

Full Length Research Paper

Quantitative sexing (Q-sexing) technique for animal sex-determination based on X chromosome-linked loci: Empirical evidence from the Siberian tiger

Samer Alasaad^{1,2,3*}, Joerns Fickel³, Ramón C. Soriguer^{1,4}, Yury P. Sushitsky⁵ and Galina Chelomina²

¹Estación Biológica de Doñana, Consejo Superior de Investigaciones Científicas (CSIC),
Avda. Américo Vespucio s/n 41092 Sevilla, Spain.

²Institute of Biology and Soil Science, Far Eastern Branch of the Russian Academy of Sciences,
690022 Vladivostok, Russia.

³Research group Evolutionary Genetics, Leibniz-Institute for Zoo and Wildlife Research,
Alfred-Kowalke-Str. 17, D-10315 Berlin, Germany.

⁴Instituto de Investigación en Recursos Cinegéticos, IREC (CSIC-UCLM-JCCM), Ronda de Toledo s/n,
13005 Ciudad Real, Spain.

⁵Khankaisky State Biosphere Nature Reserve, Russia.

Accepted 26 September, 2012

Here we report a mammal sexing procedure based on the detection of quantitative differences between females and males in the X-linked loci (quantitative sexing, Q-sexing). This novel technique was validated using samples from Siberian tigers (*Panthera tigris altaica*) whose sexes were known. The Q-sexing technique relies on the fact that amplifications proceeding exclusively from the two X chromosomes in a female mammal should reach the threshold cycle (C_T) in a real-time quantitative real time polymerase chain reaction (qPCR) assay sooner than amplifications from the single male X chromosome. Nevertheless, given that the amplification efficiency may vary between samples, results have to be calibrated to a marker that does not vary in copy number between the sexes (for example, an autosomal-linked locus). For this purpose we used quantitative real time polymerase chain reaction (RT qPCR) assays to quantify the amount of three specific Siberian tiger microsatellite markers (X/Y- and autosomal-linked loci) in individual samples in order to determine the sex of an animal. A difference of one C_T between the X and the autosome-linked loci was detected in males, but no such difference was present in female samples. The Q-sexing technique unambiguously separates female from male Siberian tigers. The future of RT qPCR is bright as technology is becoming ever more rapid, cost-effective, easier to use and capable of processing higher throughputs. Thus, we expect that our novel technique for animal sexing will have a wide applicability, although further studies are still needed to adapt it to other animal species using specific primers.

Key words: Polymerase chain reaction (PCR), quantitative real time polymerase chain reaction (qPCR), quantitative sexing, Siberian tiger.

INTRODUCTION

Animal molecular sexing techniques are usually based on polymerase chain reaction (PCR) amplification of deoxyribonucleic acid (DNA) sequences from the Y

(mammal) or W (bird) chromosome-linked loci (Aasen and Medrano 1990; Longmire et al., 1993; Ellegren, 1996; Putze et al., 2007), with concomitant amplification of X or W, respectively. Alternatively, sexing techniques are based on differences in the PCR yielded fragment of the X-Y homologous gene amelogenin from the X and Y chromosome (Sullivan et al., 1993). Nevertheless, X and

*Corresponding author. E-mail: samer@ebd.csic.es.

Z chromosome-linked loci are not usually used as stand-alone techniques for animal sex determination (Dubiec and Zagalska-Neubauer, 2006).

Sexing technique methods are usually based either on the presence or absence of PCR amplification products, on the differences in band lengths and/or the resulting banding patterns on gel, and/or on differences in sequences ('qualitative sexing'), which enable researchers to distinguish between X and Y chromosomes (mammals) or W and Z chromosomes (birds) [restriction fragment length polymorphisms (RFLPs, Sacchi et al., 2004), amplified fragment length polymorphism (AFLPs, Griffiths and Orr, 1999), single strand conformation polymorphism (SSCPs, Ramos et al., 2009), microsatellite alleles (Nesje and Roed, 2000) and oligonucleotide-microarrays: Wang et al., 2008; Kalz et al., 2006)].

