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A methodological approach for non-invasive sampling for population size estimates in wild boars (*Sus scrofa*)

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Abstract Composite microsatellite genotypes were determined at five loci from 35 tissue-sampled wild boars and used as reference genotypes to estimate both allelic drop-out rate and false allele rate in comparison to genotypes from scats and hair strands of the same animals. These rates allow to assess the genotyping reliability when only non-invasively collected material is available. Polymerase chain reaction (PCR) amplification from scats was often corrupted by inhibitors and worked poorly, whereas genotyping success in hair samples was high. Body region of hair origin had no influence on PCR suitability, whereas the type of hair had. We recommend the use of bristles. PCR conditions were optimized for single-hair (bristle) genotyping.

Keywords Microsatellite · Genotypes · Reliable single-hair typing · PCR optimization

Introduction

Forest and wildlife management measures often require information on the size of wildlife populations, which, for many species, is difficult to obtain. For wild boar, we register a growing concern regarding the rapidly increasing population, both in terms of its potential to cause agricultural damage as well as to its potential to function as disease reservoir (Briedermann 1990; Sáez-Royuela and Telleria 1986; Stubbe 1996; Artois et al. 2002). A widely used method to estimate wild boar population sizes is the capture-mark-recapture (CMR) approach, where animals are usually marked with ear tags. However, CMR in wild boars is time-, cost- and labour-intensive and prone to age class and sex bias because capture probability of experienced females is low (Baber and Coblentz 1986; Sweitzer et al. 2000; Baubet 1998). This weakens population estimates significantly.

Within the last two decades, DNA extraction methods were developed, which allowed individual identification by using genetic markers via determination of haplotypes (Higuchi et al. 1988; Höss et al. 1992) and microsatellite genotypes both from hair (Wilson et al. 1995; Foran et al. 1997; Goossens et al. 1998; Sloane et al. 2000) and faeces (Constable et al. 1995; Taberlet et al. 1996, 1997; Frantzen et al. 1998). The prominent advantage of these methods is that in contrast to tissue or blood sampling, which require experienced and skilled personnel, hair and faecal samples can be collected without capture and often without visual contact. Thus, non-invasive genotyping carries great potential as feasible censusing method (Sloane et al. 2000; Kohn et al. 1999) because it reduces potential sampling bias.

However, drawbacks of remotely collected samples are (a) that species determination of both hair and faecal samples may be confused even by experienced collectors (Davison et al. 2002) and (b) extracts from field-collected hair and faeces usually yield little target DNA and may (especially in faeces) contain polymerase chain reaction (PCR) inhibitors, potentially leading to false allele amplification and/or failure of alleles to amplify (allelic drop-out). The latter two types of error are particularly problematic for genetic census studies, because a single error in a multilocus genotype generates a false individual, leading to either underestimation (a false genotype resembles an already scored genotype, thus being considered a re-sampled individual) or overestimation (a false genotype is scored as new individual) of the true population size (Creel et al. 2003).

Thus, aims of this pilot study were twofold: (a) development of a robust non-invasive sampling method for

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wild boar and (b) establishment of analytical tools to enable reliable population size estimates in wild boar by means of genotyping.

Materials and methods

This study was performed in Rhineland-Palatinate and was carried out in two phases: (I) genotyping suitability tests for hair strands and faeces and (II) procedure optimization to limit the amount of sample material necessary for successful and reliable genotyping.

All extractions and PCRs were carried out in a separate laboratory that never before had come in contact with porcine DNA. In addition, DNA work was performed under a special UV-radiation-equipped hood, aerosol-resistant pipette tips (Biozyme, Germany) were used and negative controls were employed in each DNA-handling step.

