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TECHNICAL NOTE

A reliable, single-step method for gender determination in black rhinoceros from low-copy template DNA

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Abstract We describe an accurate, single-step 5′exonuclease assay for gender determination of black rhinoceros *Diceros bicornis*, which targets a single nucleotide polymorphism in exon 4 of the sex-linked zinc finger homologues in this species. This simple, high-throughput method facilitates the multiple-tubes approach that is necessary when using genetic material derived from non-invasive samples such as faecal material.

Keywords Black rhinoceros · Gender determination · Non-invasive sampling · 5′exonuclease assay · ZFX/ZFY

The black rhinoceros (*Diceros bicornis*) has experienced one of the most dramatic declines of any large vertebrate. Extant populations are small and fragmented and will require translocations between populations to maintain diversity (Emslie and Brooks 1999). A minimum requirement for effective metapopulation strategies is baseline information on population size and sex ratios, which for some populations of black rhinoceros are absent due to the species' cryptic nature and the dense vegetation in areas they inhabit (Mulama and Okita 2002). Molecular identification and sex determination from non-invasive samples, such as DNA derived from faecal material, is potentially a key aspect to this species' conservation management (Foose 2001; Okita-Ouma et al. 2007). However the phenomena of allelic dropout associated with genotyping low-copy, poor-quality DNA, typical of that extracted from faeces, necessitates a "multiple tubes" approach, whereby the same sample is genotyped multiple times (Taberlet et al. 1999); this is particularly important for molecular sexing where the failure to detect a Y-chromosome marker in males will lead to incorrect gender assignment (Broquet and Petit 2004). Thus, an inexpensive, high-throughput and yet accurate method for sex determination of black rhinoceros from faecal DNA is desirable. An existing two-step STR profiling approach for molecular sex determination in the black rhinoceros could be bettered if it was a single-step approach which reduced not only the time and cost of gender profiling, but also the chances of contamination (Peppin et al. 2010). We report a 5′exonuclease assay that utilizes TaqMan® probes to detect a single nucleotide polymorphism (SNP) that distinguishes the sex-linked zinc finger homologues (ZFX/Y) in the black rhinoceros.

Tissue and faecal samples of wild black rhinoceros of known sex were collected from 36 animals from 6 populations across Kenya, with tissue samples from six males and six females used to provide material to obtain a 604 bp sequence from exon 4 of the ZFX and ZFY homologues. DNA extractions from tissue were performed using the Qiagen DNeasy Blood anTissue Kit (Qiagen), with DNA extracted from faecal samples using a QIAamp® DNA Stool Mini Kit (Qiagen).

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ZFX and ZFY multiplex PCRs were undertaken in 50 µl volume containing 1× AccuPrime™ *Pfx* buffer (Invitrogen), 100 µM each dNTP, 0.5 µM of ZFY0097 [5′-CATC CTTTGACTGTCTATCCTTG-3′ (Palsbøll et al. 1992)] and P2-3EZ [5′-GCACTTCTTTGGTATCTGAGAAAGT-3′ (Aasen and Medrano 1990)], 1.5 µ AccuPrime™ *Pfx* DNA polymerase (Invitrogen), 0.1 µg/µl BSA and 50 ng template DNA. Thermal cycling conditions were: 94 °C for 45 s, followed by 25 cycles of 94 °C for 45 s, 59 °C for 45 s, 72 °C for 1 min 15 s, and 72 °C for 10 min on a MJ Research PTC-200 thermal cycler. PCR amplicons were purified using QIAquick PCR purification kit (Qiagen) and cloned using TOPO® TA Cloning Kit®(Invitrogen); recombinant plasmids were isolated, purified and then sequenced using BigDye Terminator v.3.1 chemistry (Applied Biosystems) and electrophoresis on an ABI3100 (Applied Biosystems). All sequences were aligned and the plasmid DNA excised in silico using Geneious Pro v.4.04.

