Molecular Ecology (2000) **9**, 1233–1240

Highly reliable genetic identification of individual northern hairy-nosed wombats from single remotely collected hairs: a feasible censusing method

M. A. SLOANE,† P. SUNNUCKS,* D. ALPERS,‡ L. B. BEHEREGARAY† and A. C. TAYLOR*
**Department of Biological Sciences, Monash University, Victoria, 3800, Australia, †Department of Biological Sciences, Macquarie University, New South Wales, 2109, Australia, ‡Department of Ornithology, Academy of Natural Sciences of Philadelphia 1900 Benjamin Franklin Pkwy, Philadelphia, PA 19103, USA*

Abstract

The highly endangered northern hairy-nosed wombat (*Lasiorhinus krefftii*) is extremely difficult to study in the wild, and its numbers correspondingly difficult to estimate. Disturbance to the animals caused by trapping and radio-tracking may not only constitute an excessive risk to the population's viability, but may also yield biased data. The results of a pilot study are presented, which clearly show noninvasive genotyping to be a highly feasible and reliable alternative censusing method for *L. krefftii*. The protocol can identify individual wombats from single hairs collected remotely at burrow entrances, using: (i) a panel of microsatellite markers giving individual-specific genotypes; and (ii) a Y-linked sexing marker in combination with a single-copy X-linked amplification control. Using just the eight most variable microsatellites (of 20 available), only one in 200 pairs of full-sibs are predicted to share the same genotype. From 12 wombat hair samples collected on tape suspended over burrow entrances, three known female, two known male and an unknown wombat of each sex were identified. The approach will allow censusing of individuals that evade capture, and will also reveal some otherwise problematic aspects of the behaviour of this elusive animal.

Keywords: conservation, hair follicles, microsatellite, noninvasive sampling, population censusing, Y-linked sexing

Received 2 December 1999; revision received 16 March 2000; accepted 25 March 2000

Introduction

Recent history and current status of the northern hairy-nosed wombat

The historic range of the northern hairy-nosed wombat (*Lasiorhinus krefftii*) extended through a region over 3000 km in length (Strahan 1995). This large, burrowing, herbivorous Australian marsupial is now highly endangered, known only from a single remnant population in Epping Forest National Park, central Queensland (Johnson & Crossman 1991; Crossman *et al*. 1994). The species is currently estimated to consist of 75 individuals (A. Horsup, personal communication). Its dramatic range reduction and population decline has been attributed to a combination of habitat destruction, drought and grazing competition with sheep and cattle (Crossman *et al*. 1994).

Correspondence: A. C. Taylor. Fax: +61 3 9905 5537; E-mail: andrea.taylor@sci.monash.edu.au

Population monitoring

Given the precarious state of the Epping Forest wombat colony, a form of population monitoring is desirable that imposes minimal disturbance whilst supplying important population and demographic data. Collection of such data for this species has been hampered by its nocturnal and fossorial lifestyle, and its extreme wariness (Taylor *et al*. 1998). Much of the available information on historical trends in the population has come from local landholders (Crossman *et al*. 1994). Limited knowledge on burrow sharing, home ranges and dispersal patterns has been deduced from signs of wombat activity, radio tracking, frequent trapping and genetic relatedness structure (Gordon *et al*. 1985; Johnson & Crossman 1991; Taylor *et al*. 1997).

Trapping is currently the only method used to estimate population size and sex ratio, but this entails trauma, possible injury or death of wombats, necessitates construction around burrows and is time-consuming and unreliable (Crossman *et al*. 1994; Hoyle *et al*. 1995).

Application of noninvasive genetic sampling to L. krefftii

Genetic analysis of noninvasive samples such as shed hairs and faeces avoids the risks associated with blood or biopsy collection from endangered animals, but more importantly, it provides information on otherwise elusive individuals or species (Morin *et al*. 1994; Gerloff *et al*. 1995; Tikel *et al*. 1996; Foran *et al*. 1997; Gagneux *et al*. 1997; Kohn & Wayne 1997; Reed *et al*. 1997; Taberlet *et al*. 1997).

Wombats are ideal for this type of research, as all individuals pass given points upon entry and exit of burrows. This allows easy collection of hairs from adhesive tape suspended across burrow entrances (Taylor *et al*. 1998). Although faecal pellets are also easily collected, they are less suitable for our purposes for several reasons. First, spatiotemporal patterns of faecal pellet deposition are unknown, demanding analysis of many more samples than may be necessary to fully census the population. Second, they may vary considerably in the quantity of DNA they yield, through the potentially stochastic nature of sloughing of epithelial cells onto pellets passing through the gut (Albaugh *et al*. 1992). Third, faeces are more likely than hairs to contain substances that either increase the incidence of polymerase chain reaction (PCR) errors, inhibit PCR altogether or induce DNA damage, thus reducing the reliability of PCR typing (Taberlet & Luikart 1999). Finally, unlike hairs, faecal pellets are not explicity tied to burrows so resultant information will not reveal individual burrow usage patterns (although the information obtained may still be valuable).

