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TECHNICAL NOTE

A sex determination protocol for the Iberian desman (*Galemys pyrenaicus*) based on a three primer amplification of *DBX* and *DBY* fragments with non-invasive samples

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Abstract We have sequenced partial fragments of *DBX* and *DBY* genes of the endangered Iberian desman (*Galemys pyrenaicus*). The sequences were used to design a sex determination protocol for non-invasive samples based on a PCR reaction, using only three primers. This protocol allows the simultaneous amplification of two fragments, one corresponding to the *DBX* gene and the other to the *DBY* gene, both differing in size. To increase sensitivity on the detection of positive amplifications and on the determination of fragment size we use a fluorescently labelled primer. The protocol has been tested in DNA samples from hair and stool, revealing major difficulties in sexing faecal samples, but unambiguous sexing of hair samples.

Keywords Iberian desman · Non-invasive samples · Sexing · *DBY* · *DBX*

Common sex determination techniques include visual identification or manual palpation of the urogenital region. As both can be difficult, molecular methods have been developed for a wide range of taxa (Bello and Sánchez 1999; Durnin et al. 2007; Mucci and Randi 2007). These methods can also be used with non-invasive samples, a very important feature when working with endangered species (Waits and Paetkau 2005).

The Iberian desman *Galemys pyrenaicus* (E. Geoffroy Saint-Hilaire, 1811) is a semi-aquatic mammal endemic of the Iberian Peninsula. It is taxonomically classified within the family *Talpidae*, being, with the Russian *Desmana moschata*, the only representatives of the the subfamily *Desmaninae* (Cabria et al. 2006). In Spain, populations are in regression and it is considered endangered (Palomo et al. 2007).

Although demographic census of desmans can be performed through faeces (Bertrand 1992), sex determination is difficult due to the lack of sexual dimorphism: testes are intraabdominal and both males and females have a peniform organ (González-Esteban et al. 2003). Moreover, in some genus of the *Talpidae* family intersexual females are common (Jimenez et al. 1993), a feature that could also exist in desmans (Jiménez et al. 1997).

To overcome these difficulties, we have designed a set of three primers which amplify two regions belonging to intron 8 of *DEAD Box X isoform* (*DBX*) and *DEAD Box Y isoform* (*DBY*) genes, because partial sequences of the *DBY* gene were already described in other members of the order Insectivora (Braendli et al. 2005).

Muscular tissue of three desmans (two females and one male) was used to obtain genomic DNA with a conventional phenol-clorophorm extraction. DNA was also obtained from 26 hair samples (belonging to captured and manually sexed individuals) with the *Nexttec*^{TM} *Genomic DNA Isolation kit* (Nexttec GmbH Biotechnologie, Leverkusen, Germany) and from 91 faeces (collected and preserved in 70% ethanol) with the *RealPure Spin Food Stool kit* (Durviz S.L.U., Barcelona, Spain).

The alignment of human (ENSEMBL ENSG00000067048), mouse (GenBank BC051046) and white-toothed shrew (GenBank AY918414) *DBY* sequences was used to design conserved primers (DBF, 5′-CCACTTCCACCAAGTGAACG-3′ and DBR, 5′-ACAAGGACGAACTCTAGATCGG-3′) located in exon 5 and exon 10, respectively. Amplification reactions had 1.5 mM $MgCl_2$, 200 μM dNTPs, 0.2 μM of each primer, 25 ng of genomic DNA

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and 0.3 U Taq DNA polymerase (Ecogen S.R.L, Barcelona, Spain) in a final 30 µl volume. Thermal profile was 94°C 5′ followed by 35 cycles of 94°C 30″, 50°C 1′30″ and 72°C 1′30″. PCR products were purified with the *illustra*^{TM} *GFX PCR DNA and Gel Band Purification kit* (GE Healthcare, Barcelona, Spain) and sequenced with the *BigDye*® *Terminator v1.1 Cycle Sequencing kit* (Applied Biosystems, Madrid, Spain).

Amplification of tissue DNA with primers DBF and DBR yielded one fragment in the two females (1.9 kb, GenBank acc: FJ638890) and two fragments in the male (1.9 and 1.7 kb, FJ638891). Subsequent sequencing allowed the identification through the NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) of these fragments, which corresponded to *DBX* (1.9 kb) and *DBY* (1.7 kb) genes. Interestingly, although conserved primers DBF and DBR were designed using only *DBY* sequences, we were able to amplify both *DBX* and *DBY* fragments.

Human *DBX* and *DBY* transcripts (ENSEMBL ENST00000399959 and ENSEMBL ENST00000336079) were used to compare and identify exon-intron boundaries, and both *Galemys* sequences span from exon 8 to exon 9, including intron 8.

As expected, the alignment of exonic regions of *G. pyrenaicus DBX* and *DBY* fragments showed a high degree of similarity (82%), which is at the same level as in humans (86%). In contrast, the intronic region was highly divergent and it has different lengths (488 bp in *DBY* and 131 bp in *DBX*).

This size variation was suitable for the design of a sexing protocol based in the simultaneous amplification of *DBX* and *DBY* intron 8 with one set of primers. However, PCR sizes (from 200 to over 500 bp) could handicap the reliability of the test when using low yield samples such as hair or faeces (Waits and Paetkau 2005).

