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Reliable microsatellite genotyping of the Eurasian badger (*Meles meles*) using faecal DNA

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Abstract

The potential link between badgers and bovine tuberculosis has made it vital to develop accurate techniques to census badgers. Here we investigate the potential of using genetic profiles obtained from faecal DNA as a basis for population size estimation. After trialling several methods we obtained a high amplification success rate (89%) by storing faeces in 70% ethanol and using the guanidine thiocyanate/silica method for extraction. Using 70% ethanol as a storage agent had the advantage of it being an antiseptic. In order to obtain reliable genotypes with fewer amplification reactions than the standard multiple-tubes approach, we devised a comparative approach in which genetic profiles were compared and replication directed at similar, but not identical, genotypes. This modified method achieved a reduction in polymerase chain reactions comparable with the maximum-likelihood model when just using reliability criteria, and was slightly better when using reliability criteria with the additional proviso that alleles must be observed twice to be considered reliable. Our comparative approach would be best suited for studies that include multiple faeces from each individual. We utilized our approach in a well-studied population of badgers from which individuals had been sampled and reliable genotypes obtained. In a study of 53 faeces sampled from three social groups over 10 days, we found that direct enumeration could not be used to estimate population size, but that the application of mark–recapture models has the potential to provide more accurate results.

Keywords: bovine tuberculosis, mark–recapture models, maximum-likelihood model, molecular scatology, multiple-tubes approach, population size estimation

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Introduction

Over the last two decades, the incidence of bovine tuberculosis infection (BTB, *Mycobacterium bovis*) in UK cattle herds has been rising steadily and spreading geographically (Krebs *et al*. 1997). A variety of evidence suggests that the Eurasian badger (*Meles meles*) constitutes a significant wildlife reservoir of BTB infection and that badgers transmit the disease to cattle (Krebs *et al*. 1997). However, despite a substantial amount of research, the causal link between *M. bovis* infection in badgers and outbreaks of tuberculosis infection in cattle herds has not been proven. One reason for this is that it has been impossible to examine the relationship between badger population density and frequency of BTB infection in cattle herds, owing to the difficulty of accurately measuring local badger population densities (Krebs *et al*. 1997).

Recent developments in molecular genetics have created new methods that allow populations to be censused through noninvasive DNA sampling, using microsatellite loci to establish a 'genetic profile' (a multilocus genotype unique to individual animals; Palsbøll 1999; Taberlet & Luikart 1999). DNA has been extracted from faecal samples and used for individual identification in a variety of mammals, including carnivores (e.g. Kohn *et al*. 1999; Bayes *et al*. 2000; Ernest *et al*. 2000). By applying these methods to badgers, it should be possible to determine the number of different individuals defecating within a

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particular area and, hence, the population density. Because of the nonephemeral and conspicuous nature of badger latrines (Roper *et al*. 1993; Neal & Cheeseman 1996), faeces should provide a plentiful noninvasive source material that is easy to collect and to attribute to an individual animal. The primary aim of our study therefore was to develop a reliable protocol for obtaining genetic profiles from badger faeces. In addition, we present a preliminary test of the accuracy of this method in estimating badger population size.

Accurate 'genetic profiles'

Several studies have shown that the amplification success of microsatellite loci from faecal DNA extracts can depend on both the faecal preservation method and the extraction method (Wasser *et al*. 1997; Frantzen *et al*. 1998; Murphy *et al*. 2000). Optimal preservation techniques can vary between species and ecological conditions (Frantzen *et al*. 1998), making it necessary to perform trials for each new faecal study. Accordingly, our first objective was to optimize a technique for extracting DNA from badger faeces. We achieved this by comparing the efficacy of three different storage methods and two extraction methods.

The multiple-tubes approach

Faecal DNA extracts are generally of low quantity and quality, which causes a high prevalence of errors such as 'allelic drop-out' (ADO; Gagneux *et al*. 1997; Bayes *et al*. 2000) and 'false alleles' (FA; Taberlet & Luikart 1999). The multiple-tubes approach proposed by Taberlet *et al*. (1996) is the standard protocol used to obtain reliable genotypes from faecal DNA (see for example Goossens *et al*. 2000; Constable *et al*. 2001; Garnier *et al*. 2001) and assumes a worst-case scenario for allelic drop-out (hereafter referred to as worst-case rule, WCR, following Miller *et al*. 2002). Assuming each allele is equally likely to drop out, in the worst-case scenario every amplification of a heterozygous locus will give rise to one allele only, or, in other words, the probability of obtaining only one of the two alleles of a heterozygote is 0.5 in each reaction. Reliable genotypes are obtained by recording an allele only if it has been observed at least twice (in at least three amplification reactions) and by only recording an individual locus as homozygous if a certain number of positive amplification reactions gave rise to the same allele (for a single locus $n \geq 8$ for 99% confidence; Miller *et al*. 2002). This approach is reliable but very conservative, requiring large numbers of amplifications to obtain correct genotypes. In practice, few researchers working on low-concentration DNA strictly follow the WCR (Gagneux *et al*. 1997; Gerloff *et al*. 1999; Kohn *et al*. 1999).

Recently, Miller *et al*. (2002) published a maximum-likelihood method (hereafter referred to as MLR, following Miller *et al*. 2002) to assess genotype reliability and strategically optimize the number of polymerase chain reaction (PCR) replicates used. When allelic drop-out rates were low, the method was shown to have the potential to reduce the number of PCR amplifications by up to 50% of that required using the WCR approach. The MLR model is based on three assumptions: (i) both alleles at a heterozygous locus are equally likely to drop out, (ii) allelic drop-out rates are even across loci, and (iii) all false alleles can be detected and eliminated from the data set. However, in order to avoid erroneous genotypes, it is important to know whether these assumptions are met in the data set under investigation. Our second objective therefore was to modify the multiple-tubes approach of Taberlet *et al*. (1996) so as to achieve a reduction in the number of amplifications without a significant reduction in power. In addition, we compared in retrospect the efficiency of our new approach with that of the WCR and MLR approaches, and tested the assumptions of the MLR approach.

Estimation of population size

Once individual genetic profiles have been obtained, mark–recapture models can be applied to assess population size. As with the direct trapping or observation of animals, molecular profiles obtained from faecal DNA must meet the assumptions of the relevant mark–recapture models for accurate estimates to be obtained. It is important that the size of the population remains constant during the study period. Demographic closure may be reasonably assumed by collecting faeces over a short period, while violation of geographical closure can be minimized by collecting faecal samples from latrines close to setts well within the territorial boundary of social groups.

