ZFX AND *ZFY* GENE SEQUENCES: USE FOR MOLECULAR SEXING EUROPEAN RABBITS, EUROPEAN BROWN HARES AND MOUNTAIN HARES AND PERSPECTIVES FOR SEX DETERMINATION OF OTHER LEPORID SPECIES

Fontanesi L.[1]*, Tazzoli M.[1], Pecchioli E.[2], Hauffe H.C.[2], Robinson T.J.[3], Russo V.[1]

[1]DIPROVAL, Sezione di Allevamenti Zootecnici, University of Bologna, Via F.lli Rosselli 107, 42100 Reggio Emilia, Italy
[2]Centro di Ecologia Alpina, Viote del Monte Bondone, 38040 Trento, Italy
[3]Dept. of Botany and Zoology, Evolutionary Genomics Group, University of Stellenbosch, PB X1, Matieland 7602, South Africa
*Corresponding author: luca.fontanesi@unibo.it

ABSTRACT

We have developed a new molecular sexing method for three leporid species (*Oryctolagus cuniculus*, *Lepus europaeus* and *Lepus timidus*) based on the analysis by PCR-RFLP of point mutations that differentiate the *ZFX* and *ZFY* gene sequences. Polymorphic positions on the X and Y chromosomes of male samples were recognizable as double peaks in the sequencing chromatograms. Comparison of these sequences with those obtained from the females of *O. cuniculus*, *L. europaeus* and *L. timidus* allowed us to deduce the sequence of the Y fragment for these species. Analysis of the obtained sequences revealed an *Alu*I restriction site in the female but not in the male sequences and a *Bgl*II restriction site in the male but not in the female sequences. A *Hinf*I restriction site was present only in male *O. cuniculus* sequences but not in the female fragment. Digestion of the amplified fragments obtained in all collected samples for these three species produced the expected fragments for all analysed samples (70 European rabbits, 37 European brown hares and 24 mountain hares), for which sex was recorded during their collection proving a high accuracy of the methods. The method described here enables the identification or confirmation of gender of tissue samples from three species of *Leporidae* using restriction enzymes that cut male or female fragments. The specificity of this method means that the probability of incorrect sex identification due to polymorphisms within species that might involve the two gender specific restriction sites is low enough to be ignored. Then, we obtained sequence information for the *ZFX/ZFY* loci for other 6 leporid species (*Bunolagus monticularis*, *Lepus americanus*, *Pentalagus furnessi*, *Romerolagus diazi*, *Sylvilagus floridanus* and *Sylvilagus nuttallii*) including a few threatened taxa. Several polymorphic sites can be identified between sexes and among species. However, as few samples were available for these species further investigation should be carried out to develop and evaluate appropriate sexing protocols.

Key words: Sex determination, *ZFX*, *ZFY*, *Leporidae.*

INTRODUCTION

Autochthonic European rabbit (*Oryctolagus cuniculus*), European brown hare (*Lepus europaeus*) and mountain hare (*Lepus timidus*) populations across Europe, as well as on other continents to which some of these species have been introduced, are experiencing diverse and often rapid changes in their ranges and dynamics (Zenger *et al*., 2003; Smith *et al*., 2005; Fredstel *et al*., 2006) underscoring the importance of developing a quick, reliable and rapid means of sex determination and sex ratios for monitoring their status and understanding the reasons of their expansion or decline. Moreover, since leporid species are notoriously difficult to live-trap, many studies rely on the collection of tissue samples from road kills or by hunters, when sex cannot be identified accurately further emphasising the usefulness of a genetic method of gender identification.

Molecular sexing in animals with a heteromorphic sex chromosome system relies either on the PCR-amplification of fragments specific to the Y or W chromosomes or the amplification of homologous fragments from both sex chromosomes. In eutherian mammals, the first approach is usually based on the amplification of the Y-specific *SRY* locus (Griffiths and Tiwari, 1993). As an amplified product is only expected in males that carry the Y chromosome, the *SRY* gene test can be problematic because it is not possible to distinguish the failure of a PCR amplification from the correct identification of the samples as females. To overcome this problem, a duplex PCR analysis which involves the simultaneous amplification of an *SRY* gene fragment and an autosomal DNA fragment in the same tube can be used as an internal PCR quality control. Unfortunately, amplification of the autosomal DNA region does not guarantee the amplification success of the sex specific locus. Hence, co-amplification of orthologous sexual chromosome genes such as amelogenin X and Y genes (*AMELX* and *AMELY*) or zinc finger protein *ZFX* and *ZFY* genes using the same pair of PCR primers has been suggested to improve the precision and quality of the sexing assay (Aasen and Medrano, 1990; Sullivan *et al*., 1993).

For the European rabbit and brown hare, a method for amplifying an *SRY* gene fragment together with a portion of the autosomal transferrin gene has been previously described by Wallner *et al.* (2001). More recently, Putze *et al*. (2007) used length polymorphism within introns 9 and 10 of the *ZFX* and *ZFY* genes to sex European rabbits testing the efficiency of this protocol also in few brown hare and mountain hare samples; however, it was not clear from this study why multiple fragments were obtained for both female and male animals raising some questions about primer specificity. Furthermore, the efficiency of these methods were evaluated using few samples of known sex.

