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A forensic STR profiling system for the Eurasian badger: A framework for developing profiling systems for wildlife species

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Abstract

Developing short tandem repeat (STR) profiling systems for forensic identification is complicated in animal species. Obtaining a representative number of individuals from populations, limited access to family groups and a lack of developed STR markers can make adhering to human forensic guidelines difficult. Furthermore, a lack of animal specific guidelines may explain why many wildlife forensic STR profiling systems developed to date have not appropriately addressed areas such as marker validation or the publication and analysis of population data necessary for the application of these tools to forensic science. Here we present a methodology used to develop an STR profiling system for a legally protected wildlife species, the Eurasian badger *Meles meles*. Ten previously isolated STR loci were selected based on their level of polymorphism, adherence to Hardy–Weinberg expectations and their fragment size. Each locus was individually validated with respect to its reproducibility, inheritance, species specificity, DNA template concentration and thermocycling parameters. The effects of chemical, substrate and environmental exposure were also investigated. All ten STR loci provided reliable and reproducible results, and optimal amplification conditions were defined. Allele frequencies from 20 representative populations in England and Wales are presented and used to calculate the level of population substructure (θ) and inbreeding (f). Accounting for these estimates, the average probability of identity (PI_{ave}) was 2.18×10^{-7}. This case study can act as a framework for others attempting to develop wildlife forensic profiling systems.

Keywords: Wildlife forensics; Individual identification; Badger; Validation; DNA

1. Introduction

The application of forensic genetic techniques to non-human species is an increasingly recognised approach in criminal investigations [1–3]. When evidence samples lack identifying morphological characters these techniques can provide an alternative method of identification. The method of DNA sequencing can be used to determine which species an evidence sample originates from by observing species specific differences at mitochondrial genes [4,5]. DNA sequencing of validated genetic markers [6,7] has been used to infer species identity in cases of illegal trade and wildlife persecution [e.g. 8,9]. In certain circumstances it is also necessary to determine which individual a biological sample originates from. One technique that establishes this link is short tandem repeat (STR) profiling which provides a statistical probability that the two biological evidence samples belong to the same individual. Generating the statistical probability of a match requires allele frequency data generated from profiling representative populations [10–12].

Forensic bodies such as the Scientific Working Group on DNA Analysis Methods (SWGDAM), the European DNA Profiling Group (EDNAP) and the International Society of Forensic Genetics (ISFG) provide a suite of guidelines for the development and application of STR profiling tools including methods of marker validation, the publication of population data and the generation of forensic match probabilities [10–14]. The adherence to forensic guidelines is strongly recommended and although these guidelines are devised for application in human forensic casework they can be applied to non-human

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species alongside recent recommendations in certain areas of forensic animal identity testing [15].

STR profiling systems have been developed in certain domesticated and commercial species including cats [16], dogs [17] and pigs [18]. These have subsequently been used in forensic casework where biological material was transferred from domestic species to humans. Trace evidence transfer of this type has been used in a variety of investigations including murder [19], armed robbery [20] and animal cruelty (Royal Society for the Prevention of Cruelty to Animals (RSPCA) versus Draper). Forensic STR profiling systems have also been designed and created for use in wildlife species [e.g. 21–23] although the developmental methodology in many of the published articles do not show evidence of following the necessary suit of guidelines mentioned above [e.g. 24–26]. Commonly overlooked forensic recommendations include confirmation of Mendelian inheritance of loci, assessment of cross species amplification, validation of case type samples and thermocycling parameters, the development of an allelic ladder and the publication of population allele frequency data. Lack of adherence to these guidelines may be explained by certain inherent difficulties encountered when working with wild species as opposed to domesticated and commercial species. For example, pedigree data for determining both Mendelian inheritance and mutation rates of STRs exist in domestic and commercial species [e.g. 27] while these data may be limited or absent in wild species. Additionally the number of individuals genotyped in population studies detailing human allele frequency data is generally 100–300 and can exceed 1000 [e.g. 28]. Such sample sizes may be easily accessible when working with domestic and commercial species whereas these numbers may be difficult to obtain if the animal species is of conservation interest.