In a natural population, it is unlikely that individuals have equal probabilities of being captured. Three causes of variation in capture probability have been identified: behavioural responses to capture, variation over time (with constant trapability for all individuals) and individual heterogeneity (Otis *et al*. 1978). Different models of estimating population sizes that allow relaxation of the assumption of equal capture probability have been developed (Otis *et al*. 1978; Chao *et al*. 1992; Lee & Chao 1994). It has been shown that badger latrines were used equally by the two sexes and by individuals of all age groups (Wilson *et al*. 2003). Variation in 'capture' probability, however, may still occur through other means. It is now widely accepted that changing environmental conditions, especially differences in humidity or exposure to sun or shade, have an effect on the quality of faecal DNA (Farrell *et al*. 2000; Goossens *et al*. 2000). There will therefore be variation in extraction success, and hence capture probability, due to time effects. Furthermore, it is believed that the length of the interval between deposition and collection of

a dropping has an effect on DNA extraction success (Dallas *et al*. 2000; Goossens *et al*. 2000; Jansman *et al*. 2001; but see Palomares *et al*. 2002). In this case, in addition to time effects, some individual heterogeneity will be introduced by differences in the time of defecation. Finally, given the importance of time of deposition and of exposure to the elements, the location of a faecal sample would be expected to affect its extraction success, introducing additional individual heterogeneity.

These considerations suggest that models allowing for individual heterogeneity, time effects and a combination of both should be used in the analysis. Furthermore, Mills *et al*. (2000) have suggested that the Jackknife estimator for model M_h (which allows for individual heterogeneity) should be used with noninvasive population size estimation because this estimator would produce the least biased results when faced with a 'shadow effect', i.e. failure to identify different individuals with identical profiles. Our third objective, therefore, was to assess the potential of faecal DNA profiles to census badgers accurately, using different mark–recapture models. For this part of the study, we used faeces collected from the adjacent territories of three social groups of wild badgers. The size of these social groups was estimated independently from extensive live-trapping and video observation (Wilson *et al*. 2003).

Materials and methods

Sample collection and preservation

Fresh faeces were collected from badger latrines in Woodchester Park, Gloucestershire, UK. Faecal material was taken from the surface of individual droppings using toothpicks and was immediately placed in 1.5 or 2.0-mL screw-cap microfuge tubes used for DNA extraction (see below), to minimize the handling of samples. In order to allow fresh faecal deposits to be identified, all droppings at the relevant latrines were dusted with builder's chalk (Stanley Tools) on the previous day. In addition, samples were collected prior to 10.00 h, in order to avoid prolonged exposure to the atmosphere (Jansman *et al*. 2001).

For the storage and extraction trials, samples were collected from 16 badger droppings in July 2001. Six aliquots were taken from each sample in order to test three different storage methods in combination with two DNA extraction protocols. The storage methods were: (i) buffering in DETs (20% DMSO, 0.25 M EDTA, 100 mM Tris, pH 7.5 and NaCl to saturation; Seutin *et al*. 1991); (ii) buffering in 70% ethanol; and (iii) freezing at –20 °C. Samples were frozen immediately after collection but had to be transported during a 3 h journey from the field site to the laboratory.

Each day during a 10-day period in October 2001, a sample was taken from every overnight dropping ($N = 53$) deposited at latrines close to the active setts in three adjoining social groups: Parkmill (34 samples), Kennel (9 samples) and Nettle (10 samples). The locations of these three social groups in the Woodchester Park population can be found in Tuyttens *et al*. (2000) and Delahay *et al*. (2000). In accordance with the results of the storage and extraction trials (see below), aliquots of the faecal samples were stored in 70% ethanol and extracted using the guanidine thiocyanate (GuSCN)/silica method. To verify the results from the faecal study, hair or blood DNA was also extracted from 36 individuals that had previously been captured in the social groups from which the faeces were collected.

DNA extraction

In order to avoid contamination of the faecal samples, all extractions were performed in a separate laboratory that was free of concentrated badger DNA or PCR product. Aerosol-resistant pipette tips were used in all manipulations. Negative controls were included in each extraction to monitor contamination. Faecal samples that had been frozen or stored in a DETs solution were potentially infected with *Mycobacterium bovis*. These samples were extracted in a Category 3 containment laboratory. In order to evaporate the supernatant, samples stored in 70% ethanol and DETs solution were placed overnight in a heating block at 45 °C. Two faecal extraction methods were tested: the GuSCN/silica method (Boom *et al*. 1990; Höss & Pääbo 1993) and extraction with the QIAamp DNA Stool Mini-kit (Qiagen). The Qiagen kit is an adaptation of the GuSCN/silica method.

For the GuSCN/silica method, between 400 and 600 mg of wet faecal material was suspended in 1 mL of extraction buffer (5 M GuSCN, 0.1 M Tris–HCl pH 6.4, 0.02 M EDTA pH 8.0 and 1.3% Triton X-100) and incubated overnight at room temperature with rotation. Extracts were then centrifuged for 10 min at 13 000 *g*, the supernatant was added to 20 μL of silica matrix and the mixture was vortexed and incubated for 10 min at room temperature with agitation. The silica matrix was washed twice with 500 μL of washing buffer (5 M GuSCN, 0.1 M Tris–HCl pH 6.4, 0.02 M EDTA pH 8.0) and twice with 500 μL of ethanol washing buffer (10 mM Tris–HCl pH 7.5, 100 mM NaCl, 1 mM EDTA and 50% ethanol). The pelleted silica was dried in a heating block at 56 °C for 15 min and the DNA was eluted by incubation with ddH_2O for 10 min in a heating block at 56 °C. The extractions with the Qiagen kit were carried out according to the manufacturer's instructions. The only modification to the recommended protocol was that, instead of 180–220 mg, between 400 and 600 mg of faecal sample was added to the ASL buffer.

DNA was extracted from hair or blood samples of the badgers captured in the three social groups under investigation. Hair samples had been stored in 70% ethanol and

were extracted using a chelex protocol (Chelex100, Bio-Rad; Walsh *et al*. 1991). At least 10 hairs were used in each extraction (Goossens *et al*. 1998). Blood samples were extracted using a slightly modified version of the phenol:chloroform method (Sambrook *et al*. 1989; Bruford *et al*. 1998).