X and Z chromosome-linked loci have not yet been used alone for animal sex determination since they are present in both genders in mammals and birds (females: XX or WZ; males: XY or ZZ). In light of the advances in real time qPCR techniques and the increased affordability of RT qPCR instruments, our aim was to develop a method that would apply this speedy approach to the sexing of mammal (and avian) species. Our prediction was that a RT qPCR addressed to mammalian X-chromosomal genes/segments should yield a lower C_T value (the cycle at which the fluorescence exceeds a predetermined threshold level) in females than in males given that females have twice as much template of these genes/segments as males. However, as amplification efficiency can vary among samples, results should be calibrated to a marker that does not vary in copy number between the sexes (autosomal-linked loci).

A similar assumption can be applied to birds since markers from male birds (ZZ chromosomes) should reach the threshold sooner than those of females (WZ chromosomes). Using the differences in their quantity in females and males, X/Z chromosome-linked loci could be used for animal sex determination. The applicability of this technique, for which we have coined the term 'quantitative sexing' or 'Q-sexing, was tested using samples from the threatened Siberian tiger (*Panthera tigris altaica*).

MATERIALS AND METHODS

Blood or tissue samples from 15 Siberian tigers (five females and 10 males) were collected from wild-born animals throughout its distribution range in the Primorsky region of the Russian Far East between 1999 and 2000 (Alasaad et al., 2011b). The DNA was extracted following the standard phenol/chloroform procedure (Sambrook et al., 1989).

We used the following sets of primers: (i) FCA651F and FCA651R, (ii) FCA139F and FCA139R (Menotti-Raymond et al., 2003) and (iii) DBY7-PF and DBY7-PR (Rozhnov et al., 2009). FCA651 targets an X chromosome-linked microsatellite and gives amplicons with lengths of 133 and 135 bp; DBY7 targets an Y chromosome-linked microsatellite, generating amplicons of 156 bp

length (this primer set was added to corroborate our assumption); and FCA139 targets an autosomally linked microsatellite with amplicon lengths of 132 and/or 134 bp. DNA-extracts were amplified in duplicates and control samples (DNA of one male Siberian tiger) as well as RT qPCR blanks (no template control, NTC) were added in triplicates in all assays. Each run contained two replicates per sample of the three microsatellite markers. All RT qPCR assays were repeated twice. All runs were carried out in an Mx-3005P cycler (Stratagene). Data were analysed with the software package MxPro v4.00 (Stratagene).

Amplification reactions (20 μ l) contained 300 nM of each primer per pair, 1x GoTaq® qPCR Master Mix (Promega), 2 μ l (between 50 and 100 ng/ μ l) of DNA template (replaced by water in NTCs) and nuclease free-water. Cycling conditions for the 2-step PCR consisted of a 2 min start-up denaturation step at 95°C, followed by 40 cycles of denaturation for 15 s at 95°C and 60 s amplification at 58°C. In order to check for primer dimers and other PCR artefacts, a final dissociation analysis was performed with 10 s at 95°C followed by 5 s at 65°C and an incremental increase of 0.1°C/s to 95°C.

PCR efficiency was considered and tested during primer selection and in all reactions lay between 90 and 110% (software statistics). To perform relative quantification, the delta-delta-threshold-cycle method ($\Delta\Delta C_T$) was used (Livak and Schmittgen 2001). Reference ranges (2 x standard deviation, SD) for X-, Y- and autosomally linked $2^{\Delta\Delta C_T}$ values from females and males were tested using χ^2 tests ($P < 0.05$).

