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Reliability of noninvasive genetic census of otters compared to field censuses

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Abstract Conservation and management actions are often highly dependent on accurate estimations of population sizes. However, these estimates are difficult to obtain for elusive and rare species. We compared two census methods for Eurasian otter: snow tracking and noninvasive genetic census based on the genotyping of faecal samples. With the noninvasive genetic census we detected the presence of almost twice as many otters as with snow tracking (23 and 10–15, respectively), and mark-recapture estimates based on the genetic census indicated that the real number of otters could be even higher. Our results indicate that snow tracking tends to underestimate the number of individuals and also that it is more susceptible to subjective assessment. We compared the strengths and weaknesses of the two methods.

Keywords Individual identification · Faeces · Monitoring · Population size · Snow tracking

Introduction

Accurate estimates of population sizes are fundamental in wildlife conservation and management. However, estimating population size may be very difficult for elusive and nocturnal species. For carnivores, population size is most often measured by field censuses, such as counting tracks and active dens, or direct observation (Gese 2001). Capture-mark-recapture techniques are also being used (Gese 2001) and these methods are often regarded as highly reliable, but depend on directly handling the animals. During the last few years, noninvasive genetic approaches have become an alternative to these capture-mark-recapture methods, giving the opportunity to identify individuals in an area without the need to capture or even disturb the animals. DNA is sampled from what the animals leave behind (e.g. faeces, urine, hair, feathers, sloughed skin; Taberlet et al. 1996, 1999; Taberlet and Luikart 1999). Intensive sampling of an area results in the possibility of genotyping and differentiating between the individuals and, hence, to estimate population size (Kohn et al. 1999; Ernest et al. 2000, 2003; Wilson et al. 2003; Frantz et al. 2004; Hung et al. 2005). The main drawback of the noninvasive genetic census is its relatively high cost and for this reason it is normally applied only to small areas or small and/or isolated populations.

Population size estimates based on noninvasive genetic monitoring can suffer from different biases. The availability of samples can potentially affect the estimation. For example, in the case of Eurasian otters (*Lutra lutra*), different individuals are likely to have heterogeneous capture probabilities. These depend on the ability to detect and collect spraints from the individuals, which may vary if one sex or age class does not scent-mark with the same frequency or deposits the faeces in less conspicuous places or in the water. Also, samples may belong to individuals dead or to vagrants that do not reside in the area if the population is not

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closed, especially if old faeces are collected. Additionally genotyping errors can lead to overestimation of the number of individuals and careful precautions are needed to minimize this problem (Taberlet et al. 1996; Taberlet and Luikart 1999; Creel et al. 2003).

Tracking (footprints in snow or mud) is considered a cheap and potentially effective census method for otters, especially in low-density populations (Ruiz-Olmo et al. 2001). In areas that are covered with ice and snow in the winter, snow tracking has often been used as the preferred census method (e.g. Reid et al. 1987; Sidorovich and Lauzel 1992; Aronson 1995; Sulkava 1995; Sidorovich et al. 1996). The method is based on finding, separating and counting tracks from different otter individuals on snow. It can, however, be difficult to separate individuals as they often (especially when the snow is deep) step in each other's tracks, one individual is likely to use the same path on multiple occasions, and tracks emerge from and vanish into the water (making it difficult to judge if all of them correspond to the same individual). Additionally, otters can spend a substantial amount of time, even several days, under the ice when there is a space with air between the ice and the water, being totally invisible to the census (Aronson 1995; Sulkava 1995). Finally, monitoring success is highly dependent on weather conditions during the previous days.

The aim of this study was to compare the result of a noninvasive genetic census with population size estimates obtained from snow tracking. Ideally, the two methods provide comparable and, as far as can be asserted, accurate population size estimates. We also investigated the factors affecting the genetic census in order to design a sampling strategy that would facilitate gathering reliable results.

Materials and methods

Study area

The northern and central parts of Sweden are inhabited by a more or less continuous (although often sparse) otter population. Snow tracking and collection of faeces for genetic analyses were conducted in an area of about 2500 km^2 in central Sweden. The study area consisted of eutrophic rivers and lakes surrounded by forested or open landscapes in both agricultural as well as urban areas. The area and its surroundings support a resident otter population that suffered the severe decline common to many European countries in the 1950's–1980's (Mason and Macdonald 1986), and started its recovery in the 1990's (Hammar 2006). The study area mainly included entire watersheds. However, otters were likely to move between neighboring watersheds and cross the limits of the study area. Consequently, the study population was not isolated or closed, which implies that some transient individuals may have been detected in the surveys.