The 604 bp ZFX sequences (Genbank accession: EU284593) were invariant for males and females, and ZFY sequences (Genbank accession: EU284594) were invariant for males. Gene identity was checked by aligning with orthologous sequences for *Homo sapiens* (Genbank accession: M26946, J03134). Primers and probes for the 5′ exonuclease assay were designed according to published guidelines (Morin et al. 1999; Smith et al. 2002). The assay targeted a 94-bp region of the ZFX-ZFY sequences with a synonymous nucleotide difference (C to T) at nucleotide 402. The forward primer (ZFXY_Rhino_12F) was 5′-GTC CACAGCAAGAACTTTCCTCATA-3′ and the reverse primer (ZFXY_Rhino_12R) was 5′-CAGTATGGATTCG-CATGTGCTTTT-3′. Probes were synthesised with a 5′ reporter dye (FAM for ZFX_Rhino; HEX for ZFY_Rhino) and a 3′ quencher (NFQ). The probe sequences were (ZFX_Rhino) 5′-AAACCTTTACCACACTCC-3′ and (ZFY_Rhino) 5′-ACCTTTACCGCACTCC-3′ (Fig. 1). The 5′exonuclease assays were performed in 25 µl reactions containing 1–20 ng of template DNA, 1× PCR buffer (Qiagen), 200 µM each dNTP, 1.25 µ HotStar*Taq* polymerase (Qiagen), 0.4 µg/µl BSA and 1× TaqMan Assay reaction mix (Applied Biosystems). Amplifications were performed in a MX3000P real-time PCR system (Stratagene) with an initial incubation of 15 min at 95 °C followed by 50 cycles of 95 °C for 20 s and 60 °C for 1 min 30 s. The assay was validated using DNA extracted from tissue samples of 8 confirmed males and 8 confirmed females and then tested for accuracy in assigning gender from faecal DNA from another 12 males and 12 females, with the latter (faecal) samples genotyped twice (i.e. a limited multiple-tubes approach). These validations were based on different animals from Kenya than those used to design the assay (Fig. 2).

The 5′exonuclease assay method proved capable of reliable sex-determination in black rhinoceros, with a 100 % PCR success and 100 % correct gender assignment

