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Canid-specific primers for molecular sexing using tissue or non-invasive samples

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There is an increasing use of non-invasive samples for studying wild animal populations both to avoid disturbance of the animals and to facilitate the collection of samples. An important component for conservation studies is the accurate identification of the sex of individuals. However, some sexing methods encounter difficulties in the amplification of long fragments from degraded DNA and in non-specific amplification from faeces analysis in carnivores. Here I present a method for sexing of wolves by a multiplex PCR amplification that is particularly suited to non-invasive samples. This method can easily be adapted for a wide range of species.

In mammals, RFLP analysis exploiting X- and Y- chromosome-specific polymorphism has been a widely employed method of molecular sexing, for example in the *ZFX* and *ZFY* genes (Amstrup et al. 1993; García-Muro et al. 1997). However, PCR products of considerable length are used, which is not suitable for degraded DNA such as faeces analysis. An alternative approach is the amplification of short Y-specific genes using conserved primers, for example *SRY* (Griffiths and Tiwari 1993; Taberlet et al. 1993; Gowans et al. 2000) and *Ube1Y* (Sloane et al. 2000). The use of Y-chromosome sequences alone is not informative because an absence of PCR product can indicate either a female or a failed reaction. Hence, these reactions are often performed in conjunction with a fragment of mitochondrial DNA or a nuclear locus to act as a positive control for amplification, the latter being preferable due to its presence at copy-numbers more equivalent to the Y-gene being co-amplified.

Nonetheless, the use of conserved mammalian primers, while facilitating their application to many species, can lead to non-target amplification when amplifying from faeces in carnivores. For example, Murphy et al. (2003) identified as male four female bears that had been fed on male deer. Hence, PCR sexing methods that incorporate species specificity are required and have been used in the *SRY* gene in sperm whale (Richard et al. 1994), the *ZFX*/*ZFY* genes in cetaceans (Bérubé and Palsbøll 1996) and the *DBY* gene in wolverines (Hedmark et al. 2004). Here we present canid-specific primers that provide a reliable method for sexing wolves and dogs from faeces and other degraded DNA samples.

The sexing protocol in wolves employs multiplexed primers to amplify a locus with homologues (gametologues) on both the X- and Y-chromosome. Primers that exploit the conserved nature of exonic sequences are available to amplify X- and Y- specific fragments across a range of mammalian species (Hellborg and Ellegren 2003, 2004). Using these primers and PCR conditions to obtain sequence of *DBY* intron 7 and *DBX* intron 6, a sexing primer was designed for each locus across genus-specific polymorphism (Figure 1) and used in conjunction with one of the original exon primers.

For both loci, the intron primer was designed with four considerations in mind. Firstly, the primers were chosen to be canid-specific to avoid non-target amplification. Comparative sequencing in other mammalian sequences, including horse and lynx, showed that the intron sequences vary widely among genera, so placing the primer in canid-specific sequence was straightforward. For example,

(a) DBX intron 6

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           DBX6B   10         20   *     30         40         50         60         70         80         90         100
DBX6wolf   ATGCTGCAGT TTTTCCAGAC CCTATTT-AA GAGAAAGCAT AATCTAATTC AACCACAGAA AAACCTGCTC ATTATTTTTT AATAAGTATT GGTAGATGAG
DBX6 horse .......... .......... ...G...T.. .......... .T.-..C.-. .......... ...T..A..- ---------- ---------- ----------

                  110        120        130        140        150        160        170        180        190        200
DBX6 wolf  TTTAACTGCC CTATTACTAA TGTTTTACTT TGCACCCAAA TAAATTTTCT AAAAATTAAC ACCAAAGTTA AAAATACCAA GAAGTGACTT CTTTGACCCA
DBX6 horse ---------- ---------- ---------- ---------- ---------- ---------- ---------- --------T. .---------- ----.-...-

                  210        220        230        240 DBX6Iv 250        260        270        280
DBX6 wolf  TCTGTAGGTC AGATGTAACA GAAGTCAAGA AACTAAGACC CAGCGTATTT ATCTTTGTAA ATAAAGCTTT GTTGGAATTC
DBX6 horse ---------- ---------- --...----- ----------. .-----.... .......... ........C. AA......AT
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(b) DBY intron 7