PCR amplification

PCRs were prepared using aerosol-resistant pipette tips in a laboratory that was free of concentrated badger DNA or PCR product. Reagents were always tested for contamination by including a PCR negative control. Frantzen *et al*. (1998) have shown that amplification success of faecal DNA will be reduced for microsatellite loci with alleles longer than 300 bp. From the 39 microsatellite loci published by Carpenter *et al*. (2003), seven loci with alleles shorter than 250 bp were chosen for this study: *Mel*-102, *Mel*-105, *Mel*-106, *Mel*-109, *Mel*-111, *Mel*-113, *Mel*-117. The microsatellite loci were amplified in a 25-μL volume, each containing 5 μL of DNA extract. The final reaction concentrations consisted of 75 mM Tris–HCl (pH 8.8), 20 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 0.15 μg/mL bovine serum albumin (BSA), 0.01% of Tween, 100 μM of each dNTP, 0.2 μM of primer and 0.6 units of *Taq* DNA polymerase (ABgene).

Microsatellite loci were amplified either using a touchdown profile (Don *et al*. 1991; *Mel*-102, *Mel*-106, *Mel*-109, *Mel*-111, *Mel*-113) or with a specific annealing temperature (*Mel*-105, *Mel*-117). All PCRs started with a 5-min denaturation at 95 °C. This was followed by either touchdown cycles of 95 °C for 30 s, annealing at 64–52 °C for 30 s and 72 °C for 30 s, decreasing the annealing temperature by 2 °C every other cycle for 14 cycles then 30 cycles of holding the annealing temperature at 50 °C; or 55 cycles with a specific annealing temperature (*Mel*-117: 55 °C; *Mel*-105: 56 °C). PCRs ended with a final extension at 72 °C for 5 min. Reactions were performed using a Hybaid Touchdown Thermal Cycler. Primers were end-labelled with a fluorescent dye and amplification products were separated on a 5% polyacrylamide gel using an ABI 377 DNA sequencer, and sized with a TAMRA 500 or ROX 500 size marker with bands of known size every 50 bp. All gels were analysed using GENESCAN ANALYSIS 2.0, and GENOTYPER 1.1 software.

Comparison of storage and extraction methods

For the 6 trials of combined storage and extraction methods, each of the 6 aliquots from each of the 16 samples was extracted. Each aliquot was amplified once with the seven different primers and the proportion of these seven reactions that produced a PCR product was calculated. To compare the six trials, a two-way analysis of variance (ANOVA) was applied on ranked data, using the Scheier-Ray-Hare extension of the Kruskal–Wallis test (Dytham 1999), with the proportion of successful amplifications (of seven) as the dependent variable and the storage methods and extraction techniques as factors. Amplifications were deemed successful if a PCR product of the expected size was present, even if the genotype may not have been reliable.

Probability of identity

When using microsatellite loci to establish a genetic profile, it is possible for different individuals to have identical profiles if an insufficient number of loci has been used. Mills *et al*. (2000) showed that, in order to be useful in population size estimations, genetic profiles should consist of enough microsatellite loci to distinguish between individuals with 99% certainty. Estimating the required number of loci can be achieved by computing probability of identity (P_{ID}) statistics. A number of P_{ID} equations have been derived, but where there is the potential for relatives to be present in the sample, it is best to use an estimate of P_{ID} among siblings ($P_{ID\text{-}Sib}$: Evett & Weir 1998; Woods *et al*. 1999; Waits *et al*. 2001). The overall $P_{ID\text{-}Sib}$ is the upper limit of the possible ranges of P_{ID} in a population and thus provides the most conservative number of loci required to resolve all badgers, including relatives. $P_{ID\text{-}Sib}$ was calculated using a data set of genotypes obtained from the blood or hair DNA of 36 badgers captured in 2000 and 2001 in 3 social groups. $P_{ID\text{-}Sib}$ values were estimated using the program GIMLET 1.0.1 (Valière 2002), after arranging loci in order of decreasing value of $P_{ID\text{-}Sib}$. PROB-ID5 (G. Luikart unpublished) was used to estimate the observed P_{ID} ($P_{ID\text{-}Obs}$) by computing the proportion of all possible pairs of individuals that had identical genotypes.

Comparative multiple-tubes approach

Faecal samples were scored using a comparative method, based on the WCR approach (Taberlet *et al*. 1996; see Fig. 1). We retained the rule that an allele was accepted only if it had been recorded at least twice. However, rather than initially performing three positive PCRs, samples were amplified twice. Loci that gave rise to the same heterozygous genotype twice were then accepted. After this, a stepwise amplification was introduced until each allele was observed at least twice. This stepwise process was continued for a maximum of seven positive PCRs and contrasted with the standard multiple-tubes approach where, after the first three positive amplifications, a further four PCRs were performed as a block. In the ambiguous case where, after seven positive amplifications, a locus yielded one heterozygous result and the same homozygote for the six other reactions, we followed the suggestion of Miller *et al*. (2002) and counted it as a half-locus occupied by the allele observed in the homozygote.