Although complex, the development of STR profiling systems in wild animal species would provide an additional tool in criminal investigations. The Eurasian badger, *Meles meles*, is protected against persecution in the United Kingdom (UK) under a number of legislative acts including the Protection of Badgers Act (1992) and the Wildlife and Countryside Act (1981). Despite this protection, instances where badgers are unlawfully killed in the UK are believed to be increasing [29]. Activities include badger baiting, where dogs are provoked into attacking badgers, illegal snaring, poisoning and sett destruction. Evidential samples recovered from these activities are typically blood swabs or hairs which have previously been used to provide species identification results in forensic casework (e.g. RSPCA versus Billington 2006). The ability to match two samples, effectively linking a suspect to a crime scene, would provide investigating agencies with stronger forensic evidence than species identification alone.

This study describes the methodology followed to develop a validated STR profiling system for calculating match probabilities in the badger. Current human forensic guidelines and animal forensic recommendations are used as a basis for the developmental protocol.

2. Materials and methods

2.1. Choice of STRs

From a published set of 39 polymorphic STR loci developed for *M. meles* [30] ten loci were selected based on the observed level of polymorphism and their adherence to Hardy–Weinberg expectations (Table 1). Other desirable characteristics of STRs included small fragment length and the ability to be poolplexed.

2.2. Sample collection

For the validation studies, blood from a deceased badger was provided by the RSPCA and previously generated STR profiles from two family groups were provided to verify Mendelian inheritance (Rafart, unpublished data). The allele frequency database was constructed from a combination of existing STR profile data and profiles generated from this study. Contributors of samples and pre-existing data were the University of Sheffield, the RSPCA, the National Federation of Badger Groups (NFBG), and the Department for the Environment Food and Rural Affairs (DEFRA). Twenty geographically coherent populations in England and Wales were defined totalling 1083 individuals (map available as supplemental data Figure 1).

2.3. DNA extraction and polymerase chain reaction (PCR) amplification

DNA from ear snip samples collected from deceased badgers and blood was recovered using the QIAGEN DNeasy tissue kit and was quantified against a known standard using the fluorescent dye PicoGreen (Molecular Probes, Inc.), and Galaxy Fluostar apparatus (BMG Labtechnologies Ltd.). PCR [31] reaction conditions for single locus amplification and developmental validation studies were defined as: a standard 10 μl reaction containing 0.25 units ABgene Thermo-Start® DNA Polymerase, 1× Thermo-Start® reaction buffer, 2.5 mM $MgCl_2$, 0.2 mM each dNTP, 20 pmol primer and 0.5 μl of template DNA (2–10 ng/μl). PCR was performed on a PTC-200 MJ Research thermocycler using the following standardized cycling parameters: 15 min denaturation step at 95 °C; 35 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s; 5 min elongation step at 72 °C. Amplification products were visualised under UV light following electrophoresis on an ethidium bromide stained 2% agarose gel. Positive controls (available on request) and negative controls were used throughout. Amplification product was genotyped on a Beckman Coulter CEQ8000 following standard protocols using vendor recommended analysis parameters.

2.4. Validation studies

Validation studies were performed on individual loci following the guidelines provided by the Scientific Working Group on DNA Analysis Methods (SWGDAM) (http://www.cstl.nist.gov/div831/strbase/validation/SWGDAM_Validation.doc).

Table 1
Ten loci selected for the profiling system with GenBank accession numbers and repeat sequence

