



Short communication

Non-invasive PCR sexing of rabbits (*Oryctolagus cuniculus*) and hares (*Lepus europaeus*)

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Genetic sex verification has important implications for population studies of free-ranging animals relying on the knowledge of reproductive status and sex ratio of the animals. In the brown hare (*Lepus euroaeus*) a continuous population decline has been reported in many European countries (FLUX and ANGERMANN 1990). The reason for the decrease is under debate (MCLAREN et al. 1997; PANEK and KAMIENIARZ 1999; REYNOLDS and TAPPER 1995), and population studies of this species are therefore highly needed. The collection of blood causes stress due to trapping and handling of animals (JESSUP 1993) that could affect the parameters under investigation, particularly in a highly irritable species like the brown hare. Previous studies have demonstrated the potential for faeces collected in the field as a suitable source of DNA for genotyping and sexing free-ranging mammals (TABERLET et al. 1997). Since no sex-specific DNA sequences are known for the brown hare, we initially developed a PCR test for sex determination in rabbits (*Oryctolagus cuniculus*) and adapted it for sexing hares (*Lepus europaeus*). The assay co-amplifies a part of the Y-chromosomal Sry and the autosomal rabbit transferrin gene, which is used as an internal amplification control.

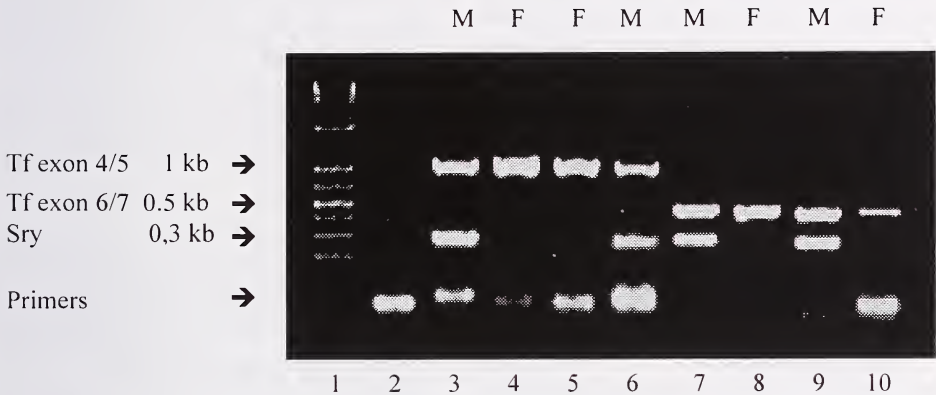
Because most sex-identification methods are not species specific, some precautions

have to be made to be aware of possible contamination with extraneous DNA, especially when animal remains such as hair or faeces are used as source for DNA analysis (TABERLET et al. 1997). In contrast to this universal primer approach, primers described in this report are placed in rabbit-specific sequence regions. To test the specificity of the assay, we amplified DNA from human, mouse, horse, and sheep, but none of these species amplified even under low stringency conditions (data not shown).

We first verified the accuracy of the assay by analysing genomic DNA from a total of 78 rabbits. Genomic DNA was isolated from 200 µl EDTA-blood from 24 adult males and 27 females of different rabbit breeds (GEMMELL and AKIYAMA 1996). For 27 new born rabbits, buccal swabs sampled with Q-Tips were used for sexing in order delete to apply a minimal invasive technique. The cut cotton-wool end of the Q-tip was placed in a 1.5 ml vial containing 600 µl digestion buffer (GEMMELL and AKIYAMA 1996) and stored at room temperature. Genomic DNA was obtained as described above by digestion of the whole swab with proteinase K (80 µg) for 2 hours at 56 °C. DNA was then extracted from the supernatant (GEMMELL and AKIYAMA 1996). Primers (Tab. 1) amplifying a fragment from the Sry region were designed accord-