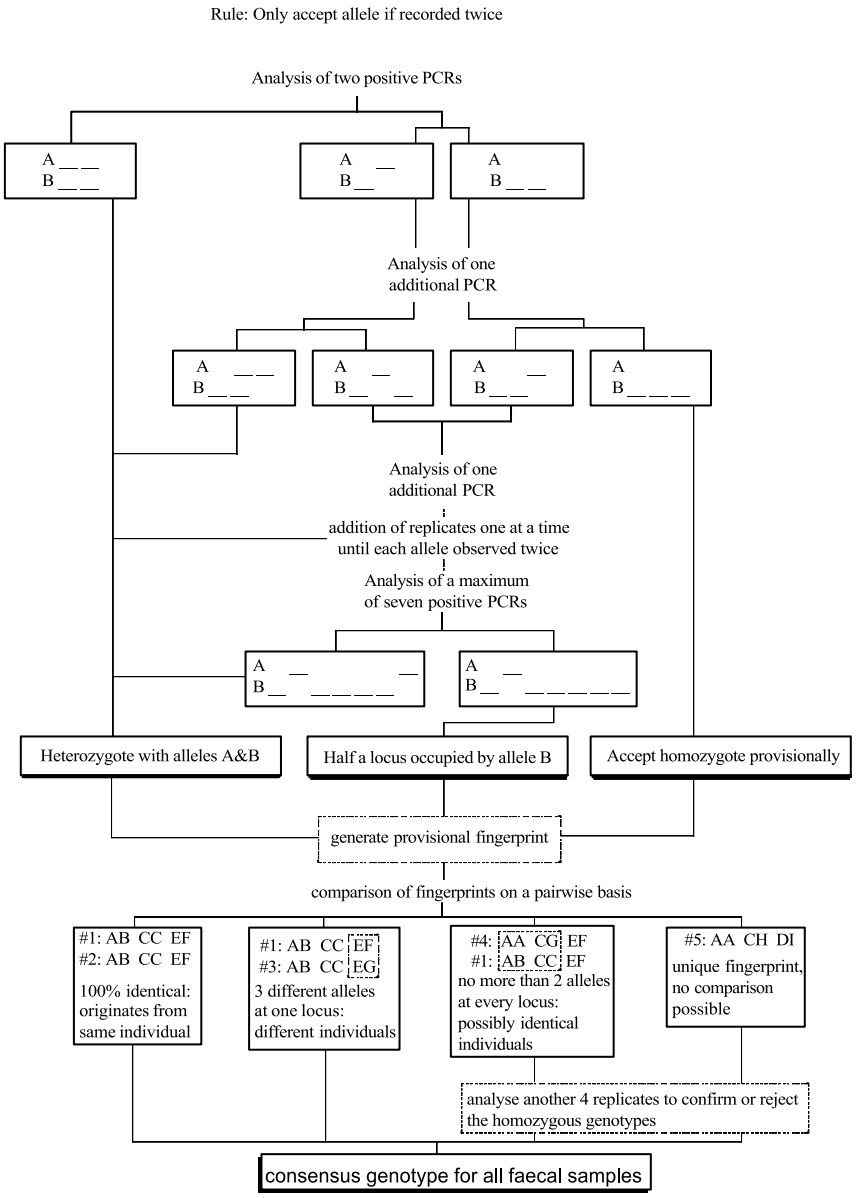

Fig. 1 Flow chart of the comparative multiple-tubes approach used in this study, modified from Taberlet *et al*. (1996).

The main difference between the two approaches lay in the second rule that dealt with homozygote genotypes. In our study, a number of faecal DNA extracts will originate from the same defecator, allowing genetic profiles to be compared to identify typing errors. Given this, a homozygote was *provisionally* accepted after three positive PCRs gave rise to the same allele. We then compared these provisional profiles using the program GIMLET 1.0.1 (Valière 2002). Provisional profiles that were shown to be 100% identical were grouped together and classified as originating from the same individual. Incomplete profiles were only considered if a consensus genotype was obtained at, according to the $P_{\text{ID-Sib}}$ statistics, the most informative locus (*Mel*-105). This made it possible to match them by hand to the only possible candidate group. Although incomplete profiles may have belonged to a new individual, we grouped them to matching complete ones because we preferred to obtain a conservative estimate of population size, rather than an estimate that was upwardly biased due to the identification of nonexistent individuals. The M_h-Jackknife estimator utilized for our population size estimate is robust when dealing with this type of error, or 'shadow effect'.

After grouping the genetic profiles, pair-wise comparisons of the different groups were then performed. If three different alleles were observed at a specific locus in a pair-wise comparison, the groups were declared different. If, however, in no case were there more than two different alleles, the profiles in the group could potentially originate

from one defecator (Fig. 1). In this case, the potentially homozygous loci were replicated a further four times. Assuming the worst-case scenario, the probability of falsely accepting a single locus to be homozygous after seven independent replicates gave rise to the same homozygous allele is 1.6% (Taberlet *et al*. 1996; Miller *et al*. 2002). Again assuming the WCR, if there was more than one homozygous genotype in a unique profile that consisted of seven loci that had been replicated seven times, the probability of generating a false multilocus profile due to allelic drop-out would vary from 3.1% (two homozygous genotypes) to 10.4% (seven homozygous genotypes) (see Miller *et al*. 2002). However, because the WCR is unlikely to be appropriate in a real study and in order to reduce replication, we judged seven replicate PCRs to be sufficient to confirm homozygous status at the relevant loci with an acceptable amount of error.

GIMLET 1.0.1 (Valière 2002) was used to verify the accuracy of the complete faecal profiles, and thus the power of our comparative approach, by comparing them with molecular tags obtained from hair and blood samples of 36 badgers captured in the 3 social groups under investigation. Incomplete faecal profiles were compared with the reference profiles by hand. The economy of the comparative approach relative to the WCR and MLR methods was tested by comparing the total number of reactions the three methods would require to obtain consensus multilocus profiles.

The MLR model estimates the probability that a genotype is correct, i.e. its reliability, and suggests a replication protocol if the estimate is below a certain threshold. Because the study was not designed to apply the MLR model, the technique was applied in retrospect to the data set using the program RELIOTYPE (C. Miller, unpublished). Only loci that gave rise to a consensus genotype using the comparative approach in the actual study were considered. The following steps were performed:

1 For every locus two initial replicates were added to the input file. These replicates corresponded to the first two obtained in the actual study, except in cases where three different alleles were observed. In those instances, the third replicate was compared with the other two and replaced the one that contained a false allele. In four cases, further replicates were consulted to identify and remove false alleles.
2 The reliability of the initial replicates was then estimated and a suggested replication strategy obtained. A multiple-sample correction was applied in order to limit the sample-wide number of genotype errors to < 5% with 95% probability.
3 If the estimated reliability of individual samples was below the estimated sample-wide threshold and if further replicates existed in the full data set, then the suggested or available number of replicates was added to the input file and reliability was re-estimated. This was continued until the reliability criteria were met, exceeded the threshold or until no further replicates were available in the full data.

We simulated the number of reactions needed to achieve the specified reliability given an upper confidence bound on the drop-out rate of 75%, both using reliability criteria alone and using reliability criteria in addition to the condition that alleles need to be observed at least twice before being recorded.

Even though it was not possible to always follow the replication strategy to the recommended extent, it was possible to obtain a general idea of the performance of the MLR model with a real data set.

We tested the assumptions of the MLR method for our data set on a *post hoc* basis by checking for the presence of genotyping errors in all the amplification reactions that gave rise to a consensus heterozygous genotype. Cases in which seven amplifications at a locus yielded one heterozygous result and the same homozygous during the six other reactions were excluded from this error analysis. For each locus, it was noted whether the long or the short allele did not amplify if an allelic drop-out had occurred. A Mann–Whitney *U*-test was then used in SPSS 9.0 (SPSS Inc.) to test whether both alleles are equally likely to drop out. To test whether there was a difference in the allelic drop-out rate among loci, we fitted a general linear mixed model (GLMM) using SPLUS 6.1 (Insightful). As the response variable was binary (drop-out/no drop-out), we assumed a binomial error structure. Locus was fitted as a fixed effect (seven-level factor) and sample was fitted as a random effect (46 levels).