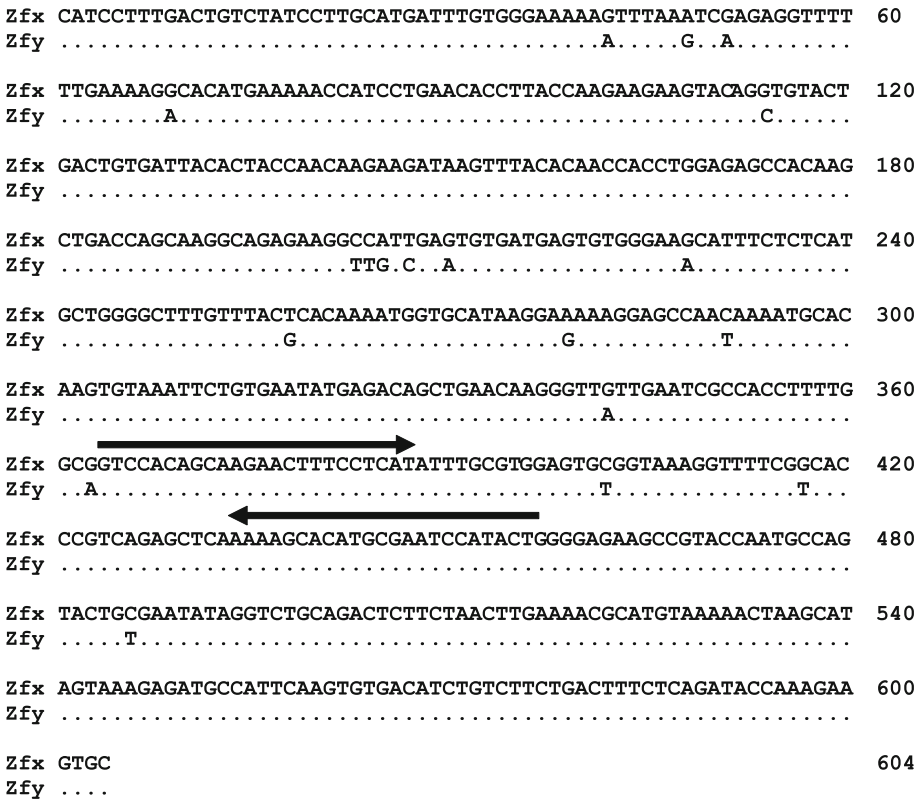

Fig. 1 Alignment of exon 4 of ZFX and ZFY of black rhinoceros detailing the region amplified using primers ZFXY_Rhino_12F (*solid arrow*) and ZFXY_Rhino_12R (*dashed arrow*). The *grey shaded* regions show the binding sites of the sex-specific Taqman® probes; and the relevant synonymous nucleotide difference is shown boxed

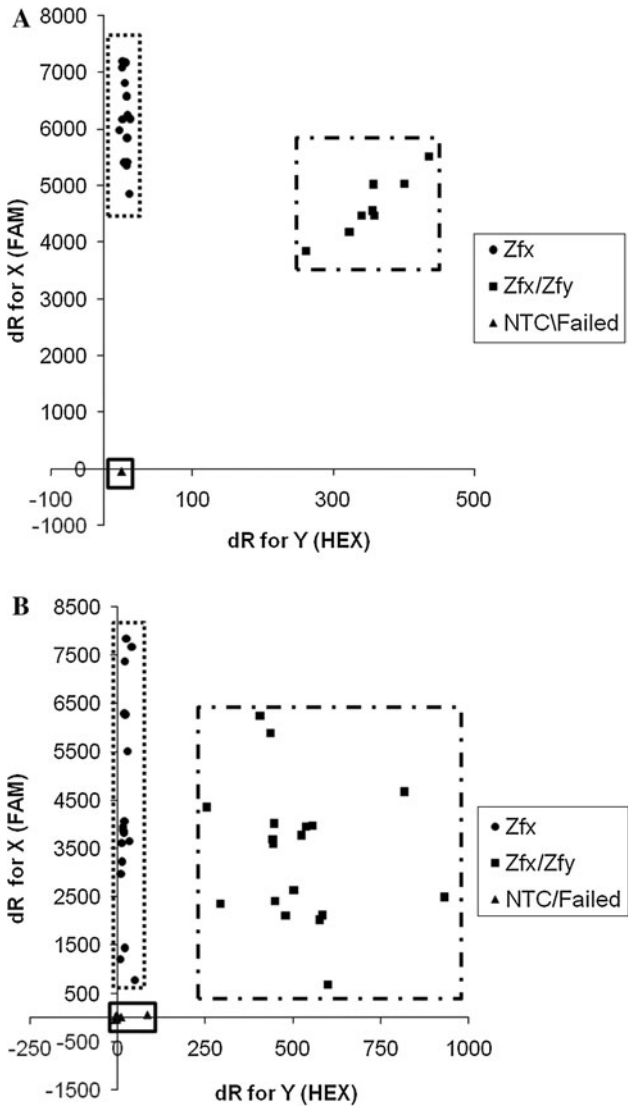

Fig. 2 Validation of molecular sexing for black rhinoceros. Plots of fluorescence values (dR) using ZFX and ZFY TaqMan™ probes and DNA derived from either **a** tissue from 8 males and 8 females or **b** faecal material from 12 males and 12 females with each reaction performed in duplicate. ZFX = female. ZFX/ZFY = male. NTC/failed = negative control or failed reaction

for all assays based on tissue samples, and ~87.5 % (42/48) PCR success rate using faecal DNA. In the 6 instances of PCR failure using DNA derived from faecal material, the multiple-tubes approach nonetheless ensured correct gender determination for all animals accept one (~4.2 %), which failed to amplify for either probe in both reactions. The extremely sensitive automated method we report has the combined benefits of facilitating a large number of replicates to be performed very easily at comparatively low cost, with an output which is proportional to PCR product amplification and clearly defined clusters according to gender (Morin et al. 2005).

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