The field study was conducted during 17 days in the winter of 2002 (January–February), when weather conditions were optimal for snow tracking. Within the study area we visited 110 places, where tracks and faeces from otters were likely to be found. Examples of such places are bridges, old mills, river confluences, river outlets, areas with open water, and conspicuous trees and rocks along the shore. We consider that the places visited could provide accurate information about all the otters living in the study area.

Snow tracking

Snow tracking was conducted following Aronson (1995) and Sulkava (1995). At each visited place we looked for otter tracks along a 100–1000 m stretch along the river or lake, depending on the local conditions and the snow and ice. When tracks were found, we tried to determine the number of otters by following the tracks and measuring footprints and the length between footprints. Tracks had to be clearly separated to be acknowledged as different individuals. Frozen lakes offered an especially good opportunity to separate individuals; the possibility of determining the number of individuals is larger when they travel long distances on the ice, as the individuals occasionally spread out on the frozen surface. The age of the tracks was also used to tell different individuals apart (fresh tracks located far from each other were likely to correspond to different individuals). When tracks could not be clearly separated, a minimum and a maximum number of individuals were registered (see below). The location of the tracks was recorded using the Global Positioning System.

Two different estimates of the number of individuals were produced from the snow tracking method. The minimum number (N_{min}) was obtained as the minimum number of otters that were tracked and could be undoubtedly separated. This number is likely to be lower than the actual number (N) if some individuals were not found. Because of the difficulties in determining the actual number of otters that have left tracks, an upper limit, the maximum number (N_{max}) of otters was also estimated. N_{max} may be an overestimation of N and included presumed individuals that could not be securely separated (for example, when tracking conditions were bad, two tracks or fresh faeces

separated by a large distance could correspond to two different animals).

Genetic sampling

To avoid cross-contamination, each individual faecal sample was collected with one pair of new disposable gloves, and put in stool vials with a disposable plastic spoon. Notes were made on the date, location, approximate age (or condition) of the spraint, and main prey content. The approximate age/condition was always estimated by the same person (J.A.) to avoid differences in appreciation between researchers: fresh (0–1 day), relatively fresh (1–3 days), old frozen (not yet dried), old relatively dry (from winter period), old dry (several days up to years). Special notes were made if spraints were found on ''spraint sites'' where remains of old spraints could contaminate the new ones. Because field work was performed in winter, most samples were obtained frozen and remained frozen (stored at –22°C) until DNA extraction.

Tissue (muscle or liver) samples (n = 20) were collected from dead otters in the study area and its surroundings between the years 1993 and 1999 in order to assess the suitability of the genetic markers to distinguish between individuals (estimation of probability of identity, see below). These samples could provide a more accurate estimate than if it was based on the faeces, as we had the certainty that each sample corresponded to a different individual and that genotyping problems (see below) were minimized.

Laboratory analyses

DNA extraction for the faecal samples was performed using QiaAmp DNA Stool Mini Kit (Qiagen) following the manufacturer's protocol. For every 15 samples one negative extraction control (without any DNA) was also processed. For extraction of DNA from the tissue samples, a small piece (approximately 10–30 μg) of the tissue was sliced with a scalpel and digested in 500 μl of extraction buffer (0.1 M Tris, 0.005M EDTA–Na_2, 0.2 M NaCl, 0.007 M SDS, adjusted to pH 8.5) with 0.3 mg of proteinase K. Digestions were incubated at 37°C overnight and genomic DNA was extracted following a conventional phenol/chlorophorm protocol (Sambrook et al. 1989). The extracted DNA was re-suspended in double-distilled water. Extractions of tissue samples were performed in a different laboratory to avoid contamination of faecal samples and only diluted extract was handled in the same lab as the faecal samples.

For the faecal samples, one microsatellite marker (locus Lut717) was initially typed three times to identify samples that did not yield any amplifiable DNA, which were excluded from further analyses. The remaining samples were genotyped for eight microsatellite markers (Lut701, Lut715, Lut717, Lut733, Lut782, Lut818, Lut832, and Lut833; Dallas and Piertney 1998). Since even a small number of genotypes allows for many pairwise comparisons (435 for 30 individuals), one additional marker (Lut902; Dallas et al. 1999) was used to obtain a higher resolution when needed. For sex determination we used a marker on the male-specific SRY gene (LutSRY; Dallas et al. 2000) with Lut715 as a positive control amplified at the same time. The amplification of Lut715 would confirm the presence of DNA in the extract, while the absence of the SRY-specific band would suggest that the sample corresponded to a female. The positive control was chosen such that it would produce a longer amplification product (Seddon 2005) than the LutSRY locus (around 200 bp compared to 70 bp). Each sex determination was replicated at least twice.

