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SHORT COMMUNICATION

Confirmation of low genetic diversity and multiple breeding females in a social group of Eurasian badgers from microsatellite and field data

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Abstract

The Eurasian badger (*Meles meles*) is a facultatively social carnivore that shows only rudimentary co-operative behaviour and a poorly defined social hierarchy. Behavioural evidence and limited genetic data have suggested that more than one female may breed in a social group. We combine pregnancy detection by ultrasound and microsatellite locus scores from a well-studied badger population from Wytham Woods, Oxfordshire, UK, to demonstrate that multiple females reproduce within a social group. We found that at least three of seven potential mothers reproduced in a group that contained 11 reproductive age females and nine offspring. Twelve primers showed variability across the species range and only five of these were variable in Wytham. The microsatellites showed a reduced repeat number, a significantly higher number of nonperfect repeats, and moderate heterozygosity levels in Wytham. The high frequency of imperfect repeats and demographic phenomena might be responsible for the reduced levels of variability observed in the badger.

Keywords: complex microsatellites, maternity, *Meles meles*, reproductive behaviour

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Introduction

In areas typified by agricultural landscapes in lowland England, the Eurasian badger (*Meles meles*) has an unusual social behaviour where groups of up to 25 animals defend a common territory, share a sett, allogroom, play and may show limited alloparental care (Neal 1986; Kruuk 1989; Woodroffe 1993). However, they forage, dig, travel and spend most of the time in solitary outside their setts (Kruuk 1989). The adaptive significance of badger facultative sociality is not fully understood (see reviews by Woodroffe & Macdonald 1993 and Johnson *et al.* 2000).

Badger groups include several mature males and females and are generally formed by recruitment of the offspring into their natal territory (Woodroffe *et al.* 1993). Litter size averages 2.4–2.7 across Europe (da Silva *et al.* 1994) and there is a high reproductive failure in social badgers compared to solitary badgers. This reproductive failure has been linked to group living and high population densities (Cresswell *et al.* 1992; Woodroffe & Macdonald 1995a). Reproductive suppression seems to be the result of female competition for resources, and there is little evidence that it is linked to co-operative care of the young (Woodroffe & Macdonald 1995a) as is common in other carnivores (Creel & Creel 1991; Creel & Macdonald 1995).

Several approaches, including behavioural observation (Cheeseman *et al.* 1987; Woodroffe & Macdonald 1995a), allozyme data (Evans *et al.* 1989; da Silva *et al.* 1994) and ultrasound scanning (Woodroffe 1992), have suggested

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that in a single reproductive season cubs can be produced by more than one female within each social group. However, multiple paternity within a litter has not been demonstrated.

Low genetic variability in badgers has been described using allozymes (Evans *et al.* 1989), multiple and single locus DNA fingerprinting (da Silva *et al.* 1994; Burke *et al.* 1996; E. Geffen personal communication), and microsatellites isolated from the Eurasian otter (*Lutra lutra*, Dallas & Piartney 1998). Random amplified polymorphic DNA markers showed moderate levels of polymorphism (P. Fakler and A. Schreiber, unpublished data). Bijlsma *et al.* (2000) described seven microsatellite markers isolated from Eurasian badgers that were moderately variable in a sample of 105 badgers from different localities in the Netherlands and Denmark. However, the markers might not be variable enough for paternity testing in a single population or in social badgers, where individuals might be closely related.

The genetic resolution for individual identification and the establishment of familial relationships can be complemented with accurate field records and behavioural observations (Mace *et al.* 1996). In this study, we use microsatellite DNA technology to detect genetic polymorphism in the badger and combine the results with behavioural and physiological data from Woodroffe (1992) to

explore baseline hypotheses of the badger mating system, such as the number of breeding animals. We also examine and discuss the demographic factors and mutation patterns that can be linked to the low genetic variability observed in badger microsatellites.

Materials and methods

We studied a badger population living in Wytham Woods, Oxfordshire, UK, a property of 6 km² belonging to the University of Oxford. The population density of badgers in this area of woodland, pastures and arable land has been increasing steadily since 1986 (Macdonald *et al.* 2002) and in 1990 and 1991 when samples for this study were collected, densities were estimated as 22.3 and 27.2 individuals (adults plus cubs) per km², respectively. Badgers live in setts (identified by two capital letters in this study) distributed across Wytham Woods (Fig. 1). The roads and water bodies around the study area present obstacles and risks for badgers to emigrate from or immigrate to Wytham Woods.