The reliability of microsatellite genotyping from the picogram amounts of DNA often obtained by noninvasive sampling has been questioned (Taberlet *et al*. 1996, 1999). Particular concerns are the potential for 'allelic dropout' (the nonamplification of alleles which may go unnoticed in heterozygotes), or the PCR generation of 'false alleles' (Taberlet *et al*. 1996; Gagneux *et al*. 1997; Goossens *et al*. 1998). Furthermore, long-lived animals and tissues may accumulate somatic mutations, as exemplified by the differing genotypes observed in hair and blood of elephants (Greenwood & Pääbo 1999). *L. krefftii* individuals live for at least 17 years in the wild and 33 years in captivity (A. Horsup, personal communication).

Despite such concerns, the potential gains from developing noninvasive genotyping as a tool for remote censusing of *L. krefftii* are so great that its feasibility warrants examination. A pilot study can achieve the dual aims of (i) ensuring there are no intrinsic barriers to the success of the technique (Taberlet *et al*. 1999), and (ii) optimizing protocols and procedures to achieve efficiency without compromising quality of information. Around 500 ng of DNA can be obtained from a single freshly plucked human hair (Higuchi *et al*. 1988). As wombat hair follicles do not differ markedly in size to those from humans, they may reasonably be expected to contain similar amounts of DNA. Indeed earlier work suggested that single hairs from this species could potentially yield sufficient DNA for reliable individual-specific genotyping, even under less than ideal conditions (particularly those relating to hair storage; Taylor *et al*. 1998). The present study therefore aimed to optimize a method of DNA extraction and microsatellite genotyping from single hairs to maximize DNA recovery and reliability of scoring, and determine the number of loci required confidently to ascribe individual identity to a sample. In particular the aim was to minimize potentially exacerbating effects such as: (i) storing hairs for long periods (Gagneux *et al*. 1997); (ii) transferring DNA through multiple tubes during the extraction process (Gerloff *et al*. 1995); (iii) using PCR primers designed for distant relatives (e.g. Morin *et al*. 1994; Gagneux *et al*. 1997); and (iv) using a small proportion of extract in PCRs (Taberlet *et al*. 1996; Goossens *et al*. 1998).

Materials and methods

Hair and blood collection

Hair collection was carried out immediately prior (in April) to trapping surveys conducted from April to September 1999. Hairs were collected by suspending strong double-sided sticky tape (TESA product No. 4970) across burrow runways (at a height of 20–30 cm) between metal posts used to align traps. Approximately 70 hair tapes were set at 16 burrows distributed throughout the park. Tapes were checked each morning for the presence of hair, and replaced with fresh tape if a hair sample was obtained. Fresh prints and faeces around the hair tapes were identified. Hairs were stored dry, still stuck to tape in plastic bottles until the DNA extractions were performed.

Hairs were also removed from five individuals during handling for blood sampling, to allow comparison of genotypes from different tissues. To simulate the event of a tape sticking to a wombat near a burrow, hairs were obtained by sticking and pulling tape off the wombat's back.

DNA extraction

As a clump of hair may represent more than one individual, it was decided to analyse single rather than pooled hairs. DNA extractions were performed on up to five replicate single hairs from tapes collected at burrow entrances. To maximize DNA yield, extractions were performed in the

field on the day of hair collection, using a method modified from Walsh *et al*. (1991). A portion of hair (1.5 cm in length) containing the follicle was placed root-end down in 200 μL of 5% (w/v in 10 mM Tris pH 8, 0.1 mM EDTA pH 8) chelex® 100 resin (Bio-Rad) and boiled for 10 min. Scissors and tweezers were ethanol flamed before handling individual hairs. Extraction blanks (chelex but no hair) were always included. Chelex extracts were stored at 4 °C for up to 2 weeks in the field, with longer-term storage in the laboratory at –20 °C.

DNA was also extracted from five hairs (from 4 to 72 h after removal from the wombats) and from EDTA-collected and then frozen blood (following Taylor *et al*. 1994) from each of five trapped wombats.