Estimation of social group size

Once individual genetic profiles had been secured, we applied mark–recapture models using the program CAPTURE (Otis *et al*. 1978; White *et al*. 1982). It was assumed that the population was closed over the 10-day period. Each collection day was considered a capture session, giving rise to ten sampling sessions with each profile assigned a '1' for a sampling session in which it was detected and a '0' when absent. Estimates of population size were obtained using the null model (M_0-Null) as well as models allowing for variation in capture probability due to individual heterogeneity (M_h-Jackknife, M_h-Chao), time effects (M_t-Chao, M_t-Darroch) and a combination of both (M_{th}-Chao).

Results

Comparison of storage and extraction methods

A two-way analysis of variance (ANOVA) was applied on ranked data, using the Scheier-Ray-Hare extension of the Kruskal–Wallis test, with the proportion of successful

Table 1 Results from an ANOVA of the ranked PCR success rate from faecal DNA obtained using three storage methods and two extraction techniques (d.f.: degrees of freedom; SS: sum of squares; MS: mean square). The ANOVA was performed using a Scheirer-Ray-Hare extension of the Kruskal–Wallis test

Source	d.f.	SS	SS/MS_{total}	*P*-value
Storage	2	34.204	0.045	0.978
Extraction	1	879.874	1.159	0.282
Storage × Extraction	2	5941.642	7.824	0.020
Error	90	65292.096		
Total	95	72147.816		

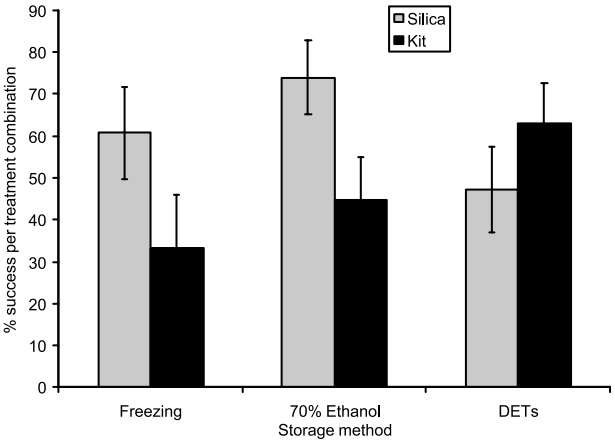

Fig. 2 Comparison of amplification success rates of different storage and extraction methods. Results are from 16 aliquots of faecal samples that were extracted using the GuSCN/silica method or the faecal DNA kit from frozen faeces or from faeces stored in 70% ethanol or a DETs solution. Each sample from each treatment was amplified once with 7 microsatellite loci and the percentage of successful amplification for each locus in each treatment was calculated by pooling of the data across the 16 samples. For each treatment, the mean of these percentages, with its standard error, is shown.

amplifications as the dependent variable and the storage methods and extraction techniques as factors. Only the interaction term was significant (Table 1), reflecting the fact that the optimal extraction method varied between samples stored in DETs and the other storage methods. The highest amplification success rate was obtained with samples stored in 70% ethanol and extracted with the GuSCN/silica method (Fig. 2). The variation in amplification success between the different primers was also smallest for this treatment (Fig. 2). This method was therefore used for the rest of the study.

Probability of identity

$P_{ID\text{-}SIB}$, calculated from the reliable genotypes of 36 badgers from the three social groups under investigation, predicted

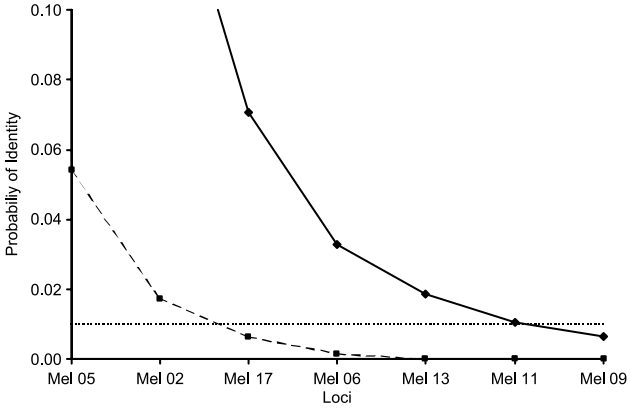

Fig. 3 Sibling probabilities of identity ($P_{ID\text{-}Sib}$) from three badger social groups at Woodchester Park. Probabilities were calculated for seven nuclear DNA microsatellite loci from a data set of 36 badgers. $P_{ID\text{-}Sib}$ for individual loci was first calculated and the loci in the data set were arranged in order of decreasing value (solid line). $P_{ID\text{-}Obs}$ gives the proportion of all possible pairs of individuals that had identical genotypes (dashed line). The dotted 1% cut-off line represents the point where enough loci are typed to distinguish between individuals with 99% certainty.

that the 7 loci used in this study were necessary, yet sufficient, to distinguish with 99% certainty between sibling badgers (Fig. 3). The observed P_{ID} showed that the proportion of individuals with identical profiles dropped to zero if the five most informative loci were used.

Comparative multiple-tubes approach and assessment

Of the 53 faecal samples collected from the latrines of 3 social groups, DNA was obtained from 47 samples (89%). Using the comparative multiple-tubes approach, 33 of these samples gave rise to complete genetic profiles with consensus genotypes at all seven loci (Table 2). A further six profiles were complete but for the presence of an ambiguous case (six homozygote, one heterozygote score). All 7 loci could therefore be amplified in 39 samples (74%). It was possible to reliably score the remaining eight samples for at least the most informative locus (*Mel*-105).

In order to analyse the reliability of the consensus genotypes, the complete faecal profiles were compared to reference profiles obtained from animals caught in the three social groups. There was a 100% match between 28 of the 33 complete profiles and the reference profiles. Faecal sample 42 matched with reference profile U61 except for one allele at locus *Mel*-102 (42 : 199 199; U61 : 195 199). Using the $P_{ID\text{-}sib}$–statistics in GIMLET 1.0.1, the probability that these two profiles represent the same individual was calculated to be 0.985 if locus *Mel*-102 was excluded from the analysis. These two profiles were therefore classified as originating from the same animal. A further four profiles could not be matched to any reference. In order to increase

Table 2 Consensus genotypes obtained using the comparative multiple-tubes approach. Genotypes containing an 'F' are those cases in which an additional allele was observed once in seven amplifications, and was therefore scored as a half-locus. A dash indicates that there were insufficient positive PCRs available to derive a consensus genotype. The last column indicates with which reference individual the faecal profile could be matched