Polymerase chain reaction (PCR) amplifications were performed in 10 μl reactions. For the faecal samples the PCR reactions contained 1× PCR buffer (Qiagen), 1.5 mM $MgCl_2$, 0.25 mM of each dNTP, 3.2 pmol of each primer (5 pmol of each LutSRY primer), 0.05 μg of bovine serum albumine (BSA, Sigma), 0.45 units HotStarTaq DNA polymerase (Qiagen), and 2 μl of template DNA. For the tissue samples the reactions contained approximately 50 ng genomic DNA, 1× PCR buffer (Qiagen), 1.5 mM $MgCl_2$, 0.20 mM of each dNTP, 2 pmol of each primer, 0.05 μg of bovine serum albumine (BSA, Sigma), and 0.25 units HotStarTaq DNA polymerase (Qiagen). Amplification conditions were as follows: initial denaturation at 95°C for 15 min, followed by 20 cycles of 95°C for 30 s, touch-down from 60°C to 50°C (decreasing 0.5°C per cycle) for 30 s, and 72°C for 30 s, followed by 20 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 30 s, followed by a final extension at 72°C for 5 min. The PCR products were visualized using an ABI 377 instrument (Applied Biosystems) and analyzed using GENESCAN and GENOTYPER software (Applied Biosystems). For all samples (tissue and faecal) pre- and post-PCR work was conducted in different laboratories.

Allelic dropout and misprinting (false alleles) can be a problem when working with low quality DNA sources such as faeces (Taberlet et al. 1996; Gagneux et al. 1997; Taberlet and Luikart 1999; Lucchini et al. 2002; Miller et al. 2002; Creel et al. 2003; Wandeler et al. 2003; Broquet and Petit 2004). To avoid incorrect

genotyping all samples were analyzed multiple times for each marker following a variant of the multiple tubes approach (Taberlet et al. 1996). Heterozygote genotypes were replicated twice and homozygotes three times (obtaining always the same homozygous result) (Flagstad et al. 2004). If three alleles (or more) were found repeatedly at a locus in a sample we assumed that it was contaminated and the sample was excluded from further analyses.

Data analyses

The faeces were grouped according to quality (condition/age and prey content) to check if this had any effect on their suitability for DNA amplification. The probability of identity for genotypes from the study population (*PI*) was calculated to assess if the number of markers in the study was large enough to separate the different individuals. Since the otters in the study area are likely to be related to some extent, we calculated the probability of identity for siblings (PI_{sibs}; Taberlet and Luikart 1999):

$$PI_{\text{sibs}} = 0.25 + \left(0.5\sum p_i^2\right) + \left[0.5\left(\sum p_i^2\right)^2\right] - \left(0.25\sum p_i^4\right)$$

where p_i is the frequency of the ith allele. The calculation was made with Mathematica 5 (Wolfram Research, Inc.).

The allelic dropout rate was calculated for each locus across all samples and for each sample across all loci. Calculations were made only from heterozygous loci, because of the difficulty in identifying dropouts in homozygotes. The genotyped samples were checked with Microsatellite Toolkit (Park 2001) for matching pairs. An estimation of population size was generated by CAPWIRE (Miller et al. 2005), which is a capture-mark-recapture program designed for noninvasive samples. The two innate rates model (TIRM) was used, as the individuals could not be assumed to have equal ''capture'' probabilities.

Results

Quality of faeces and genetic markers

A total of 150 otter faeces were collected from 42 locations (38% of the total number of sites investigated). It was possible to amplify marker Lut717 from 94 (63%) faecal DNA extracts and these samples were selected for further amplification. To evaluate the factors dictating the amplification success, the samples were divided into groups according to their approximate age/condition and main prey content (Table 1). Comparison of the groups revealed that age or condition was of large importance in the extraction success ($\chi^2 = 14.09$, d.f. = 4, $P = 0.007$) and that the groups of fresh and old frozen faeces contained amplifiable DNA more frequently than the other groups. Main prey content of the faeces did not seem to affect the success of the amplification (Table 1, $\chi^2 = 4.73$, d.f. = 2, $P = 0.094$).

Of the samples that allowed DNA amplification for Lut717, we obtained complete genotypes from 46 of them (49%). Incomplete genotypes, with between one and three loci not confirmed (not successfully replicated enough times according to our genotyping protocol), were obtained from 20 (21%) of the samples. Two samples were excluded during laboratory analyses because of evidence of cross-contamination (three alleles). None of the negative controls during extraction or PCR were contaminated and, therefore, the finding of three alleles most probably was a result of contamination on the spraint site in the field. For the rest of the samples ($n = 28$, 29%) genotypes could not be obtained for more than four markers and were therefore excluded.

The probability of identity for siblings (PI_{sibs}), based on tissue samples from dead otters from the area, for the eight microsatellite markers initially typed on the faeces was 5.47×10^{-3}. Therefore, the eight selected loci seem to have enough power to differentiate between most or all individuals in the area. When one more locus (Lut902) was added to gain higher resolution (see below), the probability decreased to $PI_{\text{sibs}} = 2.33 \times 10^{-3}$.