Microsatellite and sexing PCRs

All microsatellite markers were derived from *L. krefftii* or its very close relative *L. latifrons* (Beheregaray *et al*. 2000). It was anticipated that several nanograms of DNA would be available for each hair PCR reaction (even if only one fifth as much DNA per hair was obtained compared with Higuchi *et al*. 1988) so we accordingly employed standard amplification conditions. An aliquot of each hair extract was first PCR amplified for microsatellite Lla16CA, in order to identify hair extracts that may contain sufficient template for at least 20 microsatellites and a sexing PCR, and simultaneously confirming the hairs' wombat origin. Prior to all PCR reactions, chelex extracts were thawed, vortexed, and centrifuged at 13 000 r.p.m. for 3 min. Lla16CA reactions were performed under standard conditions, in 15 μL volumes containing 8 μL of chelex extract, 2 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.1% Triton X-100, 200 μM of each dNTP, 1 μM of each primer, and 0.4 U of *Taq* DNA polymerase (recombinant) (Fermentas). Cycling was performed in an Eppendorf Mastercycler, starting with 94 °C for 2 min, followed by a 'touchdown' PCR (30 cycles of 94 °C/15 s, annealing/30 s, 72 °C/45 s) and a final step of 72 °C for 2 min. For the first five cycles the annealing temperatures were 62, 61, 59, 57 and 55 °C, respectively, and a further 25 cycles were then carried out annealing at 55 °C. The entire reaction was electrophoresed on a 2% agarose gel containing ethidium bromide, and bands were visualized using UV light. If the first hair from a tape failed to amplify, successive hairs from the same tape were examined until one yielded visible PCR product, or the tape sample was exhausted. Hair extracts that failed to amplify were not used in further PCRs. The Lla16CA amplification products were rated by their intensity (from 1 = just visible, to 5 = very strong) in order subsequently to calibrate this rating with suitability of a hair for genotyping at all necessary loci.

Hair extracts that gave visible Lla16CA product were further analysed with the 12 most variable wombat microsatellites (Beheregaray *et al*. 2000; allele sizes and their frequencies are presented in Table 1) on denaturing acrylamide gels as described in Taylor & Cooper (1998). The PCR conditions were as described above, but dATP at only 20 μM was used, along with 0.05 μL of [α^{33} P]-dATP at 1000 Ci/mmol. Annealing temperatures used were standard for the markers, as given in Beheregaray *et al*. (2000). The total number of cycles (including touchdown steps) used for most markers was 30, but 25 cycles were carried out for Lk27, and 40 for Lk23.

Table 1 Allele frequencies and heterozygosity (in parentheses: observed/expected under Hardy–Weinberg; Nei 1978) at the 12 most variable *Lasiorhinuus krefftii* microsatellite loci, estimated from 81 captured and blood-sampled animals (Beheregaray *et al*. 2000)

Marker	Allele	Frequency
Lk32	187	0.12
(0.81/0.73)	189	0.43
	193	0.13
	195	0.05
	197	0.27
Lk9	191	0.21
(0.66/0.69)	195	0.48
	207	0.10
	209	0.21
Lla68CA	96	0.30
(0.62/0.67)	100	0.35
	102	0.35
Lla54CA	129	0.40
(0.75/0.65)	131	0.22
	162	0.38
Lk19	143	0.18
(0.66/0.64)	147	0.59
	173	0.11
	175	0.12
Ll2	120	0.59
(0.59/0.53)	132	0.41
Lla67CA	151	0.60
(0.65/0.50)	153	0.04
	165	0.36
Lk26	205	0.37
(0.64/0.49)	209	0.63
Lk23	134	0.63
(0.65/0.48)	142	0.37
Lla55A	79	0.71
(0.51/0.43)	80	0.29
Lkr102	300	0.28
(0.46/0.39)	306	0.72
Lk27	306	0.11
(0.33/0.44)	318	0.72
	322	0.17

Sexing of hair-donors was achieved by simultaneously amplifying a 175 bp region of the X-linked marsupial G6PD locus (GpdEx12 and GpdEx13R; Loebel *et al*. 1995), and 115 bp of the Y-linked *Ube1Y* gene (CY1, GGTGTTGCAGCGCCCCCAAGAGTGG; CY2, CTGTTCTGCAGGAAAGTTATGCAAGAGCTG; primers designed from *L. krefftii* sequence, Alpers 1998). Samples with only the upper X-specific band were identified as females, whereas those with both bands were identified as males. Sexing PCRs were performed as for Lla16CA, but primer concentrations of 0.16 μM each of the G6PD primers, and 0.1 μM each of CY1 and CY2 were used. Cycling conditions were: 94 °C for 3 min, followed by 40 cycles of 94 °C/30 s, annealing/30 s, 72 °C/1 (min) and a final extension at 72 °C for 5 min. For the first five cycles the annealing temperature was reduced by 2 °C per cycle from 60 °C, and the final annealing of 50 °C was used for the last 35 cycles.

Blood DNA PCRs were performed as above, using approximately 50 ng of DNA.

Probability of identity statistics

The probability of full-sib or unrelated pairs of wombats bearing the same genotype was examined using the procedures given in Taberlet & Luikart (1999) and executed by the program Prob-ID3 (G. Luikart, unpublished). Simulated identity among full-sibs gives a theoretical upper limit to the probability that pairs of individuals will share genotypes.

