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TECHNICAL NOTE

Sex identification of the extant mega mammal, the lowland tapir, *Tapirus terrestris* (Tapiridae, Mammalia), by means of molecular markers: new outlook for non-invasive samples

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Abstract The knowledge of the sex ratio of threatened populations or species is pivotal since sub-optimal sex ratios can affect negatively the population growth and resilience. The vulnerable species, *Tapirus terrestris*, is rarely studied through traditional field methods and non-invasive genetic analyses have been employed using feces as source of DNA. Here, we evaluated two molecular markers for sex identification of tapirs. Amelogenin gene (AMEL) which is shorter in the Y chromosome (−20pb), failed in the determination of the sex of tapirs, even after the design of specific primers for the species. SRY marker, co-amplified with zinc-finger gene (ZF), had successful produced the sex identity for blood and scat samples. From a total of 45 feaces, 29 had the sex identified after three PCRS (64.4%). In this way, these molecular markers are reliable tools for sex ratio determination using non-invasive samples of natural populations of this tapirs.

Keywords Amelogenin · Tapir · Sexing · SRY · ZFX/ZFY

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The knowledge of the sex ratio of threatened populations or species is pivotal since sub-optimal sex ratios can affect negatively the population growth and resilience (Peppin et al. 2010). The lowland tapir, *Tapirus terrestris* (Linnaeus 1758), is a vulnerable species, whose populations have been reduced due to habitat destruction and poaching (Cullen et al. 2000; Sanches et al. 2011). Tapirs are rarely studied through traditional field methods, and non-invasive genetic analyses have been employed using feces as source of DNA and microsatellites for the individual identification (Pinho et al. 2014). In this study we evaluated the success of two molecular markers for sex identification of tapirs, using both invasive and non-invasive samples.

The amelogenin (AMEL) gene discriminates the sex by size polymorphism, since it is shorter (−20pb) in the Y-chromosome (Peppin et al. 2010). Another class of sexing molecular marker is based on Y-chromosome specific genes, such as SRY gene, that is amplified simultaneously with other nuclear or mitochondrial gene as positive control for female identification (Pagès et al. 2009). The present experiment was initiated by testing the molecular markers repeatability using DNA from blood samples. Only those that amplified properly were tested with faecal DNA. In non-invasive genetic studies, where the quantity and quality of DNA are poor, it is often necessary to replicate individual PCRs (Wayne and Morin 2004) thereby consuming too much time and money. Therefore, our main goal was to identify a cheaper and faster method of sexing using electrophoresis in agarose gel.

DNA was extracted from stored blood samples of males and females (N = 10, 5 females and 4 males), using a

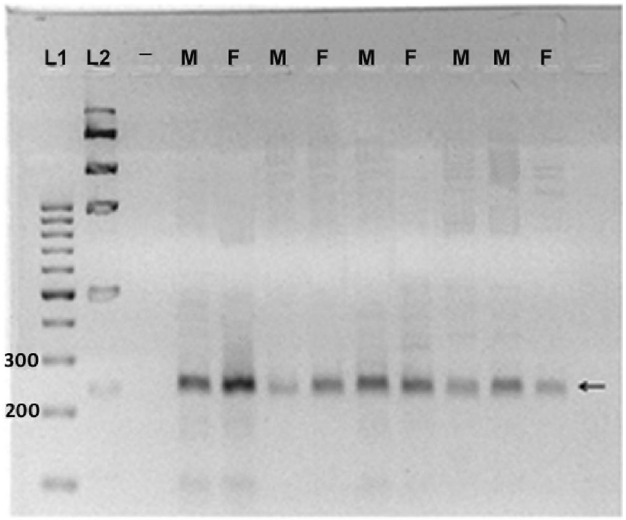

Fig. 1 Agarose gel 2,0 %, after electrophoresis at 80 V and three hours showing the result of the amplification of amelogenin gene in blood samples of *T. terrestris* using the specific primers Ttam. The *arrow* indicate the amplified fragments. *L1* 100 bp ladder, *L2* 1 Kb ladder, – negative control, *F* female, *M* male

non-enzymatic method (Lahiri and Nurnberger 1991). DNA extractions from 45 tapir faeces were performed using the "QIAmp DNA Stool Mini Kit" (Quiagen). The amelogenin sexing system was firstly tested using the KY1/KY2 (Yamauchi et al. 2000) and SE47/SE48 (Ennis and Gallagher 1994) primer pairs, which were developed for other ungulate species. These molecular markers were chosen because they produce short fragments between 165 and 280 bp, which makes them ideal for use on non-invasive DNA analysis. PCRs were carried out as described by the authors and, when required, some variations such as the concentrations of $MgCl_2$ (1.5–3 mM) and the annealing temperatures (52–66 °C) were tested.