Dropout rate and misprinting

The dropout rate differed across markers and samples. Samples that yielded complete genotypes had an average dropout rate of 7.7% (range between 0 and 33%, Table 2). When including the samples that yielded incomplete genotypes, average dropout rate increased to 13.6% (range between 0 and 82%, data not shown) due to the overall worse quality of those samples.

The allelic dropouts were not randomly spread over the eight markers ($\chi^2 = 17.34$, d.f. = 7, $P = 0.015$). Loci Lut818 and Lut715 had the highest dropout rates (Table 2, 16.7% and 13.4%, respectively). Microsatellites with longer alleles did not have a higher average dropout rate (Pearson's correlation $r = 0.21$, $P = 0.617$), but a closer look revealed that the result

Table 1 Age/condition and main prey content of the sampled otter faeces

	DNA	No DNA	Total
Sample age/condition			
Fresh	42	12	54
Relatively fresh	5	6	11
Old frozen	24	11	35
Old relatively dry	8	13	21
Old dry	15	14	29
Total	94	56	150
Main prey content			
Fish	76	47	123
Secretion[a]	12	2	14
Miscellaneous[b]	6	7	13
Total	94	56	150

The samples are grouped into two categories: those that yielded amplifiable DNA (for locus Lut717) and those that did not

[a] Excretion from anal scent glands

[b] Miscellaneous: frog, vole, bird, crayfish

was heavily affected by locus Lut818. If this locus was excluded dropout rate increased with overall marker size (Pearson's correlation $r = 0.76$, $P = 0.045$). However, when looking at heterozygote loci, longer alleles at each locus did not tend to drop out at a higher rate (Table 2, $\chi^2 = 0.018$, d.f. = 1, $P = 0.89$), indicating that allele length was not a major problem within markers. The results of these tests were very similar when including also samples that provided incomplete genotypes. Nevertheless, these analyses were conducted under the unlikely assumption that error rate was even among samples and the conclusions must be taken with caution. Only six misprintings (one occasion in six samples) were recorded in completely and incompletely genotyped samples.

Number of otters

The samples that could be completely genotyped resulted in 20 different genotypes (Fig. 1a, Table 3). The most divergent genotypes differed by 12 alleles and gender (Table 3). Genotypes of different sex differed by at least one additional microsatellite allele. Genotypes of the same sex differed by at least three alleles. In three cases the differences between two genotypes could be explained just by dropout. However, it seems unlikely that dropouts would consistently appear in all the genotyping replicates. In two of the cases the two genotypes were separated by distances of 30 and 60 km and, thus, they were most likely truly different individuals. In the third case, the two genotypes were found in sympatry, but there was no indication that any of these two genotypes were found in samples of lower quality, with unusually high dropout rates. Consequently, we consider that they also represent different individuals.

One male genotype occurred over a very large area. Since the area seemed excessive for a single individual, four of the faeces with that genotype were analyzed for an additional locus (Lut902). It was then confirmed that the samples corresponded to two different males (Fig. 1a, individuals 1 and 21).

The incompletely genotyped samples suggested the existence of two additional individuals. Individual 22 (three loci incompletely replicated) differed by five alleles from the most similar genotype (see Table 3). Individual 23 had one locus incompletely genotyped and differed by only one allele (for a completely replicated locus) from individuals 1 and 21, but individual 23 was located very distant from the two others (see Fig. 1a). The rest of the incompletely genotyped samples had genotypes that could not be differentiated from the complete genotypes. The samples that did not result in amplifiable DNA were not located far from genotyped samples and thus could not be used to imply that other otters might be present in the area. In total 23 individuals were thus found with the noninvasive genetic census, 15 males and 8 females.

The population size estimate generated by CAPWIRE, considering all genotypes (complete and incomplete), was 31 individuals, with a confidence interval between 23 and 40 otters. Three of the genotypes were regarded by the program as easier to capture and the rest (28) were harder to capture.