To construct three nonenriched libraries, whole DNA isolated from the muscle of a dead badger (w213) was cut with enzyme *AluI* or *MboI* and fragments between 100 and 500 base pairs (bp) were selected. Fragments were ligated

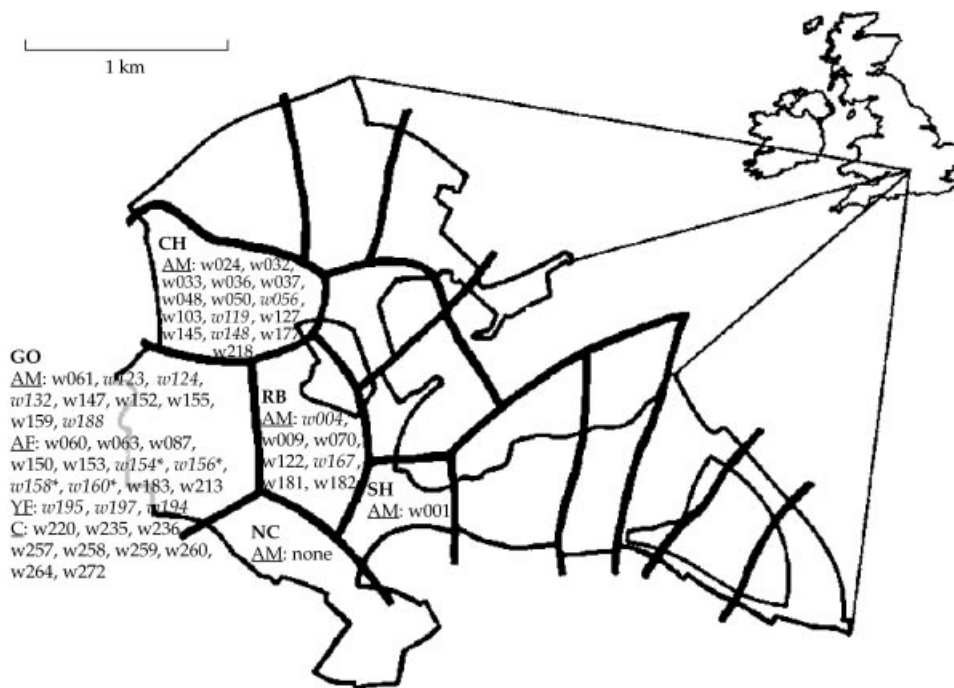


Fig. 1 Distribution of Eurasian badger territories (CH, GO, NC, RB and SH) in Wytham Woods in 1991. Thin lines demarcate the limit between wooded areas, where the core of the territories are, and pasture. Thick lines are territory borders as determined by radio-tracking and/or bait-marking. All Wytham badgers have an ID number prefaced with a 'w'. AM signifies adult males living in the territories during 1990 which are therefore candidate fathers of the offspring born in 1991. AF signifies adult females that could potentially breed; YF signifies young females and C signifies cubs born in GO in 1991. An asterisk indicates females not found to be pregnant in 1991 by ultrasound. Italics represent animals not sampled.

Table 1 Microsatellite loci and allele scores in a Eurasian badger population inhabiting Wytham Woods, Oxfordshire, and in a sample of world-wide badgers

