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TECHNICAL NOTE

Molecular sexing of pine marten (*Martes martes*): how many replicates?

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Abstract

We used primers developed for the SRY gene in otters (*Lutra lutra*) to determine sex in pine marten (*Martes martes*). The otter SRY primers worked accurately for pine marten and assigned sex correctly in most replicates. These primers can be used on tissue and non-invasively collected hair samples for the identification of the animal's sex. We found that, based on five sets of replicates, DNA extracted from leg muscle and hair gave significantly better results than DNA extracted from ear tissue. Finally, results indicate the optimum number of PCR replicates to accurately assign sex using this technique.

Keywords: mustelid, PCR, pine marten, sex identification, SRY

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Pine marten are widespread but not necessarily abundant throughout most of Western Europe and are considered rare or endangered in many southern European countries (Davison *et al*. 2001). Knowing the sex of an individual or the sex ratio of a population is important for long-term conservation and development of management plans for species. Advances in molecular sexing of individuals have enabled researchers to separate the sexes in nonsexually dimorphic species and can also be used on noninvasive samples (hair or scat) or where the dead animal's body is too badly damaged or decomposed to identify sex (Dallas *et al*. 2000; Matsubara *et al*. 2001; Pilgrim *et al*. 2005). Any molecular technique used to identify sex should be accurate, reliable, sensitive, straightforward and cheap (Griffiths 2000). Several different techniques have been developed for the identification of sex in mammals based on genes conserved on the mammalian Y chromosome, e.g. SRY, ZFY and AMGY. Only male DNA produces a band using the SRY system but sex-specific products are produced when using ZFY and AMGY. Pilgrim *et al*. (2005) found that neither of the Y-specific genes targeted (ZFY or AMGY) produced a sex-specific product for American marten (*Martes americana*), mink (*Mustela vison*), fisher (*Martes pennanti*) or wolverine (*Gulo gulo*). It is likely that this test would be inconclusive for the closely related pine marten as microsatellites developed for the American marten and wolverine have been successfully used for studying pine marten (Kyle *et al*. 2003). Woods *et al*. (1999) state that in the absence of a system that amplifies a section of the X and Y chromosome simultaneously with the same primers that the use of the SRY gene is an accurate method for sexing. Amplification of the SRY gene during polymerase chain reaction (PCR) results in a product being produced for males but not for females; thus, the absence of a product could indicate a female or PCR failure. Few authors report on errors associated with using this technique or how many replicates should be carried out prior to a sample being confirmed as either male or female (Dallas *et al*. 2000 Ortega *et al*. 2004). Here we consider the feasibility of using PCR primers developed for the SRY gene of otter (*Lutra lutra*) for sex identification in pine marten and calculate the probability of identifying males with a single round of PCR.

Total DNA was extracted from 56 individuals ($N = 35$ ear tissue and $N = 21$ leg muscle) and 79 noninvasively sampled hair samples using a QIAGEN DNeasy Tissue Kit. All samples were extracted with negative controls (no sample material added) to control for contamination. Tissue samples were taken from live captured pine martens (samples 1–28; ear biopsy: 2 mm diameter) and road traffic accidents (RTA) [samples 29–56; leg muscle ($n = 21$; 25 mg) or ear biopsy ($n = 7$; 2 mm diameter)]. Ear tissue samples were taken from RTA animals when the body was too badly damaged to obtain leg muscle or when only ear tissue was available. The sex of 47 of the animals or carcasses was known, while nine was unknown owing to the carcass

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or sample being too badly damaged or decomposed in order to sex. Of the 79 hair samples (average number of hairs per extraction: μ = 12.51, SD = 8.17, min = 1 and max = 40 and average number of days before collection: μ = 6.47, SD = 7.3, min = 1, max = 28) extracted, 55 produced DNA (average number of hairs per extraction: μ = 14.85, SD = 8.26, min = 3, max = 40 and average number of days before collection: μ = 5.53, SD = 7.18, min = 2, max = 28) and of these 44 were used for the study (amplified with microsatellite on first occasion). Hair samples were obtained from the same population as the live-trapped animals above using the hair trap design described in Messenger & Birks (2000). All samples had previously amplified during microsatellite analysis (Á. B. Lynch, unpublished data) using the primers available for pine marten which are not species specific (Kyle *et al*. 2003). The primers, Lut-SRY F and Lut-SRY R, and PCR protocol have previously been described in Dallas *et al*. (2000) [90 °C/1 min 45 s, (90 °C/15 s, 60–0.5 °C per cycle/15 s) × 20 cycles, (90 °C/15 s, 50 °C/15 s) × 15 cycles, 72 °C/1 min]. The reaction volume (10 μL) contained 1 × PCR buffer, 0.25 U *Taq* (Invitrogen, recombinant), 2.5 mM $MgCl_2$, 200 μM dATP, dCTP, dGTP, dTTP and 1 μM of each primer. A hot start PCR was performed. A 70-bp product is produced for male otters. Five replications were carried out for each set of samples (except 26 and 27, 4 replicates). Products were electrophoresed on a 1 × TBE agrose gel (2% w/v) containing 0.625 mg/mL ethidium bromide and visualized with UV light. All work was carried out by the principal author (ÁBL; female).