Table 2 Proportion of allelic dropout rates for the different loci, distributed on short and long alleles in heterozygous loci for completely genotyped samples

Locus	Dropout rate		
	Short allele	Long allele	Total
Lut701	0.053	0.039	0.092
Lut715	0.073	0.061	0.134
Lut717	0.014	0.014	0.028
Lut733	0.025	0.025	0.050
Lut782	0.041	0.033	0.073
Lut818	0.069	0.097	0.167
Lut832	0.023	0.047	0.070
Lut833	0.019	0.010	0.029
All loci	0.038	0.039	0.077

Snow tracking

Otter tracks were found in 30 places (27% of the explored sites). The minimum number (N_{min}) of individuals identified through snow tracking was 10 (Fig. 1b, A–J). Three more otters (K, L and M) and two cubs (N and O) could possibly be in the area, which gave a maximum number (N_{max}) of 15. Because

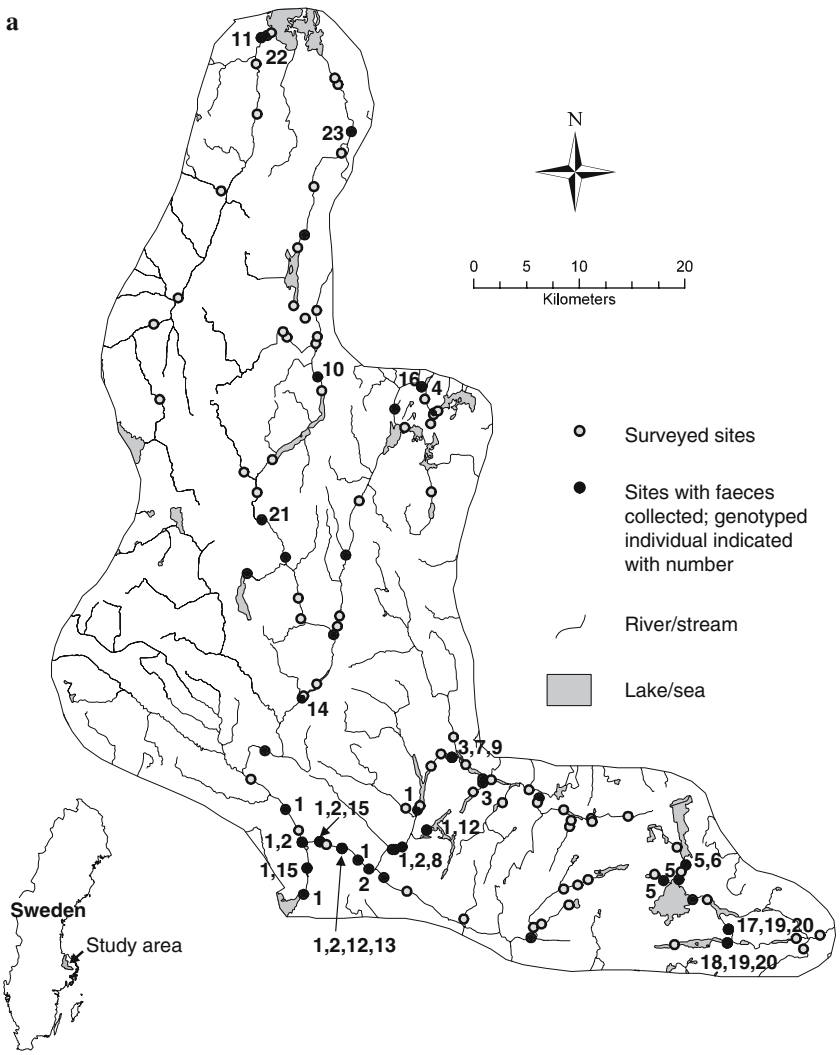


Fig. 1 Distribution of the otter individuals found: (**a**) with noninvasive genotyping (each number corresponds to a different individual); (**b**) with snow tracking (each letter corresponds to a different individual)