For phase I, we analysed tissue (skin or muscle fibers), hair strands and faeces from 35 wild boars shot in 2003 in the district of Bitburg-Prüm (kindly provided by Dr. Hoff, District Veterinary Office). For faeces, we used special Q-tips to sample the outer, intestinal mucosa-cell-containing layer. We pooled 15–20 hair follicles/animal for DNA isolation. Cell lysis and DNA extraction for all samples were carried out using the all-tissue DNA kit (GEN-IAL, Troisdorf, Germany); DNA was finally eluded in 80 µl 1× TE buffer. For DNA quality tests and to verify species origin, we amplified 495 bp of the variable mitochondrial DNA (mtDNA) control region (Anderson et al. 1981) by applying self-designed primers pigCTR22L (5′ TTCGT ATGCAAACCAAAACG 3′) and pigCTR515H (5′ GCT GATTAGTCATTAGTCC 3′). Cycling conditions were 3 min 94°C, 35× (15 s 94°C, 30 s 52°C, 90 s 72°C), 7 min 72°C. PCR mixtures (25 µl) contained 0.5 U AmpliTaq DNA polymerase (Perkin Elmer), 2.5 µl 10× PCR buffer, 1.5 mM $MgCl_2$, 200 µM deoxyribonucleotide triphosphates (dNTPs), 10 pmol of each primer and approximately 50 ng of DNA. Amplicons were sequenced bidirectionally using the BigDye cycle sequencing kit (ABI, Weiterstadt, Germany) and visualized on an ABI A3100 automated sequencer. Boar microsatellites, primers and PCR conditions were taken from Krause et al. (2002). We used loci with alleles <250 bp to avoid reduced amplification performance (Frantzen et al. 1998): UMNp09, 255, 274, 358 and 445. Samples were routinely analysed in duplicates with 5′-labelled forward primers (fluorescent dyes, BioTeZ Berlin). Homozygous loci were only scored as such when triplicate PCR and gel fragment analysis reproduced the same allele. If PCRs failed three times, data were scored as missing. Analysis was carried out on an ABI A310 sequencer.

To design a hair catcher, suitability of hair strands for microsatellite genotyping (obtained from shot wild boar) was tested according to (a) type of hair (large bristle, small bristle, wool hair) and (b) body region of hair origin (hind leg, front leg, belly, chest, front and hind back, lower and upper side, neck, cheek and snout).

In phase II, tissue-tested PCR conditions (Krause et al. 2002) were adapted to single-hair PCRs (Higuchi et al. 1988; Goossens et al. 1998). The sources for abundant hair DNA were freshly plucked hair clusters (approximately 200–250 hair follicles) from three wild boars culled in spring 2004 in Berlin (kindly provided by F. Mosch, Forstamt Friedrichshagen). Amplification protocol was 94°C 10 min, 35× (94°C 30 s, 60°C 30 s, 72°C 45 s), 72°C 30 min. The 25-µl reaction volume consisted of 50–100 ng DNA, 0.5 U Taq polymerase, 2.5 µl 10× reaction buffer, 0.5 mM dNTPs, 5 pmol forward primer, 20 pmol reverse primer and 2 µg bovine serum albumin. $MgCl_2$ concentrations were 2.5 mM for UMNp358, 2 mM for UMNp255 and 1.5 mM for all other loci. The newly developed protocol was then applied to DNA isolated from single boar bristles.

We calculated observed (H_O) and expected heterozygosities (H_E) using GENEPOP (ver. 3.1c; Raymond and Rousset 1995). Genotypic disequilibrium between loci UMNp445 and UMNp274 (both are located on chromosome 16) was

Table 1 Parameters of five microsatellite loci analysed in 35 wild boars (tissue) sampled in spring 2003 at three hunting grounds in Rhineland-Palatinate

Locus UMNp	Chromosomal location (from Krause et al. 2002)	Number of alleles scored	Allelic range	H_E	H_O	P_{ID}/locus	$P_{ID\text{-}prod}$/locus	$P_{ID\text{-}sib}$/locus	$P_{ID\text{-}sibprod}$/locus
445	16	5	175–189	0.678	0.943	0.156 (0.143)	0.156 (5.6×10^{-4})	0.459 (0.437)	0.459 (3.2×10^{-2})
358	13	8	138–162	0.660	0.875	0.169 (0.2)	2.64×10^{-2} (3.9×10^{-3})	0.471 (0.483)	0.216 (7.4×10^{-2})
255	X	3	175–181	0.664	0.143	0.181 (0.192)	4.80×10^{-3} (7.3×10^{-2})	0.471 (0.471)	0.102 (0.28)
274	16	5	125–138	0.578	0.543	0.214 (0.268)	1.03×10^{-3} (1.9×10^{-2})	0.524 (0.545)	5.34×10^{-2} (0.154)
09	1	2	105–111	0.507	0.543	0.368 (0.379)	3.79×10^{-4} (0.379)	0.594 (0.599)	3.17×10^{-2} (0.599)
Mean				0.617	0.609				

