

# Characterisation of twenty-one European badger (*Meles meles*) microsatellite loci facilitates the discrimination of second-order relatives

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**Abstract** The European badger (*Meles meles*) breeds plurally in lowland England and is important economically due to its link with bovine tuberculosis (*Mycobacterium bovis*) transmission. To understand disease transmission and facilitate effective management, it is vital to elucidate the social structure of badger groups. To improve parentage assignment and the discrimination of relatives, we isolated and characterised 21 polymorphic microsatellite loci in 24 individuals from Wytham Woods, Oxfordshire, UK. These 21 loci increased the discrimination power between full-siblings and half-siblings from 71 to 88%, when added to the existing 31 loci. Similarly, the combined non-exclusion

probability increased from  $3.0 \times 10^{-8}$  to  $5.8 \times 10^{-13}$ . Newly isolated *Mel-592* (FR745854) was X-linked, based on the genotypes of 48 known-sex individuals and will enhance the genetic sex-typing of badgers.

**Keywords** European badger · *Meles meles* · Mustelidae · Microsatellite · Parentage · X-chromosome linked locus

In the UK and Ireland, European badgers (*Meles meles*) are a wildlife reservoir for bovine tuberculosis (*Mycobacterium bovis*), and have been implicated in disease transmission to cattle, with important economic ramifications (McDonald et al. 2008). Understanding host ecology, such as the mating system and relatedness structure, is important so that patterns of disease transmission are elucidated to enable effective disease management (Pope et al. 2007). Badgers are nocturnal, breed plurally in lowland England (Carpenter et al. 2005; Dugdale et al. 2007), and cubs are raised underground (Dugdale et al. 2010); thus, parentage cannot be assigned from behavioural traits alone, and molecular genetic markers are required.

Fifty-seven microsatellites have previously been characterised for badgers (Bijlsma et al. 2000; Carpenter et al. 2003; Domingo-Roura et al. 2003; Huck et al. 2008) and 31 of these are polymorphic in the Wytham Woods population (Domingo-Roura et al. 2003; Dugdale et al. 2007; Annavi et al. unpublished data). Blouin et al. (2003) proposed that around 50 polymorphic microsatellite loci are required to differentiate second-order relatives from first-order.

As badger groups contain close relatives (Dugdale et al. 2008) we characterised 21 additional microsatellite loci from three genomic libraries (Table 1 and Supplementary Table 1). Libraries 1 and 2 were prepared as described in Carpenter et al. (2003). Library 3 was constructed from one

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**Table 1** Characterisation of 21 microsatellites for European badgers