Locus	Repeat	Primer sequences (5' > 3')	T_a (°C)	Product size (bp)	Within population		Across populations	
					n	No. of alleles	n	No. of alleles
<i>Mel1</i>	(GT) ₂₀	CTGGGGAAAATGGCTAAACC AATGCAGGCTTTGCAATTCC	60	262–274	16	1	105	5
<i>Mel3</i>	(GT) ₁₃	CTAAAACCACCACCACAATGC GTGTATAGCCTGCCAACAGG	62	128–134	16	1	105	4
<i>Mel7</i>	(GT) ₂₁	ATTCTTCCTTTTAGCTTTGGCC TCTCACAGTGTCCAGAGAAAGG	62	134–144	16	1	105	5
<i>Mel10</i>	(TG ₃ C) ₂ T ₃ CT(TA ₃) ₃ (AC) ₁₄	TGCAGAAAACGATCAGGTTC TCATTTTGGCGTGTACTTCC	58	154	40	2	12	5
<i>Mel11</i>	T ₃ CT ₇ CT ₁₃	CCTAGTTGGTAAGAGTCAAGAACAC TGGGGAATGACATGCCATAG	59	122	40	1	12	4
<i>Mel12</i>	(TTG) ₂ (TG) ₆ TA(TG) ₂₀	TCCGTTTATATTTGCTGTGCC CTGCTCAGCCTTCAAGGTAAC	53	153	40	4	11	6
<i>Mel13</i>	(AC) ₆ AG(AC) ₇	GGAGACATGACCTTGGACATCCTG CTGGGCAGTCATAGTCGTG	52	94	40	1	11	4
<i>Mel14</i>	(AC) ₁₈	GACACAAGCAAACCTTCTCTCC CATCTGGAAGTAGGCATAATG	56	188	40	4	12	7
<i>Mel15</i>	(CT ₃) ₁₄ C ₂ T ₇ A ₃ GAT ₄ (AT ₃) ₂ (GA) ₇ A(AG) ₄	ATGGAACCTCCTGGAAAGAG CAGGTTCATGATCCAGAGTC	61	270	40	4	12	5
<i>Mel16</i>	T ₅ (CT ₃) ₁₀ CT ₂ C ₂ T ₂ C(CT ₃) ₉ C(CT ₂ CT ₃) ₂	ACTGTTTCCCTAAGACTGCGT CACCTGACTTGTGAGATGAG	59	324	40	1*	12	9
<i>Mel17</i>	CT ₂ CT ₃ CT ₂ C ₄ (C ₂ T ₂) ₁₁ C ₃ T ₂ C ₂ T ₃ C ₂ T ₂ C T ₄ C ₂ T ₃ C ₂ T ₂ CT ₂ (C ₃ T) ₂ CAT ₂ CT ₂ CT ₃ (C ₂ T ₂) ₂ (C ₃ T) ₃ C ₂ AT ₆ C (C ₂ T ₂) ₂ CTCT ₂ C ₂ T(C ₃ T) ₂ CT ₃	CTTACTGCAAACACTACCA ACAGGACAGTCTTTCACAGAG	52	298	40	1	11	11
<i>Mel18</i>	(C ₂ T ₂) ₉ C(CT) ₇ T ₃	ACTGAATACCAACTACGAAC TTCCAACAGCTAATGACA	52	359	13	1	?	?

Data for *Mel1*, *Mel3* and *Mel7* are from Bijlsma *et al.* (2000) based on animals from the Netherlands and Denmark with the exception of within population data that was obtained from Wytham badgers. T_a = annealing temperature.

*No alleles within the Wytham population could be clearly differentiated in 200 × 200 mm 6% polyacrylamide gels but alleles were obtained through sequencing; ? = not available.

into plasmid vector pUC18 and recombinant plasmids were transformed into *Escherichia coli* XL1-B. Colonies containing microsatellites were detected and selected using probes (GC)₁₀, (GT)₁₀ and (AT)₁₀ (for the first library), (CA)₁₅ (for the second library) and (GAAA)₁₀ and (GATA)₁₀ for two separate hybridizations of the third library. Further details on library construction and selection have been described elsewhere (Domingo-Roura 1998, 2002). Another enriched DNA library was constructed by Genetic Identification Services as described in Kays *et al.* (2000). Sequences from these libraries for the loci presented in Table 1 have been deposited in the EMBL database under accession numbers AJ309849 and AJ309052–AJ309059.

Sequences were selected based on the microsatellite structure and a preliminary test of polymorphism of four to 20 unrelated badgers, mostly from separate geographical regions. The loci that showed variability in this initial

screening were amplified in a group of 12 badgers with the following geographical origins: Crete, Germany, Norway, Austria, northeast Spain, northcentral Spain, Japan, United Kingdom, Sweden, Finland, Ireland and Mongolia. The loci were also amplified for Wytham males captured in focal sett GO and surrounding setts (CH, NC, RB and SH) in 1990 when matings occurred and for all pregnant and/or lactating females and all offspring captured in GO in 1991. In 1991, 140 of the estimated 163 badgers living in Wytham were captured (see Macdonald & Newman 2002 for details). GO sett was selected because it was intensively observed and trapped during the study period (Woodroffe 1992), securing the sampling of all candidate mothers and cubs. *Mel18* was only tested in 13 mothers and offspring from Wytham Woods. In addition to using the markers that we developed, we also amplified and scored 16 individuals including mothers and offspring from GO sett with

primers *Mel1*, *Mel3* and *Mel7* (Bijlsma *et al.* 2000). Polymerase chain reaction (PCR) conditions for microsatellite amplification were the same as described by Domingo-Roura (2002) and the annealing temperatures are given in Table 1.