The SE47/SE48 primer pair was discarded since it failed at all for any of the tested annealing temperatures, concentrations of $MgCl_2$ and blood DNA samples. The KY1/KY2 primer pair produced inconsistent amplification patterns, but sporadically the expected X-linked fragment (~170 bp) was amplified in some blood DNA samples and then sequenced (Genbank accession number KX786248). BLAST analysis showed high similarity with *T. terrestris* amelogenin X-linked (accession no. EU168871.1). Based on the obtained sequence and its alignment with *T. terrestris* amelogenin gene (EU168871.1), a third primer pair (TtamF, 5′-CAACACCACCAGCCACACTT-3′; TtamR, 5′-CAGG TCAGGAAGCAGAGGTG-3′) was designed using Primer 3 (Untergasser et al. 2012; Koressaar and Remm 2007) for the sexing tests. PCRs were performed in a final volume of 10 µL containing 50 ng of DNA, 1× PCR buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl), 3 mM $MgCl_2$, 0.3 mM dNTPs, 5 pmol of each primer, 0.5U Taq DNA polymerase. Thermal cycling conditions were as follow: 94 °C/4 min, 35 cycles at 94 °C-30 s/56 °C-30 s/72 °C-45 s, and a final extension at 72 °C/10 min. Although, Ttam primers successful amplified an X-linked fragment (219 bp), which could be observed in a 2 % agarose gel, after electrophoresis for 3 h at 90 V (Fig. 1), the expected male pattern was still not obtained, even using blood DNA samples. All these results indicate that the expected deletion in the amelogenin gene on the Y-chromosome of *T. terrestris* is absent or it is too short to be plainly detected in agarose gels. The lack of length polymorphism between sex chromosomes has been documented for other mammal species (Fernando and Melnick 2001).

Testing SRY gene was conducted using simultaneous zinc finger gene (Zfx/Zfy) amplification as positive control (Pagès et al. 2009). We used the P1-5EZ/P2-3EZ primer pair (Aasen and Medrano 1990) for amplifying the zinc finger gene and Y53-3D/Y53-3C (Gilson et al. 1998) for the specific Y-chromosome SRY gene amplification. Firstly, PCR of blood DNA samples was performed in a final volume of 10 µL containing 50 ng of DNA, 1× PCR buffer,

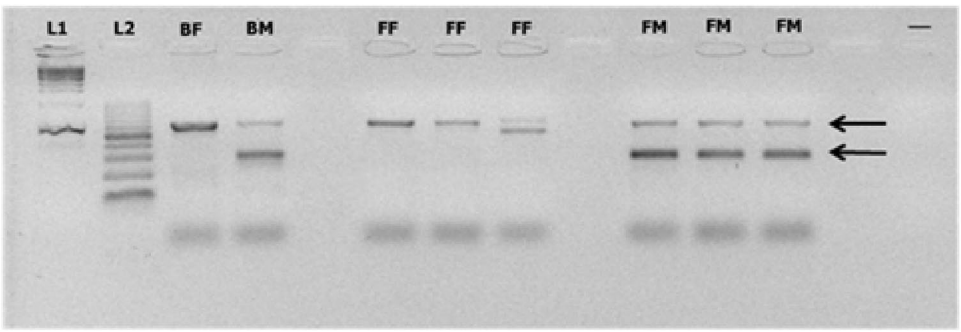

Fig. 2 Agarose gel 2,0 %, after electrophoresis at 80 V and 40 min showing the result of the amplification of Zfx/Zfy and SRY genes in samples of *T. terrestris* (2 females and 2 males). The *arrows* indicate the amplified fragments. *L1* 1Kb ladder, *L2* 100 bp ladder, – negative control, *BF* blood female, *BM* blood male, *FF* feces female (female, PCR replicate and DNA replicate, respectively), *FM* feces male (male, PCR replicate and DNA replicate, respectively)

5 mM $MgCl_2$, 0.03 mM dNTPs, 0.5 µg of BSA, 10 pmol of each primer, 0.5U Taq DNA polymerase (Invitrogen). The amplification program consisted of an initial denaturation at 94 °C/3 min, 35 cycles at 94 °C-45 s/60 °C-45 s/72 °C-1 mi n, and a final extension at 72 °C/10 min. The co-amplification of Zfx/Zfy and SRY produced band patterns expected for all tested blood samples of males and females.

Next, in order to evaluate the success of this method for lowland tapir population studies using non-invasive samples, 45 scat DNA samples were amplified. PCR of these samples was basically performed as above mentioned, but each reaction contained 4 µL of faecal DNA. All PCRs were performed at least three times for each faecal DNA sample. The results were also efficient for sexing in faecal samples, where two bands were observed for males and one for females (Fig. 2).

A total of 29 scat samples had the sex identified, presenting the same result in three independent PCRs (64.4 % of success), representing a good efficiency of sex identification using scat samples. The remaining samples presented no amplicon or inconsistence with no repeatability of the band pattern produced.

Knowledge about the sex ratio of natural populations is crucial for population monitoring and conservation plans. However, estimating the sex ratio of animals like tapirs represents a difficult task with traditional methods such as visual or camera trap observation. In this way, molecular markers using non-invasive samples are the only reliable tools for sex determination of natural populations of elusive species. The method using Zfx/Zfy and SRY genes co-amplification was efficient in determining the sex of *T. terrestris*, providing a potential tool for monitoring wild populations.

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