Locus	EMBL	Clone library	Clone	Repeat motif	Primer sequence (5'-3')	Fluorescent label (FP)	Expected/observed allele size (bp)	N	A	H <sub>o</sub>	H <sub>E</sub>	P <sub>HWE</sub>	F(null)
<i>Mel-153</i>	AJ230722	2	B18E04	(CA) <sub>12</sub>	F:ATCTGGCATCTAACAGGAGATAC R:GATCTCTGCTGTCTTTGTGTG	6FAM	309/309–313	24	2	0.29	0.31	1.00	0.02
<i>Mel-161</i>	AJ293385	1	BAD03A	(CA) <sub>15</sub>	F:TTCTATGCTTGGACCTTCTACTCTTT R:ATTACCTAAGGAAGCGGAGGA	HEX	361/355–359	24	3	0.50	0.58	0.40	0.06
<i>Mel-186</i>	FR745448	3	09D01(S)	(CA) <sub>20</sub>	F:CTCAACATCCAAGGCTGACTG R:GATCATGACCTGAGCCGAAG	6FAM	439/431–437	23	3	0.43	0.52	0.10	0.06
<i>Mel-191<sup>a</sup></i>	FR745453	3	09E03(S)	(CA) <sub>21</sub>	F:GAATTAGAGAACCCAGAAATACC R:GTTGAAATTAGCTAACAGAGTGG	HEX	273/273–275	24	2	0.08	0.08	1.00	-0.01
<i>Mel-203</i>	FR745465	3	13F01(S)	(GAAA) <sub>8</sub>	F:TGCCTGCCTTAATGTTGATG R:TCACCAAGTTGGCACTGAAG	HEX	453/441–453	24	4	0.54	0.60	0.30	0.04
<i>Mel-211</i>	FR745473	3	10A04	(AC) <sub>19</sub>	F:GGAGTCCTATGCCAAGAACAG R:AAATCTGTTACTCTCTGCTCCATC	HEX	267/265–267	23	2	0.44	0.43	1.00	-0.01
<i>Mel-213</i>	FR745475	3	10A06	(CA) <sub>15</sub>	F:CCCATAAAGGTTGAGGACAG R:AATAAGGTGAAGACCCAAAGGTG	HEX	242/246–250	24	2	0.38	0.40	1.00	0.03
<i>Mel-232</i>	FR745494	3	10C06	(CA) <sub>20</sub>	F:TGGAACCTGATGACTCACTAAACTC R:TACTTCCACTGGTCCAATCC	HEX	272/268–274	22	3	0.27	0.25	1.00	-0.07
<i>Mel-243</i>	FR745505	3	10D08	(AC) <sub>21</sub>	F:TGTTAGTCCACCTCAGATG R:ACCAATACAGAGTAAGCCTACAAACC	6FAM	275/269–273	24	3	0.50	0.67	0.17	0.14
<i>Mel-246</i>	FR745508	3	10D11	(GATA) <sub>9</sub>	F:GGCGTTAAGGTTCTCCAAAG R:CTTGCCAGCCTTTGTAACCTG	6FAM	157/151–159	24	3	0.33	0.40	0.66	0.07
<i>Mel-253</i>	FR745515	3	10E07	(GT) <sub>15</sub>	F:CACCCAAATCCACCATGAG R:CCAGAAATGCCCTTATGGTCAC	HEX	218/220–224	24	3	0.46	0.58	0.13	0.09
<i>Mel-451</i>	FR745713	3	14A05	(AAAG) <sub>11</sub>	F:CCGGTCTGAGGACAGCA R:CCCACCATAGGGCTCAAAC	6FAM	217/201–227	24	5	0.54	0.61	0.72	0.05
<i>Mel-483</i>	FR745745	3	14F03	(AAAG) <sub>12</sub>	F:CTGGATTGAGCCCTGAG R:GGTTAATGGTCAATCAAACCACT	6FAM	235/227–231	23	2	0.48	0.48	1.00	-0.01
<i>Mel-499</i>	FR745761	3	14H03	(TTTC) <sub>11</sub>	F:TGACCTGAGCCGAAGTCAG R:AAAGCCAAATCTTATTACCATCCTG	6FAM	345/345–353	24	4	0.71	0.71	0.58	0.00
<i>Mel-522</i>	FR745784	3	15C09	(AAAG) <sub>11</sub>	F:CTAATTGCTAGAATGAATGGGTTG R:TCAGTTTACCCTCCATGGTTCC	HEX	258/242–258	22	4	0.55	0.60	0.56	0.06
<i>Mel-538</i>	FR745800	3	15F01	(ATCT) <sub>9</sub>	F:CTAAGCCTTACGCCCTACATTATACAC R:TATTGGCGAGTCTAGGTCCTG	HEX	299/297–305	23	3	0.43	0.61	0.23	0.16
<i>Mel-551</i>	FR745813	3	15G11	(GATA) <sub>11</sub>	F:CCTGTAAGAGTTCATGGAG R:ACATGGTATCTACCTGGTCTCTG	6FAM	227/217–237	24	5	0.58	0.77	0.05	0.13

Table 1 continued

Locus	EMBL	Clone library	Clone	Repeat motif	Primer sequence (5'-3')	Fluorescent label (FP)	Expected/observed allele size (bp)	N	A	H <sub>0</sub>	H <sub>E</sub>	P <sub>HWE</sub>	F(null)
<i>Mel-554</i>	FR745816	3	15H03	(AAAG) <sub>15</sub>	F:TGGTGCTTATCTGAACATGAGG R:GACTGAGCCAGCCAGGTG	HEX	211/203–207	22	2	0.36	0.41	0.62	0.04
<i>Mel-558</i>	FR745820	3	16A02	(AGAA) <sub>13</sub>	F:GCAAGCACTGTGGATAATTG R:GATGCTCAACCCACTGAGTC	6FAM	255/233–245	24	4	0.75	0.72	0.52	-0.03
<i>Mel-576</i>	FR745838	3	16D05	(AAGA) <sub>9</sub>	F:ACTAGTGGCATGTTTCATATAATG R:CCTGCCTAGAGAGTTCTCTACC	6FAM	258/246–258	23	3	0.30	0.27	1.00	-0.08
<i>Mel-592<sup>b</sup></i> (X-linked)	FR745854	3	16F06	(CTTT) <sub>10</sub>	F:AGCCAAATGACCAGCAATG R:AATTGGTATGCTTATTAAGGAAGCAG	HEX	233/233–237 (Female) 233/233–237 (Male)	24	2	0.54	0.50	1.00	-0.05

bp base pairs, N number of badgers genotyped, A number of alleles observed, H<sub>0</sub> observed heterozygosity, H<sub>E</sub> expected heterozygosity, P<sub>HWE</sub> probability of deviation from Hardy–Weinberg equilibrium, F(null) estimated null allele frequency, (S) clone sequenced at NBAF–Sheffield, all other clones isolated from library 3 were sequenced at NBAF–Edinburgh