Locus	GenBank Accession	Repeat sequence	Primer sequence	Fragment size range	H_O	H_E
*Mel*103	AJ293356	$(AC)_{20}$	F: GTTTCTTCCCTGAAAGGCTATTGGGTA R: GGCTGATGCAGTTAGTCTGG	255–263	0.64	0.63
*Mel*104	AJ293352	$(CA)_{17}$	F: GTTTCTTCCTTGTGAACTCACTGCAAC R: TACACTGACACCCTCAAGTCC	315–331	0.59	0.80
*Mel*105	AJ293350	$(GT)_6G(GT)_{16}$	F: GTTTCTTGATATTCCCCTCCCACCACT R: CTCCAAGGGATCCTGGAACT	136–150	0.81	0.86
*Mel*106	AJ293355	$(CA)_{21}$	F: GTTTCTTCTGAAGCCAAATCCACTGAG R: GCCACACTGGTGCCCTAAG	220–226	0.68	0.66
*Mel*107	AJ293359	$(GT)_{22}$	F: GTTTCTTCAAGATCTCCGCAATTCTCC R: AACCCTAAATGTCTGTCAGTGG	284–288	0.37	0.36
*Mel*110	AJ293360	$(GT)_{25}$	F: GTTTCTTCATGTTTGCCATTGGAAGG R: GCCAGTGCTTGAAATAAAGTAG	324–334	0.70	0.73
*Mel*111	AJ230692	$(CA)_{15}$	F: TGCATACAGCTCCCTGAAAG R: GTTTCTTGTGGTAGATGCTGGGATAGTG	130–138	0.58	0.66
*Mel*113	AJ230713	$(CA)_{18}$	F: ATAGTTTGGGTTATTTTCTGGG R: GTTTCTTTTGAGAGGAAAGACCCTACG	120–130	0.55	0.43
*Mel*114	AJ230695	$(CA)_{15}$	F: GTTTCTTTGCTGAGAGTAGAGTGAACATG R: GAAGTGACAGAGATGAAGATAAAC	231–237	0.68	0.74
*Mel*115	AJ230703	$(TTTTC)_3(TTTC)_{15}$	F: GTTTCTTGATCAGTGCCTTCTGGTGAG R: TCCTGAGTCTGCATAACTAGCC	330–351	0.71	0.79

H_O (observed heterozygosity), H_E (expected heterozygosity) and fragment size are taken from [30] as characters used in the selection of loci.

The chromosomal location of the genetic markers was investigated using sequence similarity searches between the badger STR DNA sequence data and the most closely related species for which extensive genome data is available; the dog, *Canis familiaris*. The locations were assigned using an NCBI cross-species megaBLAST search.

Mendelian inheritance was verified by observing the genotype of each STR in a family group. Reproducibility was tested by performing five independent PCR amplifications on one individual and comparing genotype data. To test for amplification of the STR markers with non-target DNA, samples from species commonly encountered during badger persecution investigations were tested (dog, *C. familiaris* and human, *Homo sapiens*). In addition, otter (*Lutra lutra*) was used to investigate the potential for cross-species amplification in closely related species. Effects of DNA template concentration were assessed by amplifying DNA from a single individual at concentrations of 10, 5, 2.5, 1.25, 0.62, 0.31 and 0.15 ng/μl.

Amplification success of *Mel*115 (the largest locus and most susceptible to degrading agents) was used to assess the effect of chemical treatments on DNA recovery and amplification. Ten replicates of a single individual were treated with gasoline, 0.1 M sodium hydroxide, domestic bleach and liquid soap, (25 μl:25 μl undiluted chemical to blood mix). DNA was recovered from five replicates 1 h after treatment and from the remaining five replicates after 5 days' storage in ambient indoor conditions. The effect of denim, suede, wood, and metal on DNA recovery and amplification of *Mel*115 was investigated by treating a 3 cm^2 of each substrate with 25 μl of blood. DNA was recovered under the same time series described for the chemical treatments. In addition, five replicates of a single individual were subjected to environmental conditions typical of those experienced by forensic samples: swabs treated with 25 μl unpreserved blood subjected to outdoor ambient conditions sheltered from rain during January–March (winter). To investigate temporal DNA degradation and its effect on amplification efficiency of these samples, extractions were performed at weeks 0, 2, 4, and 6 before *Mel*115 was amplified via PCR and genotyped.

Single reaction components and thermocycling parameters were varied to determine windows of acceptable performance. Using DNA extracted from an individual badger the following parameters were tested: (i) annealing temperature, testing ±2, ±4 °C of the standard, (ii) cycle number, testing +5, +10, +15, of the standard, and (iii) $MgCl_2$ concentration testing 1.5 mM, 2.0 mM, 2.5 mM, 3.0 mM.

2.5. Allele sequencing

Common alleles of each STR were sequenced to verify their constituent repeat units using the same standardized thermocycling conditions as defined for the developmental validation studies. Every allele observed was named using International Society for Forensic Haemogenetics (ISFH) guidelines reported in [32,33]. Amplification products were prepared for sequencing using exonuclease I (New England Biolabs) and shrimp-alkaline phosphotase (Invitrogen). Unidirectional sequencing was carried out using BIGDYE version 1.1 chemistries on an Applied Biosystems, Inc. (ABI) 3730xl. Sequences were edited using Chromas 1.6 (Technelysium Pty Ltd.). The amplification products were used in the production of an allelic ladder following forensic recommendations [15].