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           DBY7    10         20         *30        40         50         60         70         80         90         100
DBY7 wolf  GGTCCAGGAG AGGCTTTGAA GGCTGTGAAG GTAAAGATTG ATTTCCCTCT AAAATAAGTT GTTGGCATAG AAAAAGCTTC ATAAATATAA GTT-G-AGAC
DBY7 horse .......... .......... .......... .........T C...----A. .......... T..C-.T.T. ......T.CG G.......CT ...A.G.TGG

                  110 DBY7Iv 120        130        140        150
DBY7 wolf  CTTCCTTTTA AACAATGGCA AATATGTTTT ATTTCAGTTC CTTTGAGTGT
DBY7 horse -...T..C.. .GT.T.ACG. ....GAC... G.CGTCA..T ....TGA.T.
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Figure 1. Location of primers for *DBX6* and *DBY7* canid sexing reaction. Sequences are shown in the wolf and, for comparative purposes, the horse, for intron 6 in *DBX* and intron 7 in *DBY*. The last nucleotide in the exon sequence is indicated with an asterix. Primers are shown in bold. Primers DBX6B (Hellborg and Ellegren 2004) and DBY7 (Hellborg and Ellegren 2003) have been published previously. Dots indicate identity with the top sequence; dashes indicate alignment gaps. All primers have an estimated annealing temperature of 64.5 °C.

the *DBY7* sequence of the lynx, another carnivore, shows 10 mismatches to the canid 24-mer reverse primer. Secondly, as amplification of the Y-chromosome fragment is diagnostic of a male, intraspecific variation under the *DBY7* reverse primer could lead to a false-negative result, that is, a conclusion of female. To reduce this possibility, the intron primer was placed in a region that is conserved not only among wolf and dog, but also with another canid species, the coyote. Thirdly, the PCR fragments were designed to be a maximum of 250 bp for efficient amplification of low copy number samples or samples with degraded DNA. Fourthly, because the X-chromosome locus acts as the control for the PCR reaction, it should be more difficult to amplify than the Y-chromosome locus. As smaller fragments are more readily amplified in a PCR, the X-chromosome locus was chosen as the longer PCR product.

PCR conditions for the multiplexed amplification of the *DBX6* and *DBY7* fragments are as follows: 1x Hot Star buffer (Qiagen), 0.2 mM dNTPs, 0.3 μM X primers (DBX6B and DBX6Iv), 0.2 μM Y primers (DBY7A and DBY7Iv), 2.25 mM $MgCl_2$, 0.025 units/μl Hot StarTaq DNA polymerase (Qiagen) and approximately 10 ng DNA or 2 μl faeces extraction. The PCR program had an initial denaturation of 95 °C for 15 min, 20 cycles of 95 °C for 30 sec, 60 °C for 40 sec (decreasing 0.5 °C per cycle) and 72 °C for 1 min, followed by 20 cycles of 95 °C for 30 s, 50 °C for 40 sec and 72 °C for 1 min and a final extension of 72 °C for 15 min. Following amplification, 5–10 μl of PCR product is electrophoresed together with a 100-bp ladder on a 2% agarose gel. PCR products were visualised by ethidium bromide staining. The multiplex PCR reaction amplifies a 249-bp fragment of *DBX6* and a 118-bp fragment of *DBY7*. When tested with wolf tissue samples of known sex, the amplification consistently gives two fragments in a male and one fragment in a female.

Amongst a batch of faeces samples, sex was determined in 15 of 17 samples (nine male, six female) for which microsatellite genotyping was also successful. There was amplification of only the Y fragment during PCR in at least one of three replicates for seven of the males. This indicates that the PCR conditions favours the Y fragment when present, which is the preferred balance for a conservative sexing procedure.

The sexing primers are canid-specific, providing amplification products in wolves (*C. lupus*), coyotes (*C. latrans*) and dogs (*C. familiaris*). However, no amplication was obtained when tested on hare (*Lepus europaeus*), deer (*Capreolus capreolus*) and lynx (*Lynx lynx*). The absence of amplification reduces false positives from prey DNA co-isolated

from wolf faeces, for misidentified samples and from handler contamination of low-copy number samples. Therefore, these primers allow the accurate sexing of canid samples using a method that can easily be applied to give specificity to sexing protocols in any mammalian species.

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