Thus, we designed a three-primer based protocol (Durnin et al. 2007). Two fragments, corresponding partially to intron 8 of *DBX* (fragment size of 183 bp) and intron 8 of *DBY* (fragment size of 120 bp) were simultaneously amplified in one reaction with three primers: DBXYe8F 5′-GATCTGGAAAAACTGCAGCTT-3′ (conserved both in *DBX* and *DBY* genes), DBXi8R 5′-TTG ATTGCAACA TAATTCCCTA-3′ (specific of *DBX*) and DBYi8R 5′-CCC AAAGCAAAGCCTCTCAT-3′ (specific of *DBY*) (Fig. 1). PCR conditions and thermal profile were the same as detailed above, genomic DNA quantity was adjusted to 5 ng

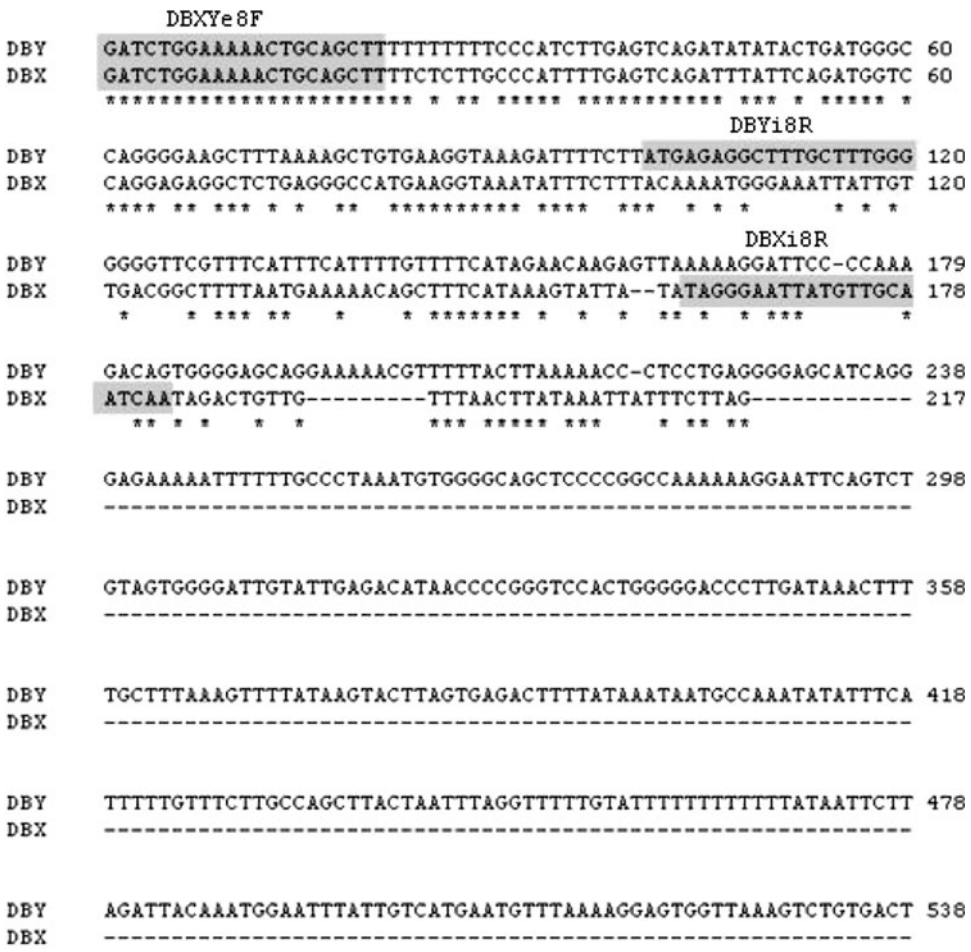

Fig. 1 Alignment of partial sequences of Iberian desman *DBX* and *DBY* genes (GenBank FJ638890 and FJ638891). The locations of three primers used for the sexing protocol (DBXYe8F, DBXi8R and DBYi8R) are overlaid

when analyzing hair and faeces. DBXYe8F1 was fluorescently labelled and a capillary electrophoreses was carried out in an ABI PRISM 3130 sequencing device (Applied Biosystems, Madrid, Spain) to detect positive amplification of both fragments.

Three tissue samples and 26 hair samples were sexed both by close observation and by genetic testing. Both methods assigned same sex in 84.6% of the hair samples (22/26). In four samples the assignation was not identical, suggesting misidentifications during palpation. As errors of assignation do not follow any clear pattern, the existence of intersexual females is not confirmed. If such individuals were present, a bias in the errors (molecular females identified as males) would be expected.

We also sexed 91 faecal samples. Of these, only 10 gave successful amplification, all showing a single fragment indicating females. The fact that we have only detected females most likely reflects problems in primer design and/or protocol optimization; however, the good results using other samples (muscular tissue, hair) indicate a high reliability for this method, when using high quality DNA. Altered sex ratios could also explain this deviation, but previous studies (González-Esteban et al. 2002) reported a 1:1 ratio in captured *G. pyrenaicus*, a proportion that we also find in our collected hair samples. All together, these data suggest problems related to DNA quality of faecal samples, which would also account for the low rate of amplification (11%).

Season of collection (cold seasons are preferred) and sample age are usually crucial for DNA quality in faecal samples (Piggott 2004). In this species, however, sample collection is restricted around summer, when individuals are more active and rivers are accessible. Thus, only age of the sample could be controlled in further studies, suggesting that frequent recollections of stools in the same area might improve the success rate.

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