	0.289
Kir-59	F	*M. meles*	–	*M. meles*	0.764	0.000	0.000	0.003	0.233	0.000	0.956	0.044
Kir-63	F	*M. leucurus*	–	*M. leucurus*	0.000	0.287	0.000	0.046	0.000	0.666	0.027	0.973
Kir-66	F	*M. leucurus*	–	heterozygote	0.000	0.000	0.194	0.140	0.097	0.569	0.280	0.720
MEL-KRV5	F	*M. leucurus*	–	*M. leucurus*	0.000	0.123	0.000	0.077	0.000	0.800	0.019	0.981
MEL-KRV6	F	*M. meles*	–	*M. leucurus*	0.000	0.000	0.000	0.175	0.000	0.825	0.071	0.929

[a] M, male; F, female.
[b] Posterior probability (*Q*) of each genotype frequency classes, *M. meles*, *M. leucurus*, F_1 hybrids (F_1), F_2 hybrids (F_2), F_1 hybrids backcross to *M. meles* (BxMm) and F_1 hybrids backcross to *M. leucurus* (BxMl) was estimated by using the NEWHYBRIDS.
[c] Posterior probability (*q*) was calculated by STRUCTURE ($K = 2$).

Some studies have reported asymmetric hybridization in family Mustelidae (e.g. Ternovsky and Ternovskaya, 1994). Cabria et al. (2011) revealed that male European polecats (*Mustela putorius*) mate with female European minks (*M. lutreola*), but that the reciprocal cross does not occur; this asymmetric hybridization was thought to be caused by differences in body size between the genders (sexual dimorphism). By contrast, our study indicated that hybridization between *M. meles* and *M. leucurus* is symmetric. For example, the NEWHYBRIDS analysis indicated that Kir-3 resulted from a BxMl cross (an F_1 hybrid backcrossed to *M. leucurus*), as this individual had *meles*-type mtDNA and *leucurus*-type Y chromosomal DNA. Kir-3 is explainable by a male *M. leucurus* mating with a female *M. meles*, followed by the F_1 hybrid mating with a male *M. leucurus*. In contrast, Kir-44 presumably resulted from a BxMm cross (an F_1 hybrid backcrossed to *M. meles*), as this individual had *leucurus*-type mtDNA and *meles*-type of Y chromosomal DNA. Kir-44 is explainable by a male *M. meles* mating with a female *M. leucurus*. One reason for symmetric hybridization may be that *M. meles* and *M. leucurus* are similar in body size: the body length of *M. meles* is 73.6 ± 5.5 cm (mean ± standard deviation) in males and

69.9 ± 5.2 cm in females (Virgós et al., 2011); that of *M. leucurus* is 70.0–78.0 cm in males and 61.0–70.0 cm in females (Heptner et al., 1967).

Hybridization typically occurs when allopatrically diverged species undergo secondary contact, or when anthropogenic habitat disturbances cause a breakdown in reproductive isolation between incipient species that have recently diverged in sympatry (Hasselman et al., 2014). The current Ural–Volga region can be a region for the secondary contact of the two badger species. Gasilin and Kosintsev (2010) reported concerted distributional changes for *M. meles* and *M. leucurus* in the Ural-Volga region, based on a morphological analysis of fossils excavated from Holocene-epoch layers. *Meles leucurus* has expanded its distribution 1500 km westward during the Holocene, reaching the Volga region 100–200 years ago, without forming extensive or long-lasting overlapping zones with *M. meles* (Gasilin and Kosintsev, 2010). Hybridization between the two species could have resulted from secondary contact due to this expansion in distribution by *M. leucurus*.

Tashima et al. (2011a) identified two female badgers MEL-KRV5 and MEL-KRV6 as *M. leucurus* and *M. meles*, respectively, based on only maternally inherited mtDNA types. By contrast, our microsatellite and *CFTR* analyses revealed that these two individuals were not pure species, but instead were hybrids. This demonstrates that information from multiple loci is necessary for precisely detecting hybridization. Rodriguez et al. (2008) reported the accurate estimation of hybridization of *Crocodylus* species based on the analyses of mtDNA, morphology and microsatellite, and showed clear evidence of the hybridization between *Crocodylus acutus* and *C. moreletii*.

Like the sympatric zone between *M. meles* and *M. leucurus* in the Volga-Kama region, hybridization zones exist between *M. leucurus* and *M. canescens* occurs in the Tian Shan Mountains, and between *M. meles* and *M. canescens* in the Caucasus Mountains (Abramov and Puzachenko, 2007, 2013). Comprehensive sampling around such zones of sympatry, and more detailed population genetic studies, will be important in revealing the direction and magnitude of hybridization among *Meles* species and also among other mammalian species.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.mambio.2018.05.003.

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