Results

Hair collection

Twenty-eight hair tape samples were obtained from eight burrows over the 11-day sampling period. Ten were identified as wombat, based on hair morphology and presence of fresh tracks and faeces around hair tapes. Hairs from a further three tapes resembled wombat hair but could not be identified with certainty. The remaining hairs were from other species known to occupy the park. There was no obvious difference in follicle size of remotely collected hairs from those of hairs taken from trapped wombats.

Blood vs. hair comparison

DNA extracts from blood samples and five single hairs from each of five wombats were subjected to an attempt at PCR amplification using 12 microsatellite loci and the sexing markers (Table 2). All 284 successful hair-extract PCRs produced genotypes identical to blood from the same individual (including those extracted 3 days after plucking) with no evidence of allelic dropout or generation of false alleles. Half of the failed PCRs (21 of 41) were due to only three hair extracts from three different wombats (Table 2).

Microsatellite genotyping and sexing from remotely collected hairs

Of 44 hair extracts from 10 tape samples identified morphologically as being from wombats, 35 (representing all tapes) gave Lla16CA products of varying intensities. Two of 20 hair extracts of unidentified species origin amplified with Lla16CA plus a further 12 and 10 loci, respectively, and were thus classified as wombat hairs. None of the 14 extracts from hairs identified as nonwombat amplified with Lla16CA.

Intensity of Lla16CA products on ethidium bromide-stained agarose gels was a good predictor of the success of genotyping from a particular extract, in terms of the number of loci for which extracts could be scored (Table 3). The minimum number of loci scored even from extracts producing the faintest of Lla16CA products, was still in excess of that required for individual identification (see results for probability of identity analysis). Importantly, even the least successful hair extracts failed for at most three loci and for the remaining loci, produced identical genotypes to other hairs on the same tape. Thus there was no indication that even the lowest quality/quantity DNA used in this study gave rise to spurious genotypes.

Multiple hairs were genotyped from several wombat tape samples (five hairs from two tapes, four from five and two from two; $n = 367$ successful amplifications), and all gave identical genotypes with the exception of two cases. For Lla67CA, one extract produced a novel allele that was two base pairs smaller (163 bp) than that observed for other extracts from the same tape sample. Upon repeat amplification, the genotype was identical to others obtained from the same tape, and the initial result discounted as PCR error. For Lk19, one extract consistently produced a PCR product four base pairs larger (the same size as known allele 147) than the equivalent allele in three other extracts from the same tape. This was recorded as a possible somatic mutation, and was not taken to indicate a separate individual. Overall, the observed rate of generation of false genotypes was 0.3% (two out of 651 successful amplifications).

Treating these two anomalies as stated, seven different genotypes were identified in the 12 wombat hair tape samples. Sexing PCR identified four of the genotypes as coming from female wombats and three from males. Two of the male genotypes matched exactly those of males M24 and M33 (trap data show the latter was very small, 9 kg at the time; A Horsup, personal communication), in a database of 81 wombat genotypes. Three known females, F80, F154 and F157, had identical genotypes to hair samples on two, four and two tapes, respectively. Four of the wombats whose genotypes matched those on hair-tapes were trapped during the next few months in the same burrows or ones near those at which relevant hairs were collected. The other, M24 was not subsequently trapped,

Table 2 Sex and microsatellite genotypes obtained from blood and 'tape-plucked' hairs from trapped *Lasiorhinus krefftii* individuals. An 'x' denotes extracts that failed to amplify at the first attempt for a particular marker. Microsatellites presented in order of decreasing heterozygosity from left to right