Loci are ranked according to their P_{ID}/locus. Corresponding P_{ID} values for ten simulated populations of 50 individuals are listed in parentheses

H_E Estimated heterozygosity, H_O observed heterozygosity, P_{ID}/*locus*, probability of identity for individual locus, corrected for small sample size, $P_{ID\text{-}sib}$/*locus* probability of identity assuming all individuals are siblings, $P_{ID\text{-}prod}$/*locus* probability of identity across loci by sequentially (according to rank) multiplying the P_{ID} values over loci; $P_{ID\text{-}sibprod}$/*locus*, probability of identity across loci by sequentially multiplying the P_{ID} value over loci assuming all individuals are siblings

likewise calculated using GENEPOP. Because all other loci were physically separated by their chromosomal location (Krause et al. 2002), they were independent per se. To estimate the reliability of hair genotyping, we calculated the number of false homozygotes, defined as the detection of only one of the two alleles of a true heterozygote [allelic drop-out rate (ADR)], and the false alleles rate (FAR), defined as alleles not present at that locus in the true genotype. ADR and FAR were calculated for each locus across samples, considering the genotypes derived from tissue samples as true genotypes. Probability of genotypic identity (P_{ID}; Waits et al. 2001) was computed based on the data set of genotypes obtained from the 35 culled wild boars using the software package GIMLET (v.1.3.3; Valière

Table 2 Composite genotypes of 35 wild boars from different hunting grounds in Rhineland-Palatinate based on alleles of 5 microsatellite loci derived from tissue and hair

ID	Loci (UMNp)									
	9 Tissue	9 Hair	255 Tissue	255 Hair	274 Tissue	274 Hair	358 Tissue	358 Hair	445 Tissue	445 Hair
139	22	22	11	11	22	22	45	45	45	55
145[a]	12	12	33	33	22	22	45	45	45	45
146	22	22	22	22	11	11	34	34	34	34
2881	12	12	22	22	22	22	45	45	15	15
2882	11	11	22	22	29	29	45	45	45	45
2883[b]	12	12	22	22	22	22	45	45	45	45
2884	12	11	13	13	24	24	45	45	45	45
2887	11	11	22	22	12	12	46	46	14	14
2889	22	22	22	22	12	12	45	45	45	55
2890	11	11	11	11	22	22	45	45	45	45
2892	11	11	22	22	19	19	22	22	15	15
2893	11	11	12	12	29	29	45	45	45	45
2894	12	12	22	22	12	12	45	45	45	45
2895	12	12	33	33	12	12	45	45	22	22
2896	22	12	33	33	22	26	14	44	15	15
2897	12	12	22	22	29	29	45	45	45	45
2898	12	12	13	13	12	12	34	34	45	45
2899	12	12	11	11	19	19	45	45	14	14
2900	12	12	11	11	12	12	45	45	45	45
16936	12	12	11	11	22	22	45	45	34	44
16937	22	22	33	33	29	29	55	55	15	15
16938[b]	12	12	22	22	22	22	45	45	45	45
16939	12	22	22	22	33	33	48	48	45	15
16940	12	22	33	33	22	22	45	45	44	**3**4
16941	22	22	33	33	12	12	78	78	34	34
16942	12	12	11	11	29	29	55	55	45	45
16943[a]	12	12	33	33	22	22	45	45	45	45
16944	22	22	33	33	22	22	45	45	45	45
16945	22	22	22	22	19	19	45	45	15	15
16946	12	12	33	33	12	12	45	45	15	15
16947	11	11	13	13	22	22	45	45	45	45
16948	11	11	13	13	29	29	88	88	15	15
16949	11	11	33	33	22	22	45	55	45	45
16951	12	12	22	22	11	11	34	44	14	14
Gees	12	12	33	33	12	12	55	55	45	45
Heterozygotes	20		5		17		30		33	
ADR (%)		15		0		0		10		9.09
FA (%)		2.875		0		2.875		0		5.71
Total error (%)		17.875		0		2.875		10		14.80

Alleles are numbered according to size
ADR Allelic drop-out rate (%), underlined; *FAR* false allele rate (%), in bold
[a]Identical composite genotypes
[b]Identical composite genotypes

2002). We also estimated $P_{ID\text{-}sib}$ (Valière 2002) because P_{ID} among full siblings provides an upper limit to the probability that pairs of individuals will share genotypes (Taberlet and Luikart 1999).