Distribution of microsatellite alleles was visually scored in $200 \times 200 \times 1.5$ mm 6% polyacrylamide gels and stained either with silver nitrate or ethidium bromide. For marker *Mel16*, different alleles were detected but could not be reliably scored since they were due to nucleotide substitutions within the microsatellites. In this case, PCR products were cleaned with QIAquick PCR Purification Kit (Qiagen) and sequenced using a dRhodamine Terminator Cycle Sequencing Kit (Perkin-Elmer) following the instructions provided by the manufacturer and were run in an ABI Prism 377 DNA Sequencer (Perkin-Elmer). Heterozygotes in the sequences were detected by the presence of two peaks of similar height for two different nucleotides in a single base position. For maternity exclusion, in case of doubt, a conservative reading assuming heterozygosity was scored. Lengths of microsatellite alleles were determined either from allele sequences or using Bio-Rad Gel Doc 2000 and QUANTITY ONE software (Bio-Rad Technical Service Department 2000).

Maternity by a specific female was excluded if she could not have contributed either allele found in the offspring. For within-population data, allele frequencies of variable loci were computed by the allele count method and their standard deviations were calculated as the square root of the variance of the corresponding binomial proportions. Maximum likelihood haplotype frequencies were estimated following Excoffier & Slatkin (1995). Diversity indices were computed following Nei (1987). Deviations of loci from Hardy-Weinberg equilibrium were conducted with an exact test using a Markov chain algorithm with 10 000 steps (Guo & Thompson 1992). Pairwise linkage disequilibrium was tested following Slatkin & Excoffier (1996) with 10 000 permutations. The power of discrimination (Pod), that is, the probability that two different individuals have different genotypes for a given loci, was calculated following Fisher (1951). The a priori probability of paternity exclusion (P_{ex}) was calculated following Chakraborty & Jin (1993).

Analysis of molecular variance (AMOVA; Excoffier *et al.* 1992) using *Mel10*, *Mel12*, *Mel14* and *Mel15* alleles was used to test for substructuring in the data set when comparing (i) adult females ($n = 7$) and cubs ($n = 9$); (ii) adult males ($n = 24$) and adult females ($n = 7$); and (iii) setts CH ($n = 12$), GO ($n = 21$), RB ($n = 6$) and SH ($n = 3$). Tests were conducted using ARLEQUIN version 2.000 (Schneider *et al.* 2000).

Microsatellites were classified according to Weber (1990) as perfect, imperfect and compound repeat sequences. The χ^2 tests were conducted to compare the number of perfect, imperfect and compound dinucleotide-based repeats obtained in this study with published results in other mus-

telids (*Lutra lutra*, Dallas & Piertney 1998; *Gulo gulo*, Davis & Strobeck 1998; Duffy *et al.* 1998; Walker *et al.* 2001; *Martes americana* and *Taxidea taxus*, Davis & Strobeck 1998; *Mustela vison* and *M. erminea*, Fleming *et al.* 1999) and in other mammalian species (humans, Weber 1990; pigs, Winterø *et al.* 1992; dogs Ostrander *et al.* 1993). The mean and standard deviation (SD) of repeat number were compared between our study and others using *t*-tests. We computed a regression line of maximum repeat length of perfect repeats against expected heterozygosity (H_E) using data from other studies on mustelids (Davis & Strobeck 1998; Fleming *et al.* 1999; Walker *et al.* 2001). All analyses dealing with microsatellite structure were conducted using spss 9.0 (SPSS Inc.) for Windows.

Results

The three nonenriched libraries combined gave around 4000 clones of which 38 were positives. Twenty-nine microsatellite regions from the nonenriched and four (*Mel15*, *Mel16*, *Mel17* and *Mel18*) from the enriched libraries were tested for variability across Europe and/or within Wytham. For locus *Mel16*, we discovered size homoplasmy in Wytham animals. Consequently, we sequenced the PCR products of GO females and offspring to determine alleles and maternity for this locus. Markers *Mel1*, *Mel3* and *Mel7*, found previously to be polymorphic in badgers from the Netherlands and Denmark, were monomorphic in 16 individuals that we tested from Wytham Woods.