Therefore, following the approach described by Fernando and Melnick (2001), we have developed a new molecular sexing method for these three leporid species based on the analysis of point mutations that differentiate the *ZFX* and *ZFY* gene sequences. Sequence information for these loci was also obtained for other leporid species opening the possibility to develop other specific sex determination methods.

MATERIALS AND METHODS

Animals and DNA Isolation

Hairs were collected from 70 (35 males and 35 females) European rabbits of different breeds (25 New Zealand White, 15 Californian, 15 Vienna Blue, 5 Burgundy Fawn, 5 Champagne d'Argent and 5 Giant Grey). Muscle tissue or ear notch samples were obtained from 37 (19 males and 18 females) European brown hares and 24 (12 males and 12 females) mountain hares. DNA was extracted using the protocols described by Fontanesi *et al*. (2006; 2007) for hair roots, a standard phenol-chloroform protocol (Sambrook *et al*., 1989) or the DNAeasy Tissue Kit (Qiagen) for muscle tissue and ear notches. DNA samples were also obtained from blood of one *Sylvilagus floridanus* male and one *L. americanus* female and from cultured fibroblast cells of one *Bunolagus monticularis* male, one *S. nuttallii* male, one *Pentalagus furnessi* female, and one *Romerolagus diazi* female using the protocol described by Sambrook *et al.* (1989).

Polymerase Chain Reactions (PCR), Sequencing and RFLP analysis

PCR primers (forward, 5'-GGTGCAGCAACATGCTCTTA-3'; reverse, 5'-TTAAAGCCTGAGGCGTCTGT-3') were designed to amplify a 432 bp of exon 11 of the *ZFX* European rabbit gene sequence (Ensembl Gene ID: ENSOCUG00000003815, database version 45.1c, http://www.ensembl.org/Oryctolagus_cuniculus/index.html**)**. PCR was carried out using a TGradient thermal cycler (Biometra) in a 20 μL volume containing 5-100 ng of genomic DNA, 0.5 U of EuroTaq DNA polymerase with 1 X *Taq* reaction buffer (EuroClone Ltd.), 2 mM $MgCl_2$, 10 pmol for each primer and 2.5 mM for each dNTP with the following profile: 95°C for 5 min; 35 cycles at 95°C for 30 sec, 56°C for 30 sec, 72°C for 30 sec; final extension at 72°C for 5 min. Amplified fragments

obtained for two males and two females of *O. cuniculus*, *L. europaeus* and *L. timidus* and for the other leporid species listed above were sequenced in forward and reverse directions with the same PCR primers using the BigDye Terminator version 3.1 Cycle Sequencing kit (Applied Biosystems), and following manufacturer's instructions. Sequences were analysed on an ABI3100 Avant sequencer (Applied Biosystems), aligned with the CodonCode Aligner software (CodonCode Corporation) and inspected manually. Digestion of the amplified fragments for *O. cuniculus*, *L. europaeus* and *L. timidus* was carried out at 37°C overnight in a 25 µL volume using 5 µL of PCR product, 5 U of *Alu*I (New England Biolabs) and either 5 U of *Bgl*II (New England Biolabs) or 5 U of *Hinf*I (Roche Diagnostics; *O. cuniculus* only) and 1 X specific reaction buffer. The resulting DNA fragments were separated by electrophoresis using 2.5% agarose or 10% 29:1 bis-acrylamide:acrylamide gels and visualised by staining with ethidium bromide.

RESULTS AND DISCUSSION

PCR amplification was successful for all species and all samples. Polymorphic positions on the X and Y chromosomes of male samples were recognizable as double peaks in the chromatograms. Comparison of these sequences with those obtained from the females of *O. cuniculus*, *L. europaeus* and *L. timidus* allowed us to deduce the sequence of the Y fragment for these species (Figure 1). Analysis of the sequences obtained for *O. cuniculus*, *L. europaeus* and *L. timidus* revealed an *Alu*I restriction site in the female but not in the male sequences and a *Bgl*II restriction site in the male but not in the female sequences. A *Hinf*I restriction site was present only in male *O. cuniculus* sequences but not in the female fragment. Digestion of the amplified fragments obtained in all collected samples for these three species produced the expected fragments (*Alu*I: male, 432 + 343 + 89 bp; female, 343 + 89 bp; *Bgl*II: male, 432 + 291 + 141 bp; female: 432 bp; *Hinf*I: male, 432 + 261 + 171 bp; female, 432 bp) for all analysed samples (70 European rabbits, 37 European brown hares and 24 mountain hares), for which sex was recorded during their collection proving its accuracy. As a matter of fact, several molecular sexing tests in wild and domestic animals do not prove their accuracy testing a sufficient number of samples with known sex (Robertson and Gemmel, 2006). The method described here enables the identification or confirmation of gender of tissue samples from three species of *Leporidae* using restriction enzymes that cut male or female fragments. The specificity of this method means that the probability of incorrect sex identification due to polymorphisms within species that might involve the two gender specific restriction sites is low enough to be ignored.