Wombat	Tissue	Sex	Lk32	Lk9	Lla68CA	Lla54CA	Lk19	LL2	Lla67CA	Lk26	Lk23	Lla55A	Lkr102	Lk27	No. failed
M5	Blood	male	187/197	195/195	100/102	129/131	143/175	120/132	151/151	x	134/134	79/79	300/306	318/318	1
	Hair 1	male	187/197	195/195	100/102	129/131	143/175	120/132	151/151	209/209	134/134	79/79	300/306	318/318	0
	Hair 2	male	187/197	195/195	100/102	x	143/175	120/132	x	209/209	134/134	x	300/306	318/318	3
	Hair 3	male	187/197	195/195	100/102	129/131	143/175	120/132	151/151	209/209	134/134	79/79	300/306	318/318	0
	Hair 4	male	187/197	195/195	100/102	129/131	143/175	120/132	x	209/209	134/134	79/79	300/306	318/318	1
	Hair 5	male	187/197	195/195	100/102	129/131	143/175	120/132	151/151	209/209	134/134	79/79	300/306	318/318	0
M32	Blood	male	187/193	209/209	96/102	129/129	147/175	120/132	165/165	x	134/142	79/79	306/306	318/318	1
	Hair 1	male	187/193	209/209	96/102	129/129	147/175	120/132	165/165	205/209	134/142	79/79	306/306	318/318	0
	Hair 2	male	187/193	209/209	96/102	129/129	147/175	120/132	165/165	205/209	134/142	x	306/306	318/318	1
	Hair 3	male	187/193	209/209	96/102	129/129	147/175	120/132	165/165	205/209	134/142	79/79	306/306	318/318	0
	Hair 4	x	187/193	209/209	96/102	129/129	147/175	120/132	165/165	205/209	134/142	79/79	x	318/318	2
	Hair 5	x	x	x	96/102	x	x	x	x	205/209	x	x	x	x	11
M43	Blood	male	189/197	191/207	100/102	129/131	147/175	120/132	151/165	209/209	134/134	79/79	306/306	318/318	0
	Hair 1	male	189/197	191/207	100/102	129/131	147/175	120/132	151/165	209/209	134/134	79/79	306/306	318/318	0
	Hair 2	x	189/197	191/207	100/102	129/131	147/175	x	151/165	209/209	134/134	79/79	x	318/318	3
	Hair 3	x	189/197	x	100/102	129/131	147/175	120/132	151/165	209/209	134/134	79/79	306/306	318/318	2
	Hair 4	x	x	x	100/102	129/131	147/175	120/132	151/165	209/209	x	79/79	x	318/318	5
	Hair 5	male	x	191/207	100/102	129/131	147/175	120/132	151/165	209/209	x	79/79	306/306	318/318	2
F77	Blood	female	193/197	195/195	100/102	129/162	143/147	120/120	151/165	209/209	134/142	79/80	306/306	318/322	0
	Hair 1	female	193/197	195/195	100/102	129/162	143/147	120/120	151/165	209/209	134/142	79/80	306/306	318/322	0
	Hair 2	female	193/197	195/195	100/102	129/162	143/147	120/120	151/165	209/209	134/142	79/80	306/306	318/322	0
	Hair 3	female	193/197	195/195	100/102	129/162	143/147	120/120	151/165	209/209	134/142	79/80	306/306	318/322	0
	Hair 4	female	193/197	195/195	100/102	129/162	143/147	120/120	151/165	209/209	134/142	79/80	306/306	318/322	0
	Hair 5	female	193/197	195/195	100/102	129/162	143/147	120/120	151/165	209/209	134/142	79/80	306/306	318/322	0
F68	Blood	female	187/189	191/207	102/102	129/129	147/175	132/132	151/165	205/209	142/142	79/79	306/306	318/318	0
	Hair 1	female	187/189	191/207	102/102	129/129	147/175	132/132	151/165	x	142/142	x	306/306	318/318	2
	Hair 2	x	187/189	191/207	102/102	129/129	147/175	132/132	151/165	x	142/142	x	306/306	318/318	3
	Hair 3	female	187/189	191/207	102/102	129/129	147/175	132/132	151/165	x	142/142	79/79	306/306	318/318	1
	Hair 4	female	187/189	191/207	102/102	129/129	147/175	132/132	151/165	205/209	142/142	79/79	306/306	318/318	0
	Hair 5	female	187/189	191/207	102/102	129/129	147/175	132/132	x	x	x	x	x	318/318	5
	No. failed	6	3	3	0	2	1	2	4	6	4	6	5	1	43

Table 3 Number of microsatellite markers for which the PCR failed at the first attempt, for 37 wombat hair extracts rated according to the intensity of Lla16CA PCR product on ethidium bromide-stained agarose gels. The extracts producing the most intense amplification products were rated '5', and the least '1'

No. failed PCRs	Lla16CA rating				
	1	2	3	4	5
0	0	2	4	6	9
1	1	2	2	1	2
2	2	2	1	0	0
3	2	1	0	0	0

but had been trapped 6 years earlier in the same burrow cluster in which his genotype was inferred on a hair tape. The remaining two genotypes differed at three out of 10 and 11 loci, respectively, from any known wombat including 40 captured and sampled during intensive trapping following our hair collections.

Probability of wombat identity

The 12 most variable microsatellites among the 20 known polymorphic ones in *Lasiorhinus krefftii* (Beheregaray *et al*. 2000) have sufficient power that only 0.08% of full-sibs would share the same genotype (Fig. 1). Even in the extreme case where the two most variable loci are unscoreable, this figure only changes to 0.30%. Scoring just the eight most variable loci ensures that only one in around 200 wombats (0.47%) would be indistinguishable from their full-sibs. These eight loci were amplifiable at the first attempt from 82% of hairs positively identified as wombat.

