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TECHNICAL NOTE

Molecular sexing eutherian mammals

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Abstract

Mammals can be molecular sexed by polymerase chain reaction (PCR) amplification of Y chromosome fragments or coamplification of homologous fragments from both sex chromosomes, which are discriminated by size polymorphism or Y-specific restriction digestion. Although coamplification of X and Y fragments is more reliable, size polymorphism in homologous fragments is uncommon and Y-specific restriction site identification requires screening with a battery of enzymes or cloning. Here we describe a simple approach, using 'double peaks' in the chromatogram upon direct sequencing of PCR products from males, to identify Y-specific restriction sites, and demonstrate its utility by application to a range of taxa.

Keywords: molecular sexing, sex identification, ZFX, ZFY

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Molecular sexing has found wide application in a number of fields including forensics (Kobayashi *et al.* 1988), archaeology (Faerman *et al.* 1995), animal husbandry (Kamimura *et al.* 1997), field biology (Palsbøll *et al.* 1992) and conservation (Griffiths & Tiwari 1995). Two approaches using polymerase chain reaction (PCR) have been used for molecular sexing mammals: (i) amplification of fragments specific to the Y chromosome; and (ii) amplification of homologous fragments from both X and Y chromosomes (Sullivan *et al.* 1993; Richard *et al.* 1994). Amplification of Y-specific fragments has been based on the Y chromosomal SRY gene, with positive amplification indicating male identity (Palsbøll *et al.* 1992; Kamimura *et al.* 1997). However, nonamplification of the target fragment does not equate with female identity as PCR amplification may fail for many reasons. Coamplification of a mitochondrial or single copy nuclear gene fragment has been used as an external control to address this problem (Palsbøll *et al.* 1992; Kamimura *et al.* 1997). However, the sensitivity and optimal conditions of different primer sets are unlikely to be identical and we have found external controls to be unreliable, especially when amplifying from suboptimal sources of DNA such as hair or faecal matter.

While amplification of X and Y homologous fragments with a single primer set should be more reliable, discrimination of the X and Y fragments requires the presence of a size polymorphism (Bailey *et al.* 1992) or a Y fragment unique restriction site (Aasen & Medrano 1990). Amelogenin and zinc finger protein (ZF) genes, both of which have copies on the X and Y chromosomes, have been used in this approach. Size polymorphism in amplified X and Y fragments from the Amelogenin gene have been used to sex primates (Bailey *et al.* 1992), but it was found to be nonapplicable to other taxa (Buel *et al.* 1995). Restriction fragment length polymorphism (RFLP) analysis of ZFX-ZFY gene fragments using the enzyme *TaqI* has been successfully applied for molecular sexing of a number of taxa including humans and ungulates (Aasen & Medrano 1990), cetaceans (Palsbøll *et al.* 1992) and canids (Garcia-Muro *et al.* 1997). RFLP analysis to differentiate X and Y fragments requires the use of Y-specific restriction sites to prevent false identification from incomplete digestion. While the highly conserved nature of the ZF sequence enables the use of 'universal' primers, the restriction sites within this sequence are not universal (Aasen & Medrano 1990; Palsbøll *et al.* 1992; present study). Therefore, to adapt this system to a new species, Y-specific restriction sites that differentiate the X and Y fragments of that species have to be identified, requiring either blind screening of amplified PCR products with a battery of restriction enzymes, or cloning and sequencing of the X and Y fragments.

Here we describe a simple method that identifies positions polymorphic between X and Y sequences based on