	Consensus alleles at the microsatellite loci under investigation							
Sample	*Mel*-105	*Mel*-102	*Mel*-117	*Mel*-106	*Mel*-111	*Mel*-109	*Mel*-113	Match
Parkmill Social Group								
2	148 148	195 197	187 189	220 222	132 132	106 106	120 120	Q36
3	148 148	195 197	187 189	220 222	132 F	106 106	120 120	
7	148 148	195 197	187 189	220 222	132 132	106 106	120 120	
8	148 148	195 197	187 189	220 222	132 132	106 106	120 120	
16	148 148	195 197	187 189	220 222	132 132	106 106	120 120	
40	148 148	195 197	187 189	220 222	132 132	106 106	120 120	
48	148 148	195 197	187 189	220 222	132 132	106 106	120 120	
49	148 148	195 197	187 189	220 222	132 132	106 106	120 120	
57	148 148	195 197	187 189	220 222	132 132	106 106	120 120	
1	138 142	199 199	174 193	220 222	132 138	106 116	120 120	X59
20	138 142	199 199	174 193	220 222	132 138	106 116	120 120	
25	138 142	199 199	174 193	220 222	132 138	106 116	120 120	
28	138 142	199 199	174 193	220 222	132 138	106 116	120 120	
29	138 142	199 199	174 193	220 222	132 138	106 116	120 120	
43	138 142	199 199	174 193	220 222	132 138	106 116	120 120	
17	138 142	199 199	174 187	220 224	130 132	106 125	120 120	D77
46	138 142	199 199	174 187	220 224	130 132	106 125	120 120	
47	138 142	199 199	174 187	220 224	130 132	106 125	120 120	
53	138 142	199 199	174 187	220 224	130 132	106 125	120 120	
9	138 142	199 199	187 187	220 220	130 132	106 106	118 120	U41
41	138 142	199 F	187 187	220 220	130 132	106 106	118 120	
44	138 142	199 199	187 187	220 220	130 132	106 106	118 120	
31	138 140	195 197	174 187	222 222	132 F	106 106	120 126	—
11	138 140	—	—	—	—	—	—	
19	138 138	197 199	174 187	220 222	132 132	106 106	120 126	—
30	138 142	199 199	174 187	220 222	132 132	106 116	120 126	—
52	136 144	—	—	—	—	—	—	Q66
54	140 144	195 197	174 187	220 224	132 132	106 106	126 126	Q65
58	138 138	199 199	174 187	222 226	132 138	106 125	120 120	H51
Nettle Social Group								
6	142 142	195 199	174 174	222 224	130 132	106 127	120 126	J68
32	142 142	195 199	174 174	222 224	130 132	106 127	120 126	
51	142 F	195 199	174 174	222 224	130 132	106 127	120 126	
4	138 142	195 199	174 174	222 224	130 132	106 106	120 126	J56
39	138 142	195 199	174 174	222 224	130 132	106 106	120 126	
5	138 142	195 199	174 187	222 224	130 132	106 106	120 126	—
33	138 142	195 199	174 187	222 224	130 132	106 106	120 126	
23	138 138	197 199	174 189	222 226	132 F	106 106	120 120	Q72
55	138 138	197 199	174 189	222 226	132 132	106 106	120 120	
56	136 140	193 193	174 187	222 F	132 132	106 127	120 120	U8
Kennel Social Group								
21	138 148	195 199	—	—	—	—	—	U62
34	138 148	195 199	187 187	222 222	130 132	106 106	120 126	
37	138 148	—	—	—	130 132	106 106	120 126	
42	144 148	199 199	174 187	222 222	130 130	106 106	126 126	U61
36	144 148	—	—	—	130 130	106 106	126 126	
13	136 142	—	—	—	—	—	—	T50
26	140 148	—	—	—	—	106 106	120 126	M58
35	148 148	—	—	—	132 132	106 106	120 120	X30

the confidence that these unique profiles were not the result of allelic drop-out, the homozygous loci from these samples were amplified a total of seven times.

Ignoring failed reactions, a total of 1009 PCRs had to be performed to obtain 293 consensus genotypes, with an average of 3.4 reactions per locus per genotype. Had the WCR approach been followed, an additional 517 positive PCRs would have had to be analysed, giving a total of 1526 reactions (5.2 reactions per locus per consensus genotype). When testing the MLR approach, a multiple-test correction was applied. For all the tests, it was found that, in order to limit the sample-wide number of genotype errors to < 5% with 95% probability, each individual sample required a reliability of 98.3%. When applying the MLR model to the data and using reliability only as a criterion, the number of recommended PCR replicates could be kept to a minimum of 1002. When, in addition to the reliability criteria, all alleles need to be observed at least twice, the minimum number of PCR replicates was estimated to be 1157.

In order to determine whether the assumptions of the MLR model were met in this study, the errors of the replicate PCRs were analysed (Table 3). Allelic drop-out occurred in 27% of the amplification reactions for heterozygous genotypes and an otherwise wrong result was obtained in 8% of these reactions so that, pooling both error types, a mean error rate for heterozygous loci of 35% was obtained. Considering both homozygous and heterozygous genotypes, i.e. all the PCRs, 19% of all the amplification reactions were erroneous. There was no difference in the drop-out rate between short and long alleles (Mann–Whitney U; N1 = 7, N2 = 7; Z = –0.321; P = 0.805). The GLMM indicated significant variation in error rates among loci (P = 0.0103) after between sample variation was taken into account. The difference among loci was mainly due to a much greater drop-out rate at locus *Mel*-102. At this locus, 47.7% of the amplifications for heterozygous genotypes experienced allelic drop-out compared with values ranging from 10.6% (*Mel*-109) to 29.7% (*Mel*-106) for the other loci.

The MLR method assumes that all false alleles can be detected and eliminated from the data set so that the only possible source of error is undetected allelic drop-out events. In order to simulate an actual study when performing the MLR in retrospect, we only eliminated false alleles that would have been recognized at each specific round of replication. There were six ambiguous cases (with six homozygote, one heterozygote score; Table 2). Of these, one (locus *Mel*-106 in sample 56) was found to be a true heterozygote through comparison with the reference genotypes, whereas the rest were homozygotes. If all alleles were accepted on the basis of reliability this would have led to five erroneous profiles. Furthermore, the initial replicates contained three false alleles that would have remained undetected based on reliability criteria, leading to a total of eight erroneous profiles. However, if in addition to a decision based on reliability criteria, alleles needed to be observed at least twice before being recorded, both the inconclusive cases and the false alleles would have been detected using the MLR method, as they were with the comparative method.