Finally, we applied our genotyping approach to ten individuals (three females and seven almost 1 year old juveniles) kept in a 1.5 ha large enclosure in April 2004. To obtain freshly plucked hair strands, the ten wild boars were lured by spread bait maize into a rectangle which was surrounded by a barbed wire with an approximate side length of 2.3 m. To avoid body-size- or age-biased sampling, we stretched the wire at different heights between 50 and 65 cm. Within 30 min, we counted 168 single wire contacts with no obvious age or size group bias. The animals left hair strands at all 95 spools. For genotyping, 35 bristles were subsampled randomly, put into a paper envelope and sent to the laboratory within 7 days.

Depending on its DNA yield, each hair strand was analysed in triplicates. To avoid cross-contaminations, sets of replicate reactions were performed consecutively. GIMLET (Valière 2002) was used to generate consensus genotypes from replicate genotypes to estimate the minimal number of individuals. The threshold for identification was set at 2, excluding alleles that only appeared once per locus across all replicates.

Results

Mitochondrial DNA could be amplified from all 35 animals, rendering the DNA suitable for genotyping. In total, we detected 23 alleles with an average number of 4.6 alleles per locus (Table 1). Locus UMNp09 had the least number of alleles (k=2) and locus UMNp358 had the most (k=8). The test for a genotypic disequilibrium between loci UMNp274 and 445 (both located on chromosome 16) was not significant (p=0.247), indicating independence of both loci. Using GEMINI (Valière 2002), we identified 33 out of 35 individuals correctly. This was confirmed by visual inspection; two pairs of individuals shared microsatellite genotypes (IDs 16938 and 2883 and IDs 16943 and 145).

Microsatellite loci could be analysed in all hair samples, whereas PCR failure rate in faeces was >75% at each locus. Thus, faecal samples were omitted from further analyses. Regarding hair type, best results in yield of DNA and genotyping (we tested loci UMNp274 and 358 only) were achieved when using large bristles (we pooled 15 hair strands of each hair type). In contrast to type, the body region from which the hair originated did not influence genotyping. Hair strands from all regions could be genotyped satisfactorily. Exceptions were leg hair strands, but from there, only wool hair strands were available. Thus, hair catchers can be designed to pluck hair strands from the flank or back of the animals.

The comparison of composite genotypes between paired tissue and hair samples is shown in Table 2. From their differences, we estimated ADR and FAR for each locus. ADR ranged from 0 to 15% and FAR from 0 to 5.7% (Table 2). Averaged over all loci, the proportions of PCR errors in genotyping hair DNA extracts were 0.068 for allelic drop-out and 0.023 for false alleles. The total error of composite genotype was approximately 39%, calculated as $1-(0.821\times1\times0.9715\times0.9\times0.852)=0.388$. For unrelated individuals, the theoretical matching probability (P_{ID}) values ranged from 0.156 (UMNp455) to 0.368 (UMNp09). However, across all loci, the probability of identity of composite genotypes ($P_{ID\text{-}prod}$) was only 3.76×10^{-4}. Even for full siblings, the probability of identity across loci ($P_{ID\text{-}sibprod}$) was still only 3.17% (Table 1).

In the single-hair analysis (35 randomly sampled hair strands from ten individuals), all hair strands but one could be typed for microsatellites. The composite genotypes were analysed separately (per single PCR run) and as consensus genotypes (across PCR replicates). Reproducibility of hair genotypes among different runs was approximately 97%. Using GIMLET (Valière 2002), the estimated number of individuals (distinguishable composite genotypes) was 14.