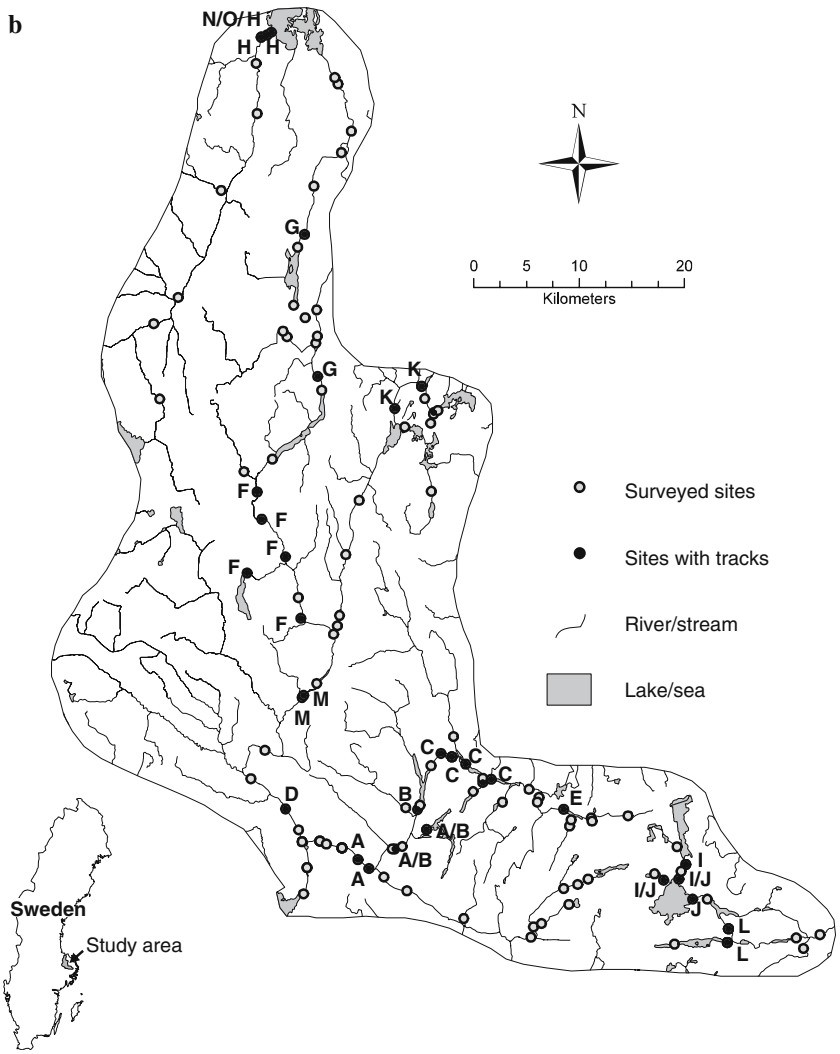
of local lack of ice and snow, individual K (see Fig. 1b) was based on fresh faeces and not on tracks. However, the distance to other tracked otters justified considering it as a different individual.

Discussion

The proportion of faecal samples from which otter DNA could be obtained (31–63%, complete genotypes and DNA amplified for marker Lut717, respectively) falls within the ranges of other studies of carnivores (e.g. Reed et al. 1997; Taberlet et al. 1997; Kohn et al. 1999; Ernest et al. 2000; Lucchini et al. 2002; Wilson et al. 2003; Bellemain et al. 2004; Hedmark et al. 2004). For otters the proportion of successful DNA extractions from faeces has proved to be equally variable, with values between 20 and 65% (Jansman et al. 2001; Dallas et al. 2003; Hung et al. 2005). The relatively low success rate and the need for a high number of replicates in noninvasive monitoring studies based on faeces imply an increase in the costs of the research. One way to optimize the economic investment and increase the success rate would be to focus the study only on higher quality samples. In our case fresh or old frozen faeces provided amplifiable DNA in a higher number of occasions, which is in concordance with previous studies on wolverines (Hedmark et al. 2004), wolves (Lucchini et al. 2002), and otters (Hajkova et al. 2006). Therefore, sampling during winter is likely to increase the success rate. However, any field sampling design that favours the location of fresh faeces may allow successful sampling in multiple seasons.

It has been suggested that the diet of the species could affect the chances of isolating DNA due to the presence of PCR inhibitors (Reed et al. 1997; Farrell et al. 2000; Murphy et al. 2003). A diet based on fish has been suggested to lower the success rate of DNA extraction and PCR amplification (Murphy et al. 2003). However, our results did not indicate a much

Fig. 1 continued



lower success rate compared to other carnivores. Also, we could not find any effect of diet on the amplification success (Table 1); however, 82% of the spraints contained fish remains and this might have limited the power of the comparison.

According to the probability of identity value, the number of loci studied here (eight) seemed to be enough to differentiate between the individuals in the area. However, in one case a ninth locus had to be used to separate samples that were suspected to come from different otters despite having identical genotypes (individuals 1 and 21, Fig. 1a). Since PI_{sibs} was calculated from tissue samples from carcasses found in the area between 1993 and 1999 and the noninvasive samples were collected in 2002, allelic frequencies might have changed in the population due to random genetic drift between the sampling periods. This could mean that the power of the markers to separate individuals was overestimated. However, the change in genetic diversity is unlikely to have been noticeable during the short time interval separating the two samples considering that the generation time for otters is around 4–5 years. Rather, the two identical genotypes could just be the result of a chance event and of the high number of pairwise comparisons involved (465 for 31 genotypes).

Although there are notable differences between the markers, dropout rate tended to increase with marker length. Therefore, it is clear that long microsatellite loci, as well as those that show increased dropout rate, should be avoided in noninvasive genetic studies (Frantzen et al. 1998). Preliminary surveys intended to select the most appropriate markers can thus result in an increased reliability of the genotypes obtained. On the other hand, small differences in the length of alleles at one locus do not seem to affect their probability of amplification.