Given the multiple test correction, we could be 95% sure that < 5% of the multilocus profiles were wrong because of undetected allelic drop-out. From 47 profiles we would expect errors for 2.35 genetic profiles at most. Consistent with this expectation, the consensus genotypes generated by both MLR models (i.e. reliability criteria alone or with the additional requirement of observing alleles twice) did not contain any undetected allelic drop-outs. The MLR model correctly indicated the need for further replication at locus *Mel*-102 of individual 42, the allelic drop-out that remained undetected using the comparative approach.

Estimation of social group size

During 2001, 29 badgers had been live-trapped in the three social groups under investigation (Wilson *et al*. 2003; though for one of these individuals a DNA profile was not

Table 3 Summary of the faecal DNA PCR errors observed in this study, by locus and type. Data are from heterozygous genotypes at seven microsatellite loci in 47 individuals. Only PCRs in which a consensus genotype was obtained were considered. Type I errors included PCRs where three alleles were obtained, as well as cases where one or two alleles were observed but one of these was false

		Loci							
		Mel-102	*Mel*-105	*Mel*-106	*Mel*-109	*Mel*-111	*Mel*-113	*Mel*-117	Total
Result type	No. individuals	23	30	31	16	22	16	30	368
Correct		43	59	63	42	50	43	68	368
	Short allele missing	30	10	17	2	8	4	13	84
Error type II									
	Long allele missing	12	13	13	3	7	3	16	67
Error type I	Wrong genotype	3	8	8	0	10	0	14	43
Total		88	90	101	47	75	50	111	562

available). Twenty different profiles were obtained from faeces collected from the three social groups. Of these, 15 could be matched to the 29 captured badgers (Table 2), and 1 genetic profile belonged to an individual trapped in 2000 but not in 2001. Thus, of 20 different genetic profiles obtained from faecal DNA, 16 could be matched to known group members. At the time of study, direct observation showed that, apart from the 16 individuals identified by their genetic profile, at least a further 8 badgers were present in the 3 social groups (see Wilson *et al*. 2003). The actual number of badgers resident in the three groups could therefore vary between 24 and 34 individuals, the maximum value being the total of the 29 live-trapped individuals, plus the 5 genetic profiles originating from animals that were not caught in the 3 social groups in 2001. Thus, from direct enumeration using the faecal DNA profiles, we sampled ≈ 47–67% of the population of the three social groups.

Mark–recapture analysis of profiles was performed using program CAPTURE, assuming a closed population during the 10-day collection period and treating each day as a capture session. Of the 53 samples collected, the daily percentage of samples from which DNA could be successfully extracted varied between 75 and 100% depending on the day of collection. Point estimates varied between 23 individuals (95% CI 21–30) for model M_t-Darroch, to 28 individuals (95% CI 23–51) using model M_{th}-Chao (Table 4). The two models that allow for individual heterogeneity both generated a point estimate of 26 individuals, with a slightly smaller 95% confidence interval for M_h-Jackknife (M_h-Jackknife: 22–40, M_h-Chao: 22–45; Table 4).

Discussion

Comparison of storage and extraction methods

In order to maximize the success of the faecal DNA extractions, various storage and extraction techniques were tested for their suitability for use with badger faecal DNA. Although all methods were successful, storage of faecal samples in 70% ethanol and extraction of DNA with the GuSCN/silica method was slightly superior. As well as being the cheapest method, this combination is also safest as storage in 70% ethanol is an effective disinfectant against *Mycobacterium bovis* (Seymour 1991). Murphy *et al*. (2002) found that storage of brown bear (*Ursus arctos*) faeces in 90% ethanol gave rise to the highest proportion of amplifiable DNA and had the longest post-collection longevity. Frantzen *et al*. (1998) found that storage in DETs solution was the most appropriate method of preserving faecal samples when fragments longer than 300 bp were amplified, but that for shorter fragments all storage methods performed similarly. It is therefore possible that the treatments would have had more of an effect on loci > 300 bp in length. It is also possible that genotyping error rates varied significantly between treatments (Flagstad *et al*. 1999), but this was not tested in our study.

Of the 53 faecal samples collected from 3 social groups of badgers (Parkmill, Nettle and Kennel) it was possible to amplify all 7 loci in 39 samples (74%), and for a further 8 samples it was possible to amplify at least the most informative locus. This resulted in 47 samples (89%) with at least partially amplifiable DNA. This success rate is high in comparison with many other studies, which report successful amplification in 48–66% of faecal samples (Gerloff *et al*. 1995; Kohn *et al*. 1999; Farrell *et al*. 2000; Jansman *et al*. 2001; Lucchini *et al*. 2002). However, a success rate of 83% (i.e. similar to ours) has been reported by Banks *et al*. (2002), and a rate of 93–95% has been reported by Flagstad *et al*. (1999). One reason for our success may be that only faeces that were less than a day old were analysed.

Probability of identity

$P_{ID\text{-}Sib}$ statistics suggested that DNA profiles consisting of the seven loci used in our study would be sufficient to distinguish between individual badgers, including siblings, with 99% certainty. This statistic was supported by $P_{ID\text{-}Obs}$,

Table 4 Estimates from six closed mark–recapture models of the population size of three badger social groups at Woodchester Park. Estimates were generated using the program CAPTURE, with the abbreviations for each model described in full in the text

Model	Source of variation in capture probability	$\hat{N}$	SE	95% CI	Average estimated recapture probabilities
M_o-Null	None	23	2.5	21–32	0.17
M_t-Darroch	Time	23	2.2	21–30	0.18, 0.22, 0.22, 0.13, 0.22, 0.22, 0.13, 0.18, 0.18, 0.09
M_t-Chao	Time	24	3.9	21–39	0.14, 0.18, 0.18, 0.11, 0.18, 0.18, 0.11, 0.14, 0.14, 0.07
M_h-Jackknife	Heterogeneity	26	4.1	22–40	0.15
M_h-Chao	Heterogeneity	26	5.0	22–45	0.15
M_{th}-Chao	Time*Heterogeneity	28	6.4	23–51	same as M_t-Chao

which dropped to zero after the five most informative loci were used. It is possible that the number of loci required would differ between populations, and perhaps even social groups (see Banks *et al*. 2002). This would need to be investigated for surveys of other badger populations, given the social nature of this species in Britain.