2.6. Analysis

Calibration between new and pre-existing data sets was achieved for each locus by genotyping twelve individuals that had been previously scored by the University of Sheffield on an ABI 3730XL with the resulting difference in allele scores being used to normalize the total data set. Data analyses and estimation of statistical parameters used in the match probability equation were performed using MSA [34], GENEPOP [35], FSTAT [36], API-CALC [37], FSTMET [38] and CODA [39].

3. Results

Tests for departures from Hardy–Weinberg (HW) equilibrium (20 populations by 10 loci) showed three significant deviations after Bonferroni correction, each of which indicated heterozygote deficiency at separate loci in different populations. Of 900 locus pair combinations across the twenty populations, eight pairs (0.9%) showed linkage disequilibrium (LD). However, as no locus combinations were consistently in linkage disequilibrium in all populations these loci are included in further analyses.

Chromosomal location results based on sequence similarity searches suggest that six of the loci are likely to be present on six different chromosomes within the dog genome. The remaining four loci returned matches on multiple chromosomes preventing precise estimation of their location. In addition, heterozygotes were observed at all loci in males, the heterogametic sex, indicating that none of the loci used were X chromosome linked. Genotype data for the two family groups demonstrated that all loci were in agreement with Mendelian inheritance which corresponds with previous observations [40].

The ten STRs gave consistent genotypes for the five independent amplifications, with *d*-values (the base pair size difference between maximum and minimum fragment lengths) less than 0.5 in all cases. The species specificity test with dog and human showed no amplification product for any STRs, while amplification product of a similar length occurred in otter for all the STR loci except *Mel*103 and *Mel*113. There was no evidence of allelic dropout at any locus as DNA template concentrations decreased from 10 to 0.15 ng/μl. The results of sample exposure to different chemicals and substrates showed that DNA was successfully recovered for all treatments although a *T*-test revealed there was a significant decline ($P \leq 0.05$) in the yield of DNA between day 0 and day 5 treatments for leather, wood, soap and gasoline. In the environmental exposure experiments DNA was successfully recovered and amplified from the blood throughout the 6-week period when stored in outdoor ambient conditions. When the amplification product from the substrate, chemical and environmental exposure studies were genotyped no variation in fragment length was observed. The thermocycling parameters study demonstrated that each STR marker can be amplified across all the annealing temperatures investigated. Nine of the ten STR markers amplified at 1.5 mM–2.5 mM $MgCl_2$, but showed no amplification product at 3.0 mM $MgCl_2$. Marker *Mel*110 only amplified cleanly at 1.5 mM. The number of PCR cycles used showed that no spurious amplification product occurs with increasing cycle number within the tested range.

Repeat unit sequence data confirmed that fragment size differences between common alleles are the result of variation in the repeat unit rather than indels in the flanking region. The inclusion of common alleles in an allelic ladder enables accurate identification of alleles in casework samples and provides an effective tool for inter-laboratory comparison.

Allele frequency data, observed and expected heterozygosities and Polymorphism Information Content (PIC) are available for individual populations as supplemental data. Allele frequency data for the entire UK population is given in Table 2. Population substructure (θ) was estimated to be 0.12. Based on this value the average probability of identity (PI_{ave}) was 2.4×10^{-7}. The inbreeding coefficient (f) was estimated to be 0.11. Inclusion of this parameter resulted in a slight decrease in the value of the PI_{ave} to 2.18×10^{-7}. The magnitude of these results suggests that the profiling system could be successfully used to identify individual Eurasian badgers in forensic casework.

4. Discussion

STR markers used in wildlife forensics are often the result of cross amplification between closely related species [21–23,41]. In other species, such as the badger, the choice is limited to markers isolated from conservation genetic and molecular ecological studies. Providing markers are validated prior to use in forensic casework, the approach of utilising existing markers from these types of study opens up the possibility of developing profiling systems for a variety of species. This approach should benefit wildlife forensic research where a wide range of species are targeted in investigations with limited financial resources. The results presented here suggest that by following human and animal forensic guidelines, pre-existing markers can be used in casework.