<sup>a</sup> *Mel-191* (FR745453) displayed high sequence similarity to many mammalian X-chromosomes; however, 125/543 male badgers (XY) were heterozygous suggesting it is autosomal in badgers (Annavi et al. unpublished data)

<sup>b</sup> *Mel-592* (FR745854) is X-linked based on the genotyping of 48 known-sex individuals

female badger (BAP1556) sampled at Wilcot, England (Ordnance Survey reference: SU141608) using the method of Armour et al. (1994) and enriched separately for di- and tetranucleotide microsatellite motifs comprising (GT)<sub>n</sub>, (CT)<sub>n</sub>, (GTAA)<sub>n</sub>, (CTAA)<sub>n</sub>, (TTTC)<sub>n</sub>, (GATA)<sub>n</sub> and their complements. These were denatured and bound to magnetic beads following Glenn and Schable (2005). Transformant colonies were sequenced in both directions without pre-screening.

Primer pairs were designed for 35 unique microsatellite loci using PRIMER3 0.4.0 (Rozen and Skaletsky 2000) with annealing temperatures in the range of 55–61°C. For each locus, we genotyped 12 males and 12 females from Wytham Woods, UK (GPS:51:46:26N;1:19:19W). To ensure individuals were as unrelated as possible we selected individuals from disparate social-groups. Genomic DNA was extracted from blood using a modified Chelex protocol (Walsh et al. 1991). Polymerase chain reactions (PCR) were performed in a 384-well Dyad Hybaid Touchdown™ thermal cycler (Thermo Hybaid, Ashford, UK). A negative control (ultrapure double-distilled water) was used to detect contamination and a positive (known genotype) control to ensure consistent allele-scoring. Each 2 µl Qiagen PCR reaction (Qiagen Inc., Valencia, USA) contained 1 µl of Qiagen master mix, 1 µl of fluorescently labelled primer mix (forward and reverse) at 0.2 µM and 50 ng of DNA. The touchdown PCR program was: 95°C for 15 min, then 30 cycles of 94°C for 30 s, 90 s at 61–55°C and 72°C for 1 min, followed by a final 30 min at 60°C. All 24 genotypes (35 loci) were derived from singleplex PCRs on an ABI 3730 DNA analyser. Alleles were scored using ROX500 size-marker and GENEMAPPER 3.7 (Applied Biosystems, California, USA). Loci were checked for sex linkage by comparing the genotypes of 24 females and 24 males.

We isolated 432 new badger microsatellite sequences: *Mel-80–Mel-611* (FR745442–FR745873). Of the 35 loci tested, 21 were polymorphic (Table 1), 6 were monomorphic and 8 failed to amplify or amplified non-specific products (Supplementary Table 1). Observed and expected heterozygosities and estimated null-allele frequencies were calculated using CERVUS 3.0 (Kalinowski et al. 2007). Means are reported ± standard errors (SE). There were 2–5 alleles per locus (mean = 3.05 ± 0.21), and expected heterozygosities ranged from 0.08–0.75 (mean = 0.44 ± 0.03). The combined probability of not excluding a single randomly-chosen unrelated individual from parentage increased from 3.0 × 10<sup>-8</sup> (31 existing loci) to 5.8 × 10<sup>-13</sup> (52 loci). KinInfor 1.0 (Wang 2006) estimated that 31 loci distinguished full-siblings from half-siblings with 71% accuracy, or 88% with 52 loci. *Mel-592* (FR745854) was X-linked; all 24 males (XY) were homozygous, whereas 11 females (XX) were heterozygous and 13 females

homozygous (Fisher's Exact Test  $P < 0.001$ ; 95% confidence interval for the odds ratio = 0.00–0.19). This locus will enhance the genetic sex-typing of badgers and due to its small product size (233–237bp) may be particularly useful for degraded samples such as those collected non-invasively (see Toouli et al. 2000).

Deviations from Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium (LD) were tested using GENEPOP 4.0.10 (Raymond and Rousset 1995). Only *Mel-551* (FR745813) deviated significantly from HWE ( $P = 0.0499$ ). Nine pairs of loci were in LD (*Mel-232–Mel-243*, *Mel-243–Mel-576*, *Mel-246–Mel-203*, *Mel-253–Mel-161*, *Mel-451–Mel-153*, *Mel-522–Mel-538*, *Mel-538–Mel-554*, *Mel-538–Mel-186*, and *Mel-153–Mel-203*) but not after adjusting for multiple testing by FDR control ( $m = 210$ ,  $\alpha = 0.05$ , adjusted  $P = 0.001–0.050$ ; Benjamini and Hochberg 1995).

These 21 polymorphic microsatellite loci will advance our capacity to resolve genealogical relationships between badgers, which will have practical application in disease management.

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