Comparative multiple-tubes approach and assessment

When testing the reliability of our comparative approach we found that in 293 consensus genotypes, 1 case of allelic drop-out was not detected. The error arose because it was not possible to compare the genotype in question with the corresponding locus from an identical profile. Although this error would not have led to an incorrect estimate of the number of individuals present (see Table 1), we determined that a further modification to our approach would reduce this error. Loci in unique profiles that, after three positive amplifications were provisionally recorded as being homozygous needed to be amplified a further four times to ensure that the genotype was scored correctly. This modification should be applied when correct profiles need to be obtained for single-sampled individuals, as would be the case when paternity analysis is performed. An outline of the complete modifications can be seen in Fig. 1. If we had performed this step, an additional 64 reactions would have been required, giving a total of 1073 PCRs. The WCR approach would require 1526 reactions; therefore our approach appears to greatly reduce PCR effort.

A mean error rate for heterozygous genotypes of 35% was obtained with, overall, an error observed in 19% of all PCRs. Allelic drop-out occurred in 27% of replication reactions for heterozygous genotypes and was therefore a more significant problem than the occurrence of false alleles. This error rate is higher than in most faecal DNA studies (e.g. Bayes *et al*. 2000; Ernest *et al*. 2000; Goossens *et al*. 2000; Constable *et al*. 2001) but similar to the error rate found when using single hairs (Gagneux *et al*. 1997; Goossens *et al*. 1998). This high error rate increases the number of PCRs required to obtain consensus genotypes but does not invalidate the use of faeces as a source of badger DNA.

We found that the MLR model, when applied in practice, will also significantly reduce the number of amplifications needed to obtain consensus genotypes compared with the WCR approach. The effectiveness of the method is dependent, however, on whether the assumptions of equal drop-out rates of alleles of different size and across loci, as well as the detection of all false alleles, can be met in a given data set. The assumption that there is no difference in the allelic drop-out rate between the longer and the shorter alleles was confirmed for our data set. This result has also been found in some other studies (Gerloff *et al*. 1995; Gagneux *et al*. 1997), but not all (Constable *et al*. 2001), and so ideally should be tested for each new data set. Although we found that the allelic drop-out rate varied significantly among loci (see also Lucchini *et al*. 2002), the overall rate was estimated to be 27%, a value low enough to expect the replication strategy suggested by the MLR model to be robust, even if an upper bound of 75% on the drop-out rate was used (Miller *et al*. 2002). In future studies, it may be worth replacing the most unreliable locus (*Mel*-102) or even removing it from the analysis if the power to distinguish between different individuals would not be too greatly reduced. Contrary to the first two assumptions, we found that by relying on reliability criteria alone (such that alleles do not need to be observed twice to be recorded) a total of eight erroneous multilocus profiles would have been obtained. Our results therefore confirmed the suggestion by Miller *et al*. (2002) that it will be necessary to not rely solely on reliability criteria, but also to observe each allele at least twice before recording it.

When utilizing the MLR model with our data set, we applied a multiple test correction in order to be 95% certain that fewer than 5% of the multilocus profiles contained undetected allelic drop-outs. Because no allelic drop-out remained undetected after following the replication strategy of the MLR model, it was concluded that more stringent reliability criteria (for example, limiting the incidence of errors to 0% with a probability of 95 or 99%) requiring more PCR replicates would not have been necessary for our study. This level of reliability should therefore be sufficient for other studies, but we recommend that the rule of observing an allele at least twice before recording always be applied when using the MLR model.

The advantage of the comparative method is that no advance knowledge of differences in the drop-out rate between large and small alleles or between different loci is required for it to be used, making it better suited for studies with limited financial resources. However, the method requires a number of identical genetic profiles that can be compared with each other, which might not be achievable with other species. The MLR model may be more appropriate where faecal samples from a single individual are less likely to be replicated. The MLR method may also be better suited to large studies in which batch PCR replication, rather than single additions, is more practical, though the comparative method could be adjusted to allow batch replication. Our method, however, seems better suited to small studies in which many faeces are expected to be obtained from single individuals.

Estimation of social group size

During the 10-day trial period, 20 different genetic profiles were obtained from the 53 faecal samples collected. Of these, 16 could be matched to known group members. The number of badgers present in the 3 social groups under investigation was estimated independently to be between

24 and 34 animals (Wilson *et al*. 2003). Therefore, this faecal sampling approach (10 consecutive days at latrines close to setts) in a high-density population would considerably underestimate population size if only direct enumeration were used. Also, the results suggest that, even though all members of a social group use latrines close to the sett (Wilson *et al*. 2003), it would be time-consuming and expensive to identify every individual member of a group using faeces.

The use of mark–recapture estimates can potentially solve the need to genotype every individual from faeces to obtain an accurate estimate of population size. The model with the point estimate closest to the 29 individuals captured in 2001 was M_{th}-Chao (28 individuals), however, it also had by far the largest 95% confidence interval (23–51). Model M_t-Darroch had the smallest 95% confidence interval (21–30), but the model is known to perform poorly when faced with individual heterogeneity (Otis *et al*. 1978; White *et al*. 1982). The M_h-Jackknife estimator works best with individual heterogeneity and is robust to some variation due to time as well as to the 'shadow effect' (Otis *et al*. 1978; White *et al*. 1982; Manning *et al*. 1995; Mills *et al*. 2000). This model therefore seems most appropriate, estimating the population size in the 3 social groups to be 26 individuals with a 95% confidence interval of 22–40 animals. The 95% confidence interval compares well with the range of the known population size (24–34 individuals), which was obtained by extensive observation by several researchers over many months (Wilson *et al*. 2003).

Even though the estimate obtained using the Jackknife estimator of M_h appears close to the one obtained from capture studies, the actual population size remains difficult to estimate because although social groups will have core members, the occurrence of transient visitors (Christian 1994; Rogers *et al*. 1998) makes it difficult to estimate the number of badgers in a small area in isolation. Although we established a technique for obtaining badger genetic profiles from faeces, further study is needed to design sampling strategies to obtain density estimates across various scales and population densities.

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Alain Frantz is a graduate student supervised by Tim Roper at the University of Sussex. Alain is currently applying the techniques presented in this study to questions relating to badger ecology in Luxembourg. Lisa Pope is a postdoctoral researcher working on badger population genetics. Lisa helped Alain with the design of the faecal genotyping approach and supervised the laboratory work. Petra Carpenter developed and optimized the badger microsatellite marker set. The laboratory work and analysis was carried out at the Sheffield Molecular Genetics Facility directed by Terry Burke at the University of Sheffield. The project was instigated by Tim Roper, Richard Delahay and Gavin Wilson, whose research focuses on the relationship between badgers and bovine tuberculosis.