Ideally, the two census methods that we compared in this study should give comparable results, but noninvasive genetic analysis of faeces do not always agree

Table 3 Genotypes obtained from the noninvasive faecal survey, grouped by individuals and the number of times (*N*) they were obtained

Ind	Sex	Lut717		Lut833		Lut832		Lut733		Lut782		Lut715		Lut701		Lut818		Lut902		*N*
1	M	186	194	155	159	181	185	167	175	176	192	204	208	194	210	180	180	149	161	2
	M	186	194	155	159	181	185	167	175	176	192	204	208	194	210	180	180			8
	M	186	194	155	159	181	185	167	175	176	192	204	208	194	210	**180**	**180**			1
	M	**186**	**194**	155	159	181	185	167	175	176	192	204	208	194	210	180	180			1
	M	186	194	155	159	181	185	167	175	176	192	204	208	194	210	**0**	**0**			3
2	F	186	186	155	155	181	193	167	175	188	192	204	208	198	198	158	180			5
	F	186	186	155	155	181	193	**167**	**175**	188	192	**204**	**208**	198	198	**158**	**180**			1
	F	186	186	155	155	181	193	167	175	188	192	204	208	198	198	**158**	**158**			1
3	F	186	186	159	163	181	193	167	167	192	192	204	204	198	206	180	180			2
	F	186	186	159	163	181	193	167	167	192	192	204	204	198	206	**0**	**0**			1
4	F	186	194	155	159	181	193	167	175	176	192	204	204	198	210	180	180			2
	F	186	194	155	159	181	193	**167**	**175**	176	192	204	204	198	210	180	180			1
5	M	186	186	155	159	185	193	175	175	188	192	204	204	198	198	158	180			9
	M	186	186	155	159	185	193	175	175	188	192	204	204	198	198	**158**	**180**			2
6	F	186	186	155	155	185	193	175	175	176	188	204	204	194	198	180	180			2
	F	186	186	155	155	185	193	175	175	176	188	204	204	194	198	**180**	**180**			1
7	M	186	194	155	159	193	193	167	175	192	192	204	204	194	198	176	180			1
8	M	186	186	155	159	181	193	167	175	188	192	204	208	198	198	158	180			1
9	M	186	194	155	163	181	193	167	167	192	192	204	204	194	206	180	180			1
10	F	186	194	155	159	193	193	175	175	188	192	204	208	198	198	158	180			1
11	F	186	190	155	163	185	193	175	183	176	192	204	208	194	206	180	180			1
12	F	190	194	155	159	185	189	167	175	176	192	204	204	194	198	176	180			1
	F	190	194	155	159	185	189	167	175	176	192	204	204	194	198	**176**	**180**			1
	F	190	194	155	159	185	189	**167**	**175**	176	192	204	204	194	198	**0**	**0**			1
13	M	190	194	155	159	185	189	167	175	176	192	204	208	194	198	176	180			1
14	M	186	186	159	159	189	193	167	167	188	192	204	208	198	198	176	180			1
15	M	194	194	159	167	181	185	175	175	176	192	208	208	194	210	158	180			1
	M	194	194	159	167	181	185	175	175	176	192	208	208	194	210	**158**	**158**			1
16	M	186	194	155	159	185	193	175	175	192	192	204	208	198	198	158	180			1
17	M	186	194	155	155	185	189	175	175	192	192	196	204	194	194	158	180			1
18	M	186	194	155	155	185	189	167	175	176	192	196	204	194	210	158	180			1
19	F	186	194	155	155	185	189	167	175	176	192	196	204	194	194	158	158			1
	F	186	194	155	155	**185**	**189**	**167**	**175**	176	192	196	204	194	194	**0**	**0**			1
20	M	186	186	155	159	185	189	167	175	188	192	204	204	194	210	180	180			1
	M	186	186	**155**	**159**	**185**	**189**	167	175	188	192	204	204	194	210	**0**	**0**			1
	M	186	186	155	159	185	189	167	175	188	192	204	204	194	194	**0**	**0**			1
21	M	186	194	155	159	181	185	167	175	176	192	204	208	194	210	180	180	145	149	2
22	M	190	194	155	163	181	185	**175**	**175**	**188**	**192**	204	212	**198**	**198**	158	180			1
23	M	186	186	155	159	181	185	167	175	176	192	204	208	194	210	**180**	**180**			1

Numbers in bold are loci that were not replicated enough times according to the genotyping protocol (incompletely genotyped samples)

Missing data are represented by 0

with estimates based on other techniques (Solberg et al. 2006; but see Eggert et al. 2003). If the two census methods gave similar results, the cheaper snow tracking method would be the preferred method. However, if the genetic census provided a more accurate estimate, and if it was possible to directly compare the results of genetic censuses and field censuses, it would be more suitable to combine both approaches to monitor larger areas at reasonable costs. In our case the two methods resulted in different estimates. Through noninvasive genetic analysis of faeces we found approximately twice as many otters in the study area as with snow tracking (23 and 10–15, respectively).