The presence of linked loci will invalidate the assumptions of the match probability equation leading to biased estimates of identity. Demonstration of independence is therefore required and is best achieved by mapping STRs in the study species. In the absence of such data cross-species genome mapping may offer an alternative method [42]. However, the inability to assign precise chromosomal positions to four loci in this study suggests the approach is limited and that accuracy is dependent on the relatedness of the species involved [43]. Given the lack of extensive sequence data in the badger and many other wild species, cross-species mapping together with familial profiling and statistical tests for linkage disequilibrium are probably the best available options for identifying unlinked loci.

Determining the mutation rate of a locus is a requirement of forensic validation and is used to set a limit on the number of intergenerational mutations permitted in cases of familial exclusion [44]. Providing this information is difficult when working with species lacking pedigree data. Based on mutation rates observed for STRs in other animals [summarised in 45],

Table 2
Allele frequency data for the total England and Wales population of badgers

Allele	*Mel*103 (N:1081)	*Mel*104 (N:1082)	*Mel*105 (N:1083)	*Mel*106 (N:1082)	*Mel*107 (N:1075)	*Mel*110 (N:1077)	*Mel*111 (N:1083)	*Mel*113 (N:1083)	*Mel*114 (N:1080)	*Mel*115 (N:1078)
6.4	–	–	–	–	–	–	–	–	–	0.0051
6.8	–	–	–	–	–	–	–	–	–	0.038
7	–	–	–	–	–	–	–	–	–	0.0543
7.2	–	–	–	–	–	–	–	–	–	0.2231
7.4	–	–	–	–	–	–	–	–	–	0.0005
7.6	–	–	–	–	–	–	–	–	–	0.026
7.8	–	–	–	–	–	–	–	–	–	0.0524
8	–	–	–	–	–	–	–	–	–	0.0775
8.2	–	–	–	–	–	–	–	–	–	0.0575
8.4	–	–	–	–	–	–	–	–	–	0.0181
8.6	–	–	–	–	–	–	–	–	–	0.1776
8.8	–	–	–	–	–	–	–	–	–	0.0176
9	–	–	–	–	–	–	–	–	–	0.2523
12	–	–	–	–	–	–	–	–	–	–
12.1	–	–	–	–	–	–	0.0005	–	–	–
13	–	–	0.0023	–	–	–	–	–	–	–
14	–	–	0.0028	–	–	–	–	0.1311	–	–
14.1	–	–	–	–	–	–	0.0886	–	–	–
15	–	–	–	–	–	–	–	0.4580	–	–
15.1	–	–	–	–	–	–	0.2895	–	0.1264	–
16	0.0301	–	0.0042	–	–	–	–	0.0259	–	–
16.1	–	–	–	–	–	–	–	–	0.7366	–
17	–	–	–	–	–	–	–	0.1057	0.1236	–
17.1	–	0.2006	–	–	–	–	–	–	–	–
18	0.4986	–	–	–	–	–	–	0.1565	–	–
18.1	–	0.0005	–	–	–	–	0.6127	–	0.0134	–
19	0.0019	–	–	–	–	–	–	0.0032	–	–
19.1	–	0.1414	–	0.0079	–	–	0.0088	–	–	–
20	0.2752	–	0.2064	–	–	–	–	0.1196	–	–
20.1	–	0.0762	–	0.0513	–	–	–	–	–	–
21	0.1711	–	0.0526	–	0.2809	0.0009	–	–	–	–
21.1	–	0.1774	–	0.6326	–	–	–	–	–	–
22	0.0208	–	0.0757	–	0.4972	0.2465	–	–	–	–
22.1	–	0.0864	–	0.1562	–	–	–	–	–	–
23	0.0023	–	0.3398	–	0.1981	0.3064	–	–	–	–
23.1	–	0.2352	–	0.1520	–	–	–	–	–	–
24	–	–	0.1630	–	0.0209	0.3171	–	–	–	–
24.1	–	0.0823	–	–	–	–	–	–	–	–
25	–	–	0.0397	–	0.0028	0.1123	–	–	–	–
26	–	–	0.0568	–	–	0.0074	–	–	–	–
27	–	–	0.0568	–	–	0.0084	–	–	–	–
28	–	–	–	–	–	0.0009	–	–	–	–

Common alleles (alleles with a frequency above 0.025) were sequenced and demonstrate that size differences between alleles are due to variation in the number of repeat units.

we suggest a conservatively high rate (10^{-3}) for use with badger profiles when exclusion is required.