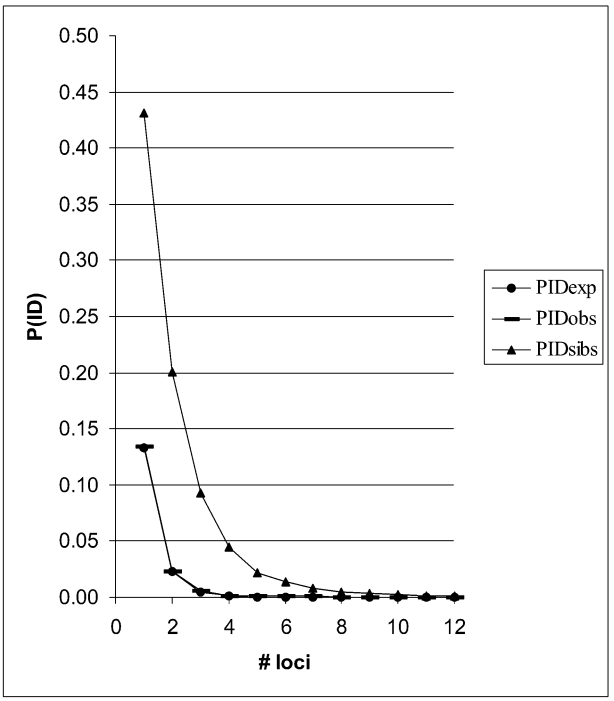

Fig. 1 Decrease in probability of identity (PID) for *Lasiorhinus krefftii* genotypes as more microsatellite markers are added in order of decreasing heterozygosity (expected; Nei 1978). PIDsibs (that simulated for full sibs) gives the upper bound.

Discussion

Although scoring of all 20 known polymorphic loci in *Lasiorhinus krefftii* from single hair follicles is technically feasible, fewer loci carry more than sufficient information to render this unnecessary for reliable and accurate individual identification. Scoring only eight loci will discriminate with high probability even full sibs, which in any case are not expected to be common in a species in which females produce at most only one young per year (Johnson 1991). The probabilities of identities are very low in comparison with the estimate of less than 100 remaining *L. krefftii* (A. Horsup, personal communication).

Reliability of single hair genotyping

Some researchers have warned against the dependence on genotypes obtained from just one PCR from single hair extracts (Goossens *et al*. 1998; Taberlet & Waits 1998), stressing the need either for many replicate PCRs (Taberlet *et al*. 1996), or the inclusion of up to 10 hairs per DNA extraction (Goossens *et al*. 1998). For example Goossens *et al*. (1998) observed the incidence of incorrect genotype assignment to be 0.3, 4.9 and 14.0%, when DNA was extracted from 10, three and one hair, respectively. Nonetheless, the current pilot study has demonstrated the feasibility of using single hairs as a source of DNA for the reliable identification and sexing of individual *L. krefftii*. There was a reasonable *a priori* expectation that wombat hairs may contain several hundred nanograms of DNA (Higuchi *et al*. 1988), and that DNA recovery could be maximized by optimizing collection, storage and extraction procedures. Standard, moderate PCR conditions were therefore employed (e.g. with respect to number of cycles) that are unlikely to have produced detectable amplification products from only a small number of template molecules (Taberlet & Luikart 1999). Thus, although the DNA used in PCRs was not quantified, it was not thought to be in the picogram range that produced errors in other studies (see Taberlet *et al*. 1999). This conclusion is supported by several lines of independent evidence. First, there was no evidence that extracts which failed in more PCRs were scored as more homozygous (through 'allelic dropout'):

of 12 informative cases in Table 2, eight extracts with some loci failing were at least as heterozygous as expected at the successful loci. Second, genotypes scored from remotely collected *L. krefftii* hairs were heterozygous at between 25 and 80% of loci, which is within the range of individual heterozygosity observed in known wombats (25–83%). Five hairs match genotypes among those obtained from blood DNA from 81 trapped wombats, which is extremely unlikely to occur if they did not come from the individuals from which they are inferred to belong (Fig. 1). Third, identical genotypes were obtained from hair and blood from the same wombat ($n = 284$) and, with only two exceptions, from multiple hairs on the same tape collected at burrow entrances ($n = 367$). The ultimate test of precision of the experimental protocol is in its ability to detect the presence of genotypes known to exist in the population. This was demonstrated by the detection of genotypes matching those of five known wombats.

In contrast to some previous studies, only a low genotyping error rate (0.3%) was observed when using single hair DNA extracts. Under the present protocol, DNA is released in a single-tube procedure from hairs plucked by hair-tapes no more than 24 h before. A high proportion (approximately 1/25th) of this DNA extract is used in each PCR employing primers specifically developed from *L. krefftii* or from its very close relative *L. latifrons*. Such favourable conditions do not necessarily pertain to previous studies that have noted difficulties in obtaining reliable genotypes from single hairs (see introduction).

The high reliability of our method renders the use of a multiple tubes approach unnecessary (Taberlet *et al*. 1996), and removes its associated expense and potentially increased rate of PCR error due to dilution of DNA extracts. Although the pooling of multiple remotely collected *L. krefftii* hairs from the same tape may yield more DNA for analysis, multiple donors may be distinguishable only with extra analysis and additional assumptions (Alpers 1998; Balding 1999). Finally, given that sufficient DNA is available for accurate PCR, approaches such as multiplexing microsatellite loci (potentially error-prone at low template quantities, Taberlet *et al*. 1999) are unnecessary.