A 70-bp band was observed for all known males (n = 22) (Table 1). No band was ever observed for any of the known females (n = 25) including the positive control (Fig. 1). In ear samples 1–28, 62.5% of the PCRs were correctly identified as males (Table 1). For samples 29–56 (known males n = 12), 96.66% of PCRs were correctly identified during the five replicates (Table 1). Of the unknown males (n = 4) 75% of reactions proved positive and the remaining five unknown animals were classified as females as no bands were ever observed for them (Table 1). Thirty-two of the 44 hair samples were designated as male and the remaining 12 as female. Of the 160 reactions performed on the male hair samples, only five failed to show a band and all samples showed bands on at least four occasions (Table 1). DNA extracted from leg muscle and hair was significantly more successful for sexing males than ear tissue (chi-square: $\chi^2 = 49.72$, d.f. = 2, $P < 0.00001$).

Table 1 Indicates the sex of each sample after five replicates of PCR. M and F refer to male and female, respectively. RTA refers to road traffic accidents

		Sex known		Sex unknown		No. of replications for 1%
N	Sample	M	F	M	F	misassignment
1–28	Live trapped	10	18	—	—	5
29–56	RTA	12	5	4	5	2
57–100	Hair	—	—	32	12	2

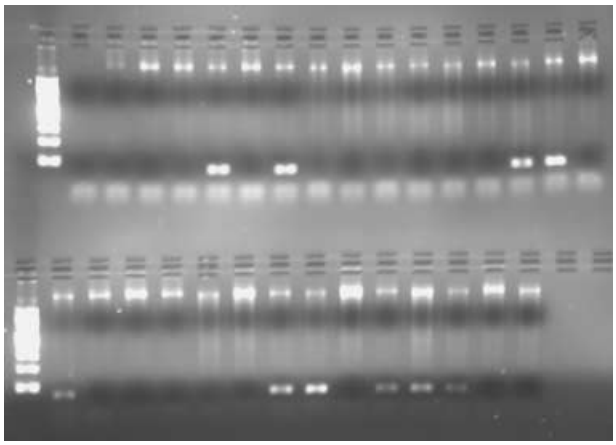

Fig. 1 Agarose gel for samples 1–28 showing DNA ladder (lanes 1 and 18, first band is 100 bp); lane 2 is the negative control and lane 32 the positive control (female). Males are shown in lanes 6, 8, 15, 16, 19, 25, 26, 28, 29 and (30). The samples in the rest of the lanes are from females.

The proof of positive PCR amplification and the number of times a sample is typed are important in determining whether an animal will be assigned as a male or not when using the SRY system as absence of a band may be indicative of either a female or a PCR failure. Dallas *et al*. (2000) suggest incorporating another marker into the PCR assay to indicate a successful PCR. However, even if another marker is incorporated into the PCR assay and co-amplification occurs, this is still not indicative of a female as PCR failure can occur for many reasons (Ortega *et al*. 2004). In this study, at least one male always amplified in each PCR replicate indicating positive PCR success. We found that DNA extracted from ear biopsies was not as successful during amplification as leg muscle or hair samples, though this may have been as a result of sampling technique. Biopsies were taken from animals while they were restrained and while working solo, and as such, the optimum sample may not have been obtained in all cases, hence lower quantities and/or quality of DNA. Ortega *et al*. (2004) found, using primers for the ZFY gene, that 85% of scats from animals of known sex and 86% of scats of unknown origin produced the correct banding pattern while the rest either produced unsatisfactory results (9.3% and 12%, respectively) or failed to produce PCR products (0.5% and 2%, respectively). Although we would have expected our noninvasively collected hair samples that are considered of poorer quality (Pilgrim *et al*. 2005), to produce more errors than ear biopsies, this was not the case, and this result stresses the importance of replication for all sample types. We would suggest that

a minimum of two replicates be carried out for all tissue and hair samples before characterization as male or female and only after prior amplification with a microsatellite. Knowledge of the sex ratio of a population or populations is important for making management decisions. The data generated from the noninvasive hair samples during this study allowed the identification of a minimum of five new animals, two (both males) of whom were located on transects where live trapping had been carried out but no males were trapped (Lynch *et al*., in press). The remaining three animals (two males and one female) were located in remote regions of the study area where live trapping was not feasible and were far enough away from other transects and from each other to be classed as new animals. When dealing with highly mobile and elusive carnivores like the pine marten, where the cost of live trapping is expensive or prohibited or restricted for whatever reason (legal protection or accessibility of the terrain), the use of molecular techniques and noninvasive samples provides beneficial tools for aiding conservation and management. We have shown here that primers developed for identifying sex in otters can be used for the same purpose in pine martens on both tissue and noninvasively collected hair samples.

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