The reproducibility of the STR markers was demonstrated through the repeated amplification and successful identification of correct genotypes. The observed *d*-values fall within the range previously used to define individual alleles [46]. To ensure correct allelic identification it is recommended that the allelic ladder be run alongside any unknown sample. Locus *Mel*115 has alleles a single base pair apart which are clearly distinguishable in the ladder, however in addition it may be preferable to sequence particular alleles at this locus to reinforce the genotype results observed.

Cross species amplification was only observed in the European otter, *L. lutra*, however previous studies have also shown amplification of badger STRs in the dog, *C. familiaris* [30]. Cross species amplification has also been noted in validated human forensic profiling systems [47] and is unlikely to result in false positives given the current wildlife forensic genetic approach of DNA species identification prior to providing an individual profile.

Individual identification in forensic genetic science relies on the ability to recover and amplify DNA from samples subjected to a variety of conditions. In this study DNA successfully amplified in all five replicates when treated with different substrate and chemicals despite previous research showing certain substrates and chemicals decrease PCR efficiency [48]. The genotyping results of the environmental exposure study agree with previous data on the stability of other genetic

markers in post-mortem tissues which observe no evidence of somatic mutations over time [49].

The range of PCR conditions investigated during the thermocycling parameter study allow these conditions to be used when faced with material such as faecal matter and hair [50]. These results also provide windows of optimal performance allowing the STR markers to be amplified in other laboratories notwithstanding variation in performance parameters of equipment or staff.

Individual identification is inferred in human forensic genetic casework through use of the match probability equation [10–12]. This refers to the probability that the DNA profile of an evidence item will match the profile of an individual, when the individual is chosen at random from the population. Importantly, the match probability equation allows the relative likelihood of the defence and prosecution hypotheses to be evaluated. An alternative approach used in wildlife forensic casework is the average probability of identity, PI_{ave}, which is the probability of observing two copies of *any* profile in the population [37,51]. This method is particularly useful for evaluating the discriminatory power of an STR profiling system and was used here to demonstrate that the badger loci provide sufficient resolution for individual identification.

The equations used to calculate both the match probability and PI_{ave} require inclusion of an estimate of population substructure, theta, to account for the natural variation in allele frequencies between sub-populations [52]. Given the effect of theta on resulting probabilities [53], this estimator is of particular importance in wildlife species which typically display higher theta values than humans due to more pronounced population structure. The estimate of theta in this study ($\theta = 0.12$) is high compared to those recorded in most human populations (0.01–0.03) [10], but similar to that of dog [17] and otter populations [54]. The theta value here indicates limited gene flow among badger populations which is in agreement with previous estimates of theta from the Eurasian badger [55].

The inclusion of an estimate of inbreeding (f) in the match probability equation has been proposed [56] but is not routinely used in human forensic casework as inbreeding values are considered to be too low. However, when working with wildlife species with certain mating systems, the value of f may be large enough to have a significant effect [51]. As f increases, so does the probability of a match at homozygous loci, therefore ignoring the effect of inbreeding can lead to bias [56]. The opposite effect is observed at heterozygote loci. In the case of badgers, we recommend that estimates of f should be used, but only incorporated into the match probability calculation at homozygote loci.

The two predominantly desirable characteristics of forensic STR profiling systems are discriminatory ability and robustness. The latter can be achieved by following the developmental and application guidelines recommended by forensic advisory groups. Previous wildlife profiling systems appear to have overlooked the importance of adhering to these guidelines and it is important to recognise that the principles of forensic genetic identity testing are the same regardless of the species under study. At the same time it should be noted that certain forensic recommendations cannot easily be followed when working with certain species. Wildlife forensic STR profiling systems should be developed with this in mind and try to maximize both discriminatory ability and robustness.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fsigen.2007.08.006.

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