RESULTS AND DISCUSSION

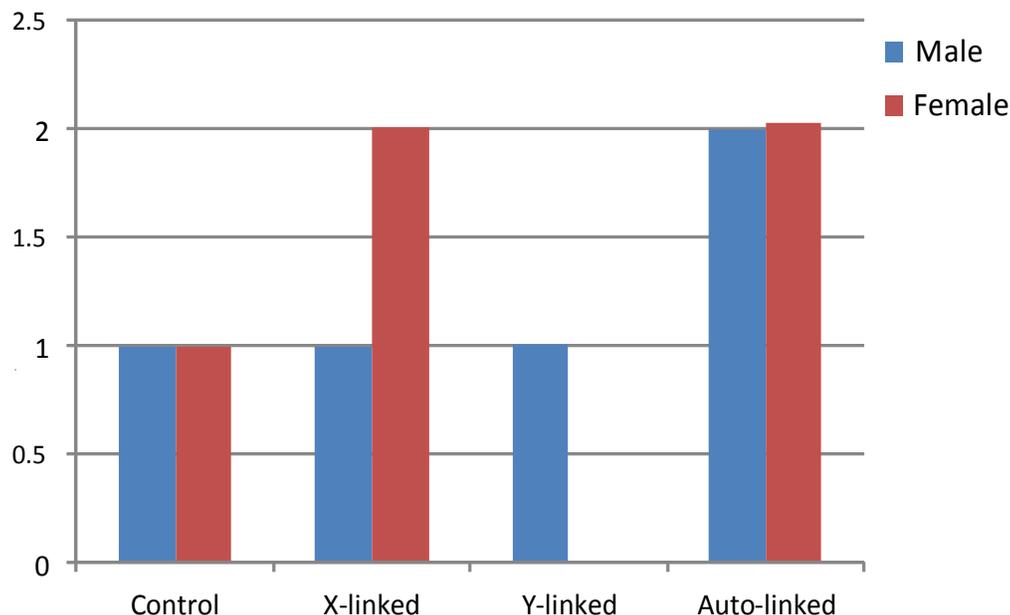
Animal sex determination: DNA based animal sex determination approaches are ideal ways of sexing animals – especially when they are young and morphologically indistinguishable – since they only require a small sample such as a drop of blood or a single hair, thereby minimizing animal trauma (Vali and Doosti, 2011). Nowadays, although several ways of animal molecular sexing exist, our novel technique aims to highlight the possibility of using a RT qPCR application for this purpose with the attendant advantages it possesses vis-à-vis traditional methods.

In our novel Q-sexing technique, the reference range (2 x SD) for the X-/Y- and autosomal-linked loci copy numbers were established as follows (assuming that an X-chromosome-linked locus has a single copy in the control Siberian tiger): (i) in males (10 Siberian tigers): 0.983 to 1.005 (one copy of the x-chromosome), 0.995 to 1.023 (one copy of the Y-chromosome) and 1.981 to 2.009 (two copies of the autosomal marker) for X-, Y- and autosomal chromosome-linked loci, respectively; (ii) in females (five Siberian tigresses): 1.997 to 2.021 (two copies of the X-chromosome), 0 (no copy of the Y-chromosome) and 1.994 to 2.064 (two copies of the autosomal marker) for X-, Y- and autosomal chromosome-linked loci, respectively. Hence, the Q-sexing technique enabled the sex of female and male Siberian tigers to be unambiguously determined ($P < 0.05$) (Table 1 and Figure 1).

Overall, a difference of one C_T was detected in males between the X and the autosome-linked loci, while no such difference was present in female samples (Figure

Table 1. Values of $2 \times \text{SD}$ of $2^{-\Delta\Delta C_T}$ for male and female Amur tigers (*Panthera tigris altaica*) across all three tested markers.

Marker	Male (n = 10)	Female (n = 5)
FCA651 (X-chromosomal)	0.983–1.005	1.997–2.021
DBY7 (Y-chromosomal)	0.995–1.023	0
FCA139 (autosomal)	1.981–2.009	1.994–2.064

**Figure 1.** Reference range ($2 \times \text{SD}$) for $2^{-\Delta\Delta C_T}$ of X-/Y- and autosomal-linked loci from the female and male Siberian tiger samples tested in our study, with one morphologically sexed male Siberian tiger used as a control.

2). As expected, in males the Y-linked locus had the same C_T value as the X-linked locus but was not present in female-derived samples, thereby corroborating the simplicity and effectiveness of Q-sexing. Although the concomitant analysis of a Y-chromosomal marker as shown here is not necessary for the sexing procedure, it was included in our study to corroborate our assumption and to validate the sexing assay.

Our new Q-sexing technique is based on RT qPCR and hence compared to existing methods has the advantage of not requiring; (i) any post-PCR sample manipulation (for example, restriction digests and gel electrophoresis) or (ii) any internal PCR control (although an initial calibration against a control is still necessary) (Vali and Doosti, 2011; Cenariu et al., 2012).

Our novel Q-sexing technique is based on only one PCR-cycling difference between males and females and could be affected by the low sensitivity of some RT qPCR machines. Thus, we recommend that PCR runs be