Quality control and census design issues

Accompanying the high reliability that has been demonstrated in the present system, additional features lead to an unusually high likelihood of detecting artefactual alleles that may rarely arise. Much of the *L. krefftii* population has been genotyped (minimum $n = 78$ for the loci reported here), and a maximum of five alleles at a locus has been found (Table 1). Because the number of animals scored is so large in comparison with the number of alleles detected, we can be confident that most or all alleles have been detected. Consequently, putative artefactual PCR products of novel sizes would be obvious. Furthermore, *L. krefftii* microsatellite allele size distributions are unusually disjunct (Taylor *et al*. 1994) (probably through stochastic subsampling during population contraction). Thus PCR errors and somatic mutations in this species are relatively likely to lead to novel 'alleles' as the relevant *in vivo* and *in vitro* molecular mechanisms usually result in small changes in PCR product/allele sizes (Goldstein & Schlötterer 1999).

We have shown that genotyping errors will be rare and most likely detectable. However the impact of any that are undetectable (along with potential somatic mutation in older animals; Greenwood & Pääbo 1999) will be negligible for censusing if we stipulate that a mismatch at only one locus does not indicate separate wombats. For example in this study, one hair produced a repeatable spurious allele at one locus (possibly the result of somatic mutation) but matched a known genotype at the other 11 loci, and would thus not lead to the false inference of an additional individual. This conservative criterion will only negligibly underestimate the number of wombats, because very few pairs are predicted to mismatch at only one locus (Fig. 1).

Conclusion

In developing the present protocol for noninvasive genotyping of *Lasiorhinus krefftii* a variety of common-sense measures were adopted to maximize quality and quantity of DNA, as well as a range of approaches to error detection (see also Taberlet *et al*. 1999). The optimization of a noninvasive genetic sampling technique is timely given the precarious state of the species. In combination with other forms of management, censusing of wombats from remotely collected hairs will greatly assist in conservation efforts (Horsup 1998). The present results give some confidence that noninvasive sampling will provide accurate census data, and repeated remote censuses will result.

Acknowledgements

The authors would like to acknowledge the assistance of A. Horsup with sample collection and exchange of ideas, and Environment Australia who funded the research in part. We are grateful to P. Taberlet for helpful comments on the manuscript, and G. Luikart for allowing the use of his unpublished P(ID) program. Our various collaborators at Macquarie University, especially D. Briscoe, N. Tait and D. Cooper, provided a supportive environment for the initial laboratory work.

References

Albaugh GP, Iyengar V, Lohani A *et al.* (1992) Isolation of exfoliated colonic epithelial cells, a novel, non-invasive approach to the study of cellular markers. *International Journal of Cancer*, **52**, 347–350.

Alpers DA (1998) *Genetic Population Structure of the Southern*

Hairy-Nosed Wombat (Lasiorhinus latifrons). PhD Thesis, University of New South Wales, Sydney.

Balding D (1999) Forensic applications of microsatellite markers. In: *Microsatellites: Evolution and Application* (eds Goldstein DB, Schlötterer C), pp. 198–210. Oxford University Press. Oxford.

Beheregaray LB, Sunnucks P, Alpers DL, Taylor AC (2000) A set of microsatellite loci for the hairy-nosed wombats (*Lasiorhinus krefftii* and *L. latifrons*). *Conservation Genetics* (in press).

Crossman DG, Johnson C, Horsup A (1994) Trends in the population of the northern hairy-nosed wombat in Epping Forest National Park. *Pacific Conservation Biology*, **1**, 141–149.

Foran DR, Minta SC, Heinemeyer KS (1997) DNA-based analysis of hair to identify species and individuals for population research and monitoring. *Wildlife Society Bulletin*, **25**, 840–857.

Gagneux P, Boesch C, Woodruff DS (1997) Microsatellite scoring errors associated with noninvasive genotyping based on nuclear DNA amplified from shed hair. *Molecular Ecology*, **6**, 861–868.

Gerloff U, Schlötterer C, Rassmann K *et al.* (1995) Amplification of hypervariable simple sequence repeats (microsatellites) from excremental DNA of wild living bonobos (*Pan paniscus*). *Molecular Ecology*, **4**, 515–518.

Goldstein DB, Schlötterer C (1999) *Microsatellites: Evolution and Application*. Oxford University Press. Oxford.

Goossens B, Waits LP, Taberlet P (1998) Plucked hair samples as a source of DNA: reliability of dinucleotide microsatellite genotyping. *Molecular Ecology*, **7**, 1237–1241.

Gordon G, Riney T, Toop J, Lawrie BC, Godwin MD (1985) Observations of the Queensland hairy-nosed wombat *Lasiorhinus krefftii* (Owen). *Biological Conservation*, **33**, 165–195.