Then, we obtained sequence information for the ZFX/ZFY loci for several other leporid species (Figure 2). The X and Y sequences in some species (*S. floridanus*, *S. nuttallii* and *B. monticularis*) for which only male samples were available, were inferred from the conserved regions of *ZFX* and *ZFY* sequences between species, thus these data should be confirmed sequencing also female samples.

However, several polymorphic sites can be identified between sexes as well as among species providing useful information for establishing sex determination protocols that could also make it possible to detect the species of origin. Moreover, the obtained sequences confirm that the leporid *ZFX* and *ZFY* genes are highly conserved but contain also useful information for phylogenetic investigation.

CONCLUSIONS

This method can be used for forensic investigations or to study the gender-ratio in wild rabbits, brown and mountain hares using tissue samples for which the sex has not been noted. Moreover, *ZFX/ZFY* loci could be suitable for molecular sexing of several other species of this family, including several threatened taxa, for which other appropriate sexing protocols should be developed and evaluated.

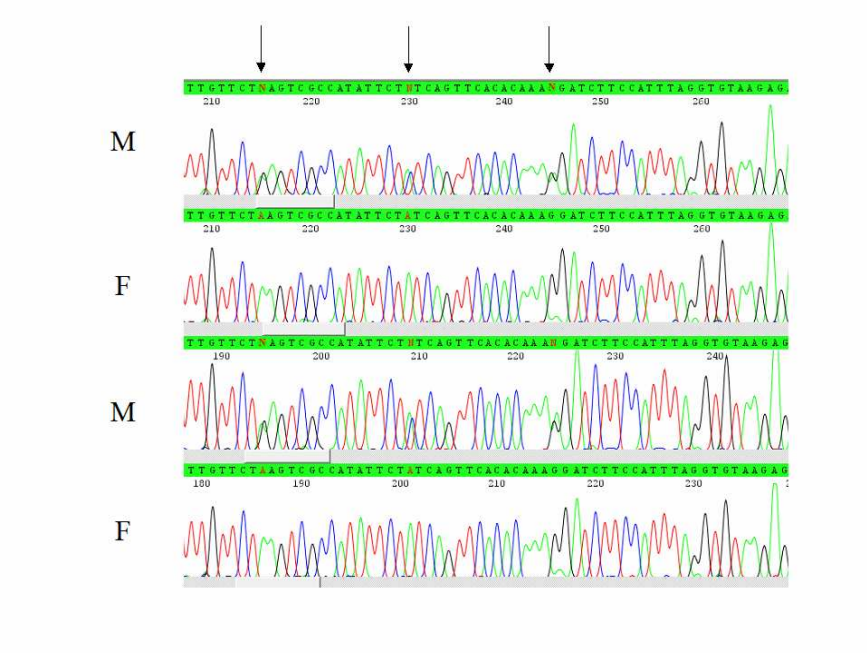

Figure 1: Sequence chromatograms of the amplification products obtained from two males (M) and two female (F) European rabbits. Arrows indicate the polymorphic sites between the *ZFX* and *ZFY* gene sequences.

	H A B	
	11111222222222333333	
	37736778911355678144778	
	13730584347279249103092	EMBL #
ZFX		
Oryctolagus cuniculus	**AGTGCCGCCCGCCATAGATGTAG**	AM778417
Lepus europaeus	**.....G.G.....**	AM778429
Lepus timidus	**G.....G.G.....**	AM778431
Lepus americanus	G.....G.G.....	AM778428
Sylvilagus floridanus	.....G.....	AM778421
Sylvilagus nuttallii	.....G.....	AM778419
Pentalagus furnessi	G.....	AM778423
Romerolagus diazi	G.....TG.....	AM778424
Bunolagus monticularis	G....T.TTT...G.CA..A...	AM778426
ZFY		
Oryctolagus cuniculus	**G..A....TT...G.CA..A...**	AM778418
Lepus europaeus	**G..A.T.TTT.T.G.CA..A...**	AM778430
Lepus timidus	**G..A.....G.CA..A...**	AM778432
Sylvilagus floridanus	G...TT.TT.A..GCG.....GA	AM778422
Sylvilagus nuttallii	GA..TT.TT....GCG....CGA	AM778420
Bunolagus monticularis	GAG.TTATTT...G.CAGCA...	AM778427

Figure 2: Comparison of *ZFX* and *ZFY* variable sites. EMBL accession numbers are reported for each sequence. Numbers indicate the position of the sites in the amplified fragments. Dots represent nucleotides identical to the *O. cuniculus ZFX* sequence. Grey highlighted positions denotes the informative positions used to developed the PCR-RFLP sexing test using *Alu*I (A), *Bgl*II (B) or *Hinf*I (H). *O. cuniculus*, *L. europaeus* and *L. timidus ZFX* and *ZFY* sequences are in bold. These sequences were determined from fragments originating from both female and male individuals. *S. floridanus*, *S. nuttallii* and *B. monticularis* sequences were obtained from males only, thus *ZFX* and *ZFY* sequences were deduced by comparison with other female sequences. For *P. furnessi* and *R. diazi* only females were sequenced.

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