The genetic census method could result in under- or overestimation of the number of individuals. Overestimation could result from contamination of samples and genotyping errors. For this reason strict controls are required for the laboratory protocols as well as an appropriate replication scheme (Taberlet et al. 1996; Taberlet and Luikart 1999; Creel et al. 2003; Bonin et al. 2004). Overestimation could also result from occasional visits from otters that are not resident in the area (also possible for snow tracking), or sampling of old faeces belonging to now dead otters. Since it is not always possible to ascertain the age of field-collected faeces, noninvasive sampling could involve the identification of individuals over a longer time scale.

Underestimation could result from differences in scent-marking behavior between the sexes or age classes (subordinate individuals, cubs, or females with cubs might not scent-mark or do it at a lower frequency), or the inability to find faeces from some individuals. The sex ratio of the genotyped individuals showed a bias towards males. Dallas et al. (2003) also found slightly more males: 1.48:1 for a study of faecal samples, and 1.41:1 for carcasses. The similarity of the sex ratio estimated from faecal samples and from carcasses suggests that both sexes may be equally detectable from their scats. Thus, the higher frequency of male faeces could indeed represent a bias in the population. Other otter studies have found sex ratios that do not differ significantly from 1:1 (Kruuk and Conroy 1991; Ruiz-Olmo et al. 1998), but when they differ, more males are found (Philcox et al. 1999, but see Hung et al. 2005). It is also evident from field observations that otter cubs do scent-mark at this time of the year (J. A., personal observations), which implies that cubs can be monitored sampling faeces.

The estimated population size that CAPWIRE generated takes into consideration the chances of not having sampled all individuals in the study area. Our samples had a skewed capture rate distribution, with a few genotypes sampled many times and many genotypes sampled only once. Therefore, it seems likely that some individuals in the area were not genotyped (e.g. individuals N and O in the snow tracking). This leads to a census estimate much larger than estimated from just counting genotypes (mean: 31, range: 23–40). However, CAPWIRE does not consider possible overestimation due to the presence of vagrants. Also, it is possible that the software will tend to overestimate the number of otters because faeces were not collected at random: samples were intended to represent the entire study area, avoiding oversampling a limited area. Therefore, it is possible that the true population size is within the lower range of the population size estimation.

The snow tracking method resulted in a range for the possible number of individuals (between 10 and 15) depending on the criteria chosen. This shows that this method is susceptible to subjective assessment. Also, our results indicate that the snow tracking might tend to underestimate the number of individuals even when the weather conditions are reasonably good (as in our case). It is difficult to track otters, especially when there are large areas of open water. This made impossible the consistent application of identification criteria. However, even the maximum number of otters estimated from the snow tracking was well below the estimate obtained from the noninvasive genetic analysis. Ruiz-Olmo et al. (2001) concluded that for mud tracking it is possible to underestimate the true number of otters where otter densities are high (>0.6 individuals/km). Although our study only gave rough measures of otter densities, it is clear that higher densities were found to the south of the study area. Accordingly, the largest differences between the two methods are also found there (see Fig. 1a, b). We therefore agree that tracking methods can be less suitable in areas with high otter densities.

The results from this study imply that the census size obtained from a genetic approach could be twice the census size obtained from snow tracking (or even more using the rarefaction approach implemented in CAPWIRE), and that this method is likely to miss a part of the population. To decrease the risk of including visiting otters in the sample, an important precaution for noninvasive genetic studies may be to avoid limiting the survey to very small areas since the ratio of periphery compared to the total area is larger, which increases the probability of detecting occasional visitors from the neighboring home ranges. Additionally, repeated sampling over time of fresh faeces could allow to identify resident otters that live in the region during a certain time. The high risk of missing individuals with the snow tracking method, gives a clear advantage to the genetic census if the genotyping protocols are carefully selected. Additionally, the genetic census delivers additional information about the population (e.g. exact identity of individuals, relatedness, genetic diversity) that can be very valuable for the design of conservation strategies. However, both methods could be used as independent measures of population size, providing somewhat complementary information.

If the costs of noninvasive genetic censuses do not allow their use over extensive areas, an alternative could be to combine field census methods (here snow tracking) with the noninvasive genetic census in a smaller area. The census methods could then be used complementary, with the field census as the cheaper background method for extensive censuses over large areas and noninvasive genetic census would provide more detailed information in smaller areas.

Noninvasive genetic censuses of carnivore populations have shown increasingly promising results during the last few years and have provided a more comprehensive view of population densities and individual home-ranges (e.g. Flagstad et al. 2004; Bellemain et al. 2005). This study shows that genetic censuses

can effectively complement otter surveys and, hence, give more detailed information about these elusive animals.

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