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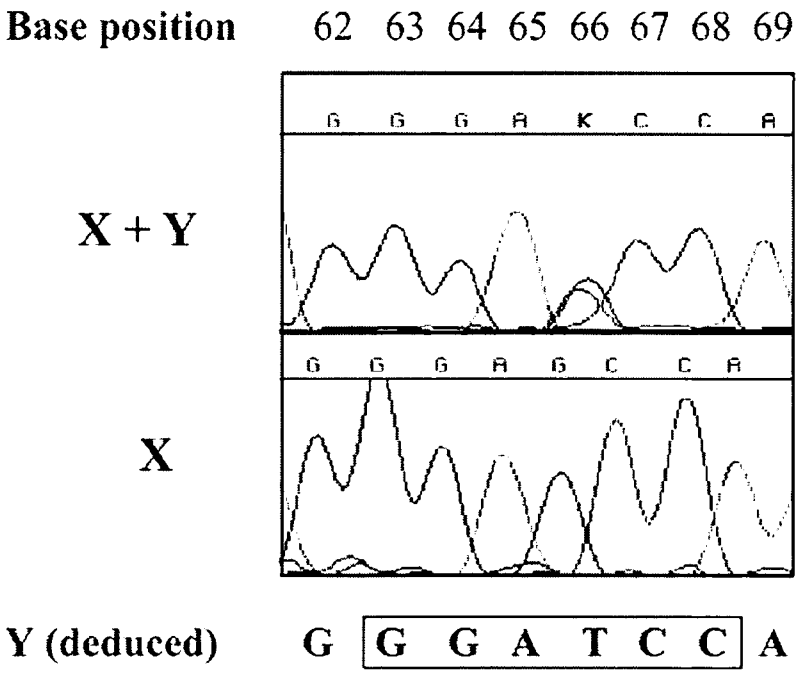

Fig. 1 Chromatograms of ZF fragment amplifications of male (upper panel) and female (lower panel) elephant DNA, demonstrating a double peak at position 66 of the Y sequence which creates a restriction site for the enzyme *Bam*HI. The deduced sequence of the Y fragment is given below the figure with the recognition sequence for *Bam*HI boxed.

the presence of double peaks in the chromatogram upon direct sequencing of PCR products from a male, enabling the recognition of unique restriction sites on the Y fragment. We demonstrate its utility by application to a range of mammalian taxa; Probocideans: Asian elephant (*Elephas maximus*), Perrisodactyls: Indian rhinoceros (*Rhinoceros unicornis*), Carnivores: domestic dog (*Canis familiaris*) and Primates: Tonkean macaque (*Macaca tonkeana*).

DNA was obtained from blood samples of four individuals (two males, two females) each, of Indian rhinoceros, domestic dogs, Tonkean macaques and Asian elephants, using a standard phenol extraction protocol (Sambrook *et al.* 1989). PCR amplification of ZFX-ZFY fragments was performed using primers P1-5EZ: 5′-ATAATCACATGGAGAGCCACAAGCT-3′ and P2-3EZ: 5′-GCACTTCTTTGGTATCTGAGAAAGT-3′ (Aasen & Medrano 1990). Reactions were run in a Perkin-Elmer 9700 thermocycler, using 1 μL DNA extract, 2 μL 100 mg/mL BSA, 2.5 μL 10× PCR buffer (100 mM Tris-HCl pH 8.4, 500 mM KCl, 15 mM $MgCl_2$), 2.5 μL 8 mM dNTP mix, 0.5 μL 10 μM primers, 0.1 μL *Taq* DNA polymerase (Perkin-Elmer), and 15.9 μL water. Samples were amplified for 35 cycles by denaturing at 93 °C for 1 min, annealing at 53 °C for 30 s and extension at 72 °C for 1 min. PCR cycles were preceded by a 93 °C, 3 min incubation step and followed by a 72 °C, 15 min extension step. PCR products were purified using a QIAGEN PCR purification kit and the manufacturer's protocol, and sequenced in forward and reverse directions using primers ZFSEQ: 5′-ATGTCACACTTGAATGGCATC-3′ and P2-3EZ. Sequences were analysed in an ABI 377 automated sequencer, and aligned and edited using the program SEQUENCHER (Gene Codes Corporation).