Greenwood AD, Pääbo S (1999) Nuclear insertion sequences of mitochondrial DNA predominate in hair but not in blood of elephants. *Molecular Ecology*, **8**, 133–138.

Higuchi R, Von Beroldingen CH, Sensabaugh GF, Erlich HA (1988) DNA typing from single hairs. *Nature*, **332**, 543–546.

Horsup A (1998) A trapping survey of the northern hairy-nosed wombat *Lasiorhinus Krefftii*. In: *Wombats* (eds Wells RT, Pridmore PA), pp. 147–155. Surrey Beatty & Sons, Chipping Norton.

Hoyle SD, Horsup AB, Johnson CN, Crossman DG, McCallum H (1995) Live-trapping of the northern hairy-nosed wombat (*Lasiorhinus krefftii*): population-size estimates and effects on individuals. *Wildlife Research*, **22**, 741–755.

Johnson CN (1991) *Behaviour and Ecology of the Northern Hairy-Nosed Wombat* Lasiorhinus krefftii. Internal report, Queensland National Parks and Wildlife Service, Rockhampton.

Johnson CN, Crossman DG (1991) Dispersal and social organisation of the northern hairy-nosed wombat *Lasiorhinus krefftii*. *Journal of Zoology*, **225**, 605–613.

Kohn MH, Wayne RK (1997) Facts from feces revisited. *Trends in Ecology and Evolution*, **12**, 223–227.

Loebel DAF, Longhurst TJ, Johnston PG (1995) Full-length cDNA sequence of X-linked *G6PD* of an Australian marsupial, the wallaroo. *Mammalian Genome*, **6**, 198–201.

Morin PA, Moore JJ, Chakraborty R *et al.* (1994) Kin selection, social structure, gene flow, and the evolution of chimpanzees. *Science*, **265**, 1193–1201.

Nei M (1978) Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics*, **89**, 583–590.

Reed JZ, Tollit DJ, Thompson PM, Amos W (1997) Molecular scatology: the use of molecular genetic analysis to assign species, sex and individual identity to seal faeces. *Molecular Ecology*, **6**, 225–234.

Strahan R (1995) *The Mammals of Australia*. Australian Museum/Reed, Chatswood.

Taberlet P, Luikart G (1999) Non-invasive genetic sampling and individual identification. *Biological Journal of the Linnean Society*, **68**, 41–65.

Taberlet P, Waits LP (1998) Non-invasive genetic sampling. *Trends in Ecology and Evolution*, **13**, 26–27.

Taberlet P, Camarra JJ, Griffin S *et al.* (1997) Noninvasive genetic tracking of the endangered Pyrenean brown bear population. *Molecular Ecology*, **6**, 869–876.

Taberlet P, Griffin S, Goossens B *et al.* (1996) Reliable genotyping of samples with very low DNA quantities using PCR. *Nucleic Acids Research*, **24**, 3189–3194.

Taberlet P, Waits LP, Luikart G (1999) Noninvasive genetic sampling: look before you leap. *Trends in Ecology and Evolution*, **14**, 323–327.

Taylor AC, Cooper DW (1998) A set of tammar wallaby *Macropus eugenii* microsatellites tested for genetic linkage. *Molecular Ecology*, **7**, 925–931.

Taylor AC, Alpers DL, Sherwin WB (1998) Remote censusing of northern hairy-nosed wombats via genetic typing of hairs collected in the field. In: *Wombats* (eds Wells RT, Pridmore PA), pp. 156–164. Surrey Beatty & Sons, Chipping Norton.

Taylor AC, Horsup A, Johnson CN, Sunnucks P, Sherwin WB (1997) Relatedness structure detected by microsatellite analysis and attempted pedigree reconstruction in an endangered marsupial, the northern hairy-nosed wombat. *Lasiorhinus Krefftii. Molecular Ecology*, **6**, 9–19.

Taylor AC, Sherwin WB, Wayne RK (1994) Genetic variation of microsatellite loci in a bottlenecked species: the northern hairy-nosed wombat *Lasiorhinus krefftii*. *Molecular Ecology*, **3**, 277–290.

Tikel D, Blair D, Marsh H (1996) Marine mammal faeces as a source of DNA. *Molecular Ecology*, **5**, 456–457.

Walsh PS, Metzger DA, Higuchi R (1991) Chelex® 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. *Biotechniques*, **10**, 506–513.

Mathew Sloane began this work as an Honours project and finished it under a contract for Queensland Department of Environment and Heritage. He was supervised by Andrea Taylor and Paul Sunnucks who carry out a range of research on molecular population genetics of vertebrates and invertebrates. Luciano Beheregaray is a PhD student working on molecular phylogeny and population genetics, and Deryn Alpers works on conservation and phylogenetics of a range of vertebrates.