Table 1. Specifications of duplex-PCRs for sex determination in rabbit and hare DNA sequence of rabbits Sry region in SINCLAIR et al. (1990). Rabbit transferrin (Acc. number. X58533). T_a: Annealing temperature

Species	Primer	5'-primer sequence – 3'	T _a	Primer conc.	Fragment length
Rabbit	Transferrin	GACCTTCTACTATGCTGTGGC	65 °C	0.5 µM	1 kb
	Exon 4/5	GTAGCCGAAATACGGCTGAAC		0.5 µM	294 bp
	Sry	AATACAGGAGGAACACGTAAAGTG CAAACCTGCGCTCTTCTGTAGGAT			
Hare	Transferrin	GCCTTTGTCAAGCAAGAGACC	60 °C	0.5 µM	0.5 kb
	Exon 6/7	CACAGCGCTCATACTGATCC		1.0 µM	294 bp
	Sry	AATACAGGAGGAACACGTAAAGTG CAAACCTGCGCTCTTCTGTAGGAT			

**Fig. 1.** Sex determination of rabbit and hares using the transferrin (Tf)/Sry duplex-PCR assay. Lane 1 Molecular size marker (Gibco); lane 2 no template control; rabbit: lane 3, 4 blood samples; lane 5, 6 buccal swabs; hare: lane 7, 8 tissue; lane 9, 10 faeces. M = male, F = female

ing to a published rabbit-human sequence alignment with their 3'-end being placed in rabbit-specific sequence regions (SINCLAIR et al. 1990). An amplicon from exons 4/5 (Tab. 1) of the rabbit transferrin gene (Tf) was used as amplification control. Duplex-PCR was carried out in a reaction mixture of 15 µl containing 20 ng template DNA, 0.5 U AmpliTaq polymerase Gold (PE Biosystems), 15 mM Tris-HCl pH 8.0, 50 mM KCl, 2 mM MgCl₂, 0.25 mM of each dNTP and primers as indicated in table 1. The cycling conditions on a GeneAmp 2400 Cycler (PE Biosystems) were: 10 min at 95 °C; 30 sec at 95 °C, 30 sec at 65 °C, and 60 sec at 72 °C for 35 cycles. Amplicons were separated by agarose gel electrophoresis. PCR on male samples amplifies two products (Sry and Tf), whereas from female samples

only one product (Tf) is obtained (Fig. 1). The PCR result was consistent with the animals' phenotypic sex in the 51 rabbit blood samples tested. When using genomic DNA isolated from 27 buccal swabs we obtained unambiguous results in 24 cases and the assigned gender was correct. In three cases we could not amplify any fragments because of degraded DNA.

The assay was then adopted for sexing brown hares using DNA extracts of tissue samples from 12 individuals. In hares the Sry region could only be amplified under less stringent conditions (Tab. 1). Primers for Tf exons 6/7 were used as internal control. Target specificity was certified by comparing directly sequenced gel-purified PCR products with published sequences. The amplified hare Sry sequence has been sub-

mitted to the GenBank database (Acc. number AF230075). Rabbit and hare Sry sequences differed at four nucleotide positions indicating that the amplified region is not completely conserved between these species. Rabbit specific primers may not perfectly match hare target sequences and thus only amplify the respective genomic regions under reduced stringency.

DNA extracted from hare faeces can be used for sex determination, but at a higher test dropout rate. We collected fresh faecal samples from 36 individually caged hares with known sex into separate plastic tubes and froze them immediately. We extracted DNA from faeces by a silica-based purification method in order to purify DNA and to break down compounds that inhibit subsequent PCR reactions (after BOOM et al. 1990; CONSTABLE et al. 1995). PCR reactions of faeces

samples contained bovine serum albumin (100 µg/ml final concentration) and were incubated for 47 cycles. Analysis of 20 out of the 36 DNA samples purified from faeces revealed the correct gender. Fifteen samples amplified no PCR product, probably because of poor DNA quality. Only one female was sexed incorrectly, possibly due to male-derived contamination of the cage.

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