PCR amplification was successful for all taxa. Three hundred and forty bp of the amplified fragments were used in the analysis (GenBank accession nos AF393751–AF393758). As both the X and Y chromosome fragments were amplified from male nuclear DNA, positions polymorphic between X and Y sequences were represented by double peaks in the chromatograms of both forward and reverse sequences. Comparison of female (which have only the X copy) and male sequences, enabled us to deduce the sequence of the Y fragment (Fig. 1) using the 'call secondary peaks' feature of the program SEQUENCHER and setting the minimum lower peak height at 75% of the upper. The program SEQUENCHER was used to identify restriction sites on the X and Y fragments and enzymes that digested the Y but not the X fragment were selected for each species (Table 1).

Table 1 Restriction enzymes selected for restriction digestion of Y fragments. Restriction positions are numbered with reference to the 1st base of the segment used for analysis

Species	Restriction enzyme	Position of restriction site
Asian elephant	*Bam*HI	63, 226
Indian rhinoceros	*Nsi*I	79
Domestic dog	*Mse*I	307
Tonkean macaque	*Hae*III	66

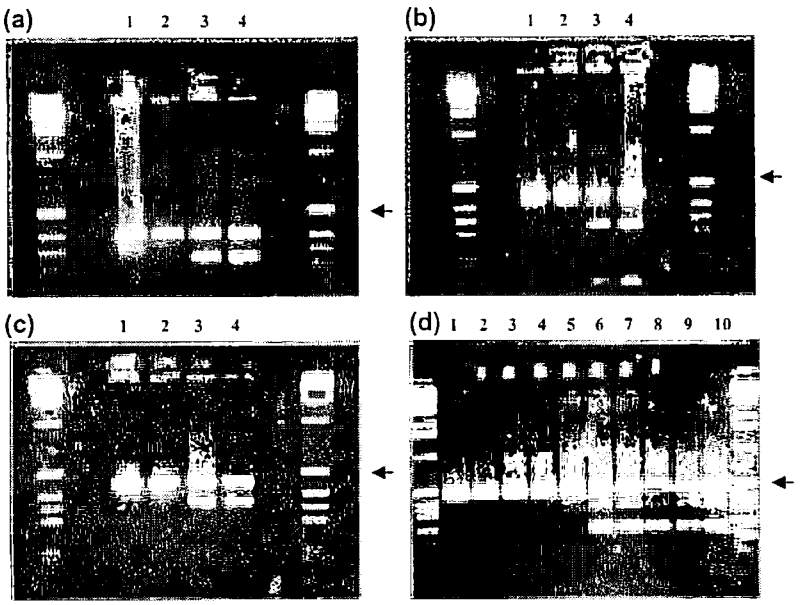

Fig. 2 Band patterns from PCR/RFLP analysis of male and female DNA. Arrows indicate the position of the 500 bp fragment of a 1 kb DNA ladder. The numbers above the figures denote lanes. (a) domestic dog; (b) Indian rhinoceros; (c) Tonkean macaque; (d) Asian elephant. Female samples: lanes 1 and 2 in (a, b, c) and 1–5 in (d). Male samples: lanes 3 and 4 in (a, b, c) and 6–10 in (d).

DNA from four individuals each (two males, two females) of Indian rhinoceros, domestic dogs and Tonkean macaques, and 10 individuals (five males, five females) of Asian elephants, was PCR amplified and products digested with 5 units of the relevant restriction enzyme following the manufacturer's protocol. Restriction fragments were electrophoresed in ethidium bromide stained 2% agarose gels and visualized with UV light. Restriction digestion and gel electrophoresis of PCR products demonstrated a single band for females and multiple bands for males (Fig. 2), allowing us to unambiguously and accurately assign sex to all individuals of all four species. The absence of intra-species polymorphism within either the X or the Y sequence in individuals we analysed indicates high intra-species conservation of the target fragment. Therefore, the probability of false identification due to an individual having a polymorphism involving the restriction site is sufficiently remote to be discounted.

The method outlined in our study enables easy and quick identification of restriction sites that enable discrimination of equal sized X and Y homologous fragments amplified with a single set of primers. As homologous segments of the ZF gene reside on the X and Y chromosomes of all eutherian mammals (Page *et al.* 1987), this system should be immediately applicable to a wide range of species. The same approach could also be applied to other genes such as Amelogenin, that have copies on both X and Y chromosomes.

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