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A NEW MARKER FOR RAPID SEX IDENTIFICATION OF RED DEER (*CERVUS ELAPHUS*)

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RIASSUNTO - ***Un nuovo marcatore per l'identificazione rapida del sesso del cervo* (Cervus elaphus)**. Il cervo (*Cervus elaphus*) è una specie cacciabile economicamente importante che risente di alcuni problemi di conservazione. La conoscenza di parametri di popolazione come il rapporto sessi è importante per la sua gestione e conservazione. In questo studio, utilizzando condizioni poco specifiche per l'amplificazione di un frammento del cromosoma Y già descritto in precedenza, descriviamo un metodo che permette una rapida e corretta identificazione del sesso in questa specie, producendo due *amplicons* diversi nei maschi e uno nelle femmine. I due frammenti, diversi per il peso molecolare, sono facilmente separabili mediante elettroforesi in gel di agarosio, permettendo l'identificazione del sesso con un unico test in campioni biologici come carcasse, embrioni recuperati da femmine abbattute e, forse, peli o escrementi (se la qualità del DNA è accettabile). Questo metodo può essere di aiuto in studi sul rapporto sessi degli embrioni, sulla mortalità associata al sesso o sul rapporto sessi in popolazioni selvatiche o semi-selvatiche, con ricadute applicative per le analisi demografiche, la gestione venatoria e la conservazione.

Parole chiave: *Cervus elaphus*, identificazione del sesso, marcatore sessuale, cromosoma Y, gestione venatoria

The red deer (*Cervus elaphus*) is a big game species with considerable economical value. It is widely distributed across the northern hemisphere and is therefore listed as Least Concern by the International Union for Conservation of Nature, although it has suffered range contractions, population fragmentations and local extinctions (Lovari *et al*., 2008). The main threats to this species are translocations (with the consequent disease spreading and subspecies intermixing), habitat loss, and over-hunting in some areas (Lovari *et al*., 2008).

The knowledge of population parameters such as sex ratios is an important basis for the management and conservation of this species. However, for deer populations living in the wild or in extensive game estates, different detectability of males and females hampers the direct estimation of the sex ratio. The sex of embryos is also

sometimes hard to determine visually. Genetic markers are thus useful for sex recognition on biological samples such as flesh from carcasses, embryos recovered from culled females, and possibly hair and faeces (where DNA quality is sufficient), with important applications in demographic and evolutionary studies and in management decisions (see, for example, Carranza *et al*., 2009).

Many mammal sex markers amplify Y-chromosome-specific sequences such as SRY (e.g. Matsubara *et al*., 2001), but this normally implies that females are identified by absence of amplification. Such an outcome is not conclusive, as it can be the result of experimental errors or PCR (polymerase chain reaction) failure caused, for example, by low DNA quality. For this reason, these sex identification tests have sometimes been complemented with the addition of a second pair of primers to the reaction, to amplify a non-sex-linked marker, as an internal positive control for DNA quality (Takahashi *et al*., 1998; Dallas *et al*., 2000). However, this increases costs, requires primer pairs with similar optimal conditions, and the amplification of the additional marker still does not guarantee the successful amplification of an eventually present Y-chromosomal sequence. Another approach consists in a two-step procedure, with co-amplification of a homologous X and Y locus followed by digestion with restriction enzymes to detect diagnostic polymerphism (García-Muro *et al*., 1997), but this procedure demands even more investment and expertise.

Simple one-reaction sex recognition has become popular with the amplification, using the same primer pair, of different-length homologue regions in the X and Y chromosomes (e.g. Pilgrim *et al.*, 2005). Male and female amplicons can thus be distinguished through agarose gel electrophoresis, although sometimes their sequences are too long to assure effective amplification in degraded DNA samples, such as those recovered from faeces or decomposing carcasses. The method is not infallible either, as mutations can result in incorrect sex assignment (Shadrach *et al*., 2004). Such sex markers have been described for mammals in general (Shaw *et al*., 2003, using primers previously publicshed by Cathey *et al*., 1998) and for red deer in particular (Pfeiffer and Brenig, 2005, with primers previously described by Ennis and Gallagher, 1994). Here we report the finding of another single-step sex marker for red deer, based on an exonic primer pair for an intron of the DBY gene, which Hellborg and Ellegren (2003) designed for a different purpose.

We extracted DNA through a salt-out procedure (Miller *et al*., 1988) from either flesh or antlers of red deer, and amplified it by PCR using Hellborg and Ellegren's (2003) DBY8 primers: CCCCAACAAGAGAATTGGCT (forward) and CAGCACCACCATAKACTACA (reverse). These primers were originally designed to bind only to Y-chromosomal sequences, and not to any homologous regions in other chromosomes. Indeed, using the conditions described by Hellborg and Ellegren (2003), only the targeted 200-bp Y-chromosomal fragment was amplified in males, and no PCR product was obtained from control females. However, we lowered the specificity of these PCR conditions by changing the reaction profile to one cycle of 95°C for 10 min; 20 cycles of 95°C for 30 s and a touchdown from 60°C to 50°C for 1 min decreasing by 0.5°C/cycle; 72°C for 1.50 min; then 30 (rather than 20) cycles of 95°C for 30 s, 50°C for 1 min and 72°C for 1.50 min; and a final extension step of 72°C for 10 min. This decrease in PCR stringency caused a second clearly larger fragment (≈350 bp) to be amplified, this one appearing also, and with higher yield, in females (Figure 1). This female fragment was purified with ExoSAP-IT (GE Healthcare) and sequenced in both dir-

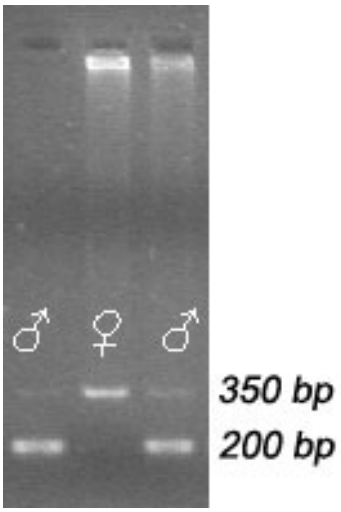

Figure 1 - Male and female products of DBY8 low-stringent amplification electrophoresed on agarose gel. Two fragments are amplified in males and one in females.

ections using the PCR primers and a BigDye Terminator v1.1 Cycle Sequencing kit (Applied BioSystems). The nucleotide sequence was recorded with an ABI3130 automated sequencer and included in GenBank (accession number EU219378).

Both amplified fragments were easily separated through electrophoresis on a 1.6% agarose gel stained with SYBR safe (Invitrogen, Barcelona) and visualized under ultraviolet light (Figure 1). The accuracy of the sexing method was confirmed by a blind test on biological samples of 30 red deer individuals (including both embryos and adults) whose sexes had been identified anatomically. The correct gender was plainly assigned in all cases. Low-stringent PCR of DBY8 thus allows quick and accurate sex identification in European red deer, with only one primer pair and a single one-step reaction. Regarding some sex markers previously described for this species (e.g. Pfeiffer and Brenig, 2005), this one has the advantage of yielding fragments with a greater difference in size, making electrophoretic separation easier, faster, and cheaper.

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