Discussion

When working with remotely collected samples, the amount of DNA available for amplification is usually very low. In addition, time span and "storage conditions" between sample deposition (hair shedding/plucking, defaecating) and its collection may reach a threshold where DNA degradation reduces genotyping reliability. Analysis of mtDNA provides a way to assess DNA quality prior to microsatellite analysis. It (a) allows to determine the species in suspicious samples (Davison et al. 2002), (b) allows the genetic follow-up on matrilines and (c) can serve as additional marker in distinguishing otherwise identical genotypes.

Once a suitable quality of DNA has been established, the total number of microsatellite reactions depends on two main criteria: (a) the minimal number of microsatellite loci necessary to be analysed to allow reliable individualization and (b) the number of PCR repetitions per locus necessary to sufficiently reduce chances of allelic drop-out and false alleles. In a study on alpine marmots, Goossens et al. (1998) determined the number of hair strands required to meet these criteria by genotyping 50 individuals at a single locus using DNA extracted from one, three and ten hair strands. They concluded that ten hair strands provide a sufficient amount of DNA to obtain consistently reliable genotyping using the single-tube approach (one PCR per sample), whereas single hair strand should undergo the multiple-tube approach (Taberlet et al. 1996) to provide reliable results (Goossens et al. 1998). Follicles of wild boar bristles provide a sufficient amount of DNA to run PCRs in multiple replicates required by the multiple-tube approach (Taberlet et al. 1996). But because consequent abiding by the multiple-tube rule requires to conduct high numbers of PCRs (the product of sample number, number of loci and number of replicates per locus–PCR), it is very costly for large samples and many loci. Therefore, alternative approaches, such as the maximum likelihood estimate (Miller et al. 2002), whereby reliability of erroneous loci is

increased by consecutive replicate PCRs of only these loci, should also be considered.

In a microsatellite genotyping study on faeces of a wolf population of known size (n=41, 13 loci) in Yellowstone National Park, Creel et al. (2003) showed that the minimum population size was greatly overestimated when based on unique genotypes. The magnitude of overestimation was greatest (approximately 5.5-fold) when all 13 loci were included. Because the opportunity for errors to create false unique genotypes increases with every additional locus used, overestimation can easily be reduced by decreasing the number of loci defining a composite genotype. Thus, minimal population size estimates should only employ the minimum number of loci necessary to avoid matching by chance (low P_{ID}) among samples from different animals (Kohn et al. 1999; Waits et al. 2001; Creel et al. 2003). In their study, Creel et al. (2003) reached the best match between true and estimated minimal population size by using only 5 out of 13 loci. Another aspect concerns the error rate per locus. Even if the error rate for a single locus is low, the probability of at least one or more errors in a composite genotype can be quite high. With a total error rate of approximately 9% (ADR+FA) per single locus, a five locus genotype has a 37.5% chance of error ($1-0.91^5=0.375$). Thus, when given a sufficiently large collection of samples, each individual has the potential to contribute one or more false genotypes to the population data set. Such errors inevitably cause both overestimation of the minimum number of individuals alive and underestimation of the resampling probability for each individual, with the latter effect also overestimating population size. However, in wild boar, the total error rate (ADR 30%+FAR 8%) was similar to the one reported for chimpanzees (ADR 31%; Gagneux et al. 1997) but higher than the one measured in alpine marmots (ADR 14%; Goossens et al. 1998). Nevertheless, the error rate led to a population size overestimation ($n_{estimate}$=14, n_{true}=10). Such estimates can be considerably improved by larger data sets used to determine allelic frequency/distribution in both true and remotely obtained genotypes.

Although five typed microsatellite loci were already sufficient to successfully distinguish individual wild boars, for censuses of much larger populations, the use of additional loci may be required (Alexander et al. 1996; Vernesi et al. 2003; Souto et al. 2004) because the proportion of relatives within such populations will be larger. It is also conceivable that sex and age/rank, i.e. prior experiences, affect the likelihood of a given individual to leave hair at baited hair catcher sites. Those factors may introduce bias into sampling and later population estimates. Thus, further research should focus on those aspects, e.g. by using video surveillance techniques at hair catcher sites.

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