None of the microsatellites that showed size polymorphism in Wytham revealed deviations from Hardy-Weinberg expectations or showed evidence of linkage ($\alpha = 0.05$). The combination of allele information from *Mel10*, *Mel12*, *Mel14* and *Mel15* in badgers from Wytham Woods (Table 2) showed a reduced number of rare alleles, a mean number of pairwise differences of 2.144, and a mean H_E of 0.536. Markers *Mel14* and *Mel15* showed a bimodal distribution of allele frequencies with no intermediate-sized alleles (Table 2).

The overall probability of two random, unrelated individuals having the same genotype for all loci, including *Mel16*, is 5.79×10^{-4} , whereas the overall probability of excluding a candidate parent when the individual is not the parent is 0.674. The probability of an animal being completely homozygous is 0.02. Genetic substructuring in the Wytham dataset gave nonsignificant results for comparisons between adult females and their offspring ($P = 0.158$) and between adult males and adult females ($P = 0.066$) but a significant substructuring of the dataset for comparisons among setts ($P < 0.001$).

Our four libraries yielded six tetranucleotide and 27 dinucleotide repeat loci. Fourteen dinucleotide loci were perfect, 11 were imperfect, and two were part of a compound microsatellite. The number of nonperfect repeat loci

Locus	<i>k</i>	2 <i>n</i>	Allele or haplotype	Frequency (SD)	<i>H</i> _O	<i>H</i> _E	Pod	Pex
<i>Mel10</i>	2	80	111	0.912 (0.032)	0.175	0.184	0.281	0.013
			113	0.087 (0.032)				
<i>Mel12</i>	4	86	116	0.395 (0.053)	0.674	0.706	0.847	0.265
			118	0.128 (0.036)				
			120	0.128 (0.036)				
			122	0.349 (0.051)				
<i>Mel14</i>	4	86	188	0.221 (0.045)	0.581	0.612	0.795	0.199
			190	0.105 (0.033)				
			196	0.570 (0.053)				
			198	0.105 (0.033)				
<i>Mel15</i>	4	86	245	0.140 (0.037)	0.814	0.695	0.851	0.265
			249	0.151 (0.039)				
			257	0.453 (0.054)				
			265	0.256 (0.047)				
<i>Mel16</i>	4	32	CTTTA	0.469 (0.088)	0.813	0.662*	0.827	0.238
			CTTTT	0.062 (0.043)				
			TCTTT	0.281 (0.079)				
			TTCCT	0.187 (0.069)				

Table 2 Genetic data on five microsatellite loci screened in 43 badgers from Wytham Woods

*The expected heterozygosity was computed from haplotype frequencies. *k*, number of allele; *H*_O, observed heterozygosity; *H*_E, expected heterozygosity; Pod, power of discrimination; Pex, paternity exclusion.

is significantly larger than the number obtained from other mustelid species (48 perfect, 10 imperfect, three compound; $\chi^2 = 6.475$, $P = 0.011$) and from other mammalian species (230 perfect, 58 imperfect, 35 compound; $\chi^2 = 4.421$, $P = 0.035$). The average largest repeat number per clone was 11.55 ($n = 29$, $SD = 4.24$, range = 6–20), which is significantly different ($t = -2.95$, $P = 0.006$) from the values obtained in the other Eurasian badger study ($n = 7$, mean = 17, $SD = 4.32$, range = 12–23), and also different ($t = -3.69$, $P < 0.001$) from the values from other mustelid species ($n = 67$, mean = 15.58, $SD = 4.87$, range = 7–29). The length of the largest repeat number per clone was not different between variable and invariable microsatellite loci in the world sample ($t = -0.938$, $P = 0.357$) but was different in the Wytham population ($t = -2.72$, $P = 0.012$).

The correlation between expected heterozygosity and the length of the longest perfect repeat in the original clone of published mustelid microsatellites was significant ($r = 0.355$, $P = 0.011$) and the regression line followed the equation $H_E = 0.326 + (0.021 \text{ repeat length})$. Only *Mel10* deviates significantly from this regression line.

Using ultrasound to detect pregnancy, we excluded four of 11 reproductive age adult females as candidate mothers in the sett GO (Woodroffe 1992). Four of the offspring (w235, w257, w260 and w272) could be assigned either to female w63 or to female w213 but not to any other candidate mother. Female w63 was ultrasound scanned in January 1991 and she was diagnosed with one foetus. Female w213 could not be ultrasound scanned but had extended teats when she was caught in April 1991, indicating that

she was lactating and, thus, a candidate mother. Therefore, these data suggest that w213 was the mother of three cubs. However, the ultrasound examination of w63 was performed at an early stage of pregnancy and additional foetuses could have been missed. In addition, offspring w264 could be assigned to mother w150 since this female was the only one who had a C nucleotide in position 41 bp for primer *Mel16*. In summary, our data suggest at least three different mothers for the nine offspring captured in GO and outliers during 1991. Finally, we typed males captured in GO and nearby setts during 1990 and 1991 (Table 2), but paternity could not be assigned confidently since some candidate fathers were not sampled (Fig. 1).

Discussion

We found low levels of genetic variability in badgers from Wytham Woods. However, implicit limitations of the study are that results are based on a reduced number of loci and a reduced number of potentially related individuals. Trends in microsatellite evolution and population genetic parameter estimates might be subject to sampling bias and should be taken with caution.

In this study we detected many short microsatellites and a high number of nonperfect repeats, characteristics that are known to be associated with low allelic variation (Weber 1990; Brinkmann *et al.* 1998). Only two out of seven of the loci described by Bijlsma *et al.* (2000) were imperfect in the original clones. Some of the results could be explained by a low stringency in the washes during library

production that can cause the relative size and number of loci isolated from genomic libraries to vary greatly among experiments and experimenters. It is always risky to compare data from different libraries and across studies but the high frequency of short, complex repeats is not likely to be only due a systematic bias in probing efficiency during library production, since in this study we produced different libraries under different conditions (see Materials and methods section).

Our results, and those of previous studies using different markers (Evans *et al.* 1989; da Silva *et al.* 1994; Burke *et al.* 1996), suggest genome-wide reductions in variability for British badgers. We found lower overall levels of variation and three loci to be monomorphic in the Wytham population that were variable in the Netherlands and Denmark (Table 1, Bijlsma *et al.* 2000). In addition, *Mel14* and *Mel15* have alleles of intermediate sizes missing (Table 2) that could have been lost by genetic drift. We also detected significant substructuring by sett in the Wytham population consistent with a strong matrilineal structure as suggested by behavioural data.

The reduced variability we found might be due to two main groups of factors, one dealing with the isolation, structure and evolution of microsatellite regions and the other linked to demographic and selective events. The two groups of factors might have additive effects since, for instance, the variance in repeat number is determined by mutation rate and by effective population size (Estoup & Cornuet 1999). The bias in library production is not likely to be the main factor responsible for this low variability; it is possible that British badgers have indeed suffered reduced population numbers in the recent past. Nevertheless, the inclusion of related animals in the sample as well as the particular badger reproductive and social system might have accentuated this reduction in polymorphism.

The multiple maternity that we found appears to have behavioural implications within setts. Aggression occurring between females or towards the offspring from subordinate females, as is usual in canids (Kruuk 1989; Girman *et al.* 1997), does not necessarily result in reproductive suppression in the badger.

Our genetic data also support previous behavioural observations suggesting that reproductive output may be skewed, for instance towards older females or females in better nutritional condition (Cheeseman *et al.* 1987; Woodroffe & Macdonald 1995a). Gestation and lactation are costly in badgers (Cheeseman *et al.* 1987; Woodroffe & Macdonald 1995b). For females in poor condition the costs of maintaining gestation and the expected cost of lactation may outweigh the potential benefit of breeding, favouring termination of their pregnancies (Clutton-Brock 1991). Additionally, badgers have delayed implantation allowing for more precise timing of development to match environ-

mental conditions (Harrison & Neal 1956; Woodroffe 1995). Because of reduced levels of genetic variation, conclusive evidence of reproductive skew and detailed mapping of kinship and reproduction in the Wytham Woods population will require an extensive effort to develop additional polymorphic loci to complement detailed demographic and behavioural data.

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This research is part of the long-term Wytham badger field project led by David Macdonald and has been a collaboration with the groups led by Terry Burke, Jaume Terradas and Bob Wayne. Rosie Woodroffe, Michael Roy and Josep Marmi are past members of WILD CRU who worked on this project. Xavier Domingo-Roura and Josep Marmi are now at the Universitat Pompeu Fabra and conduct research on the evolution and application of microsatellite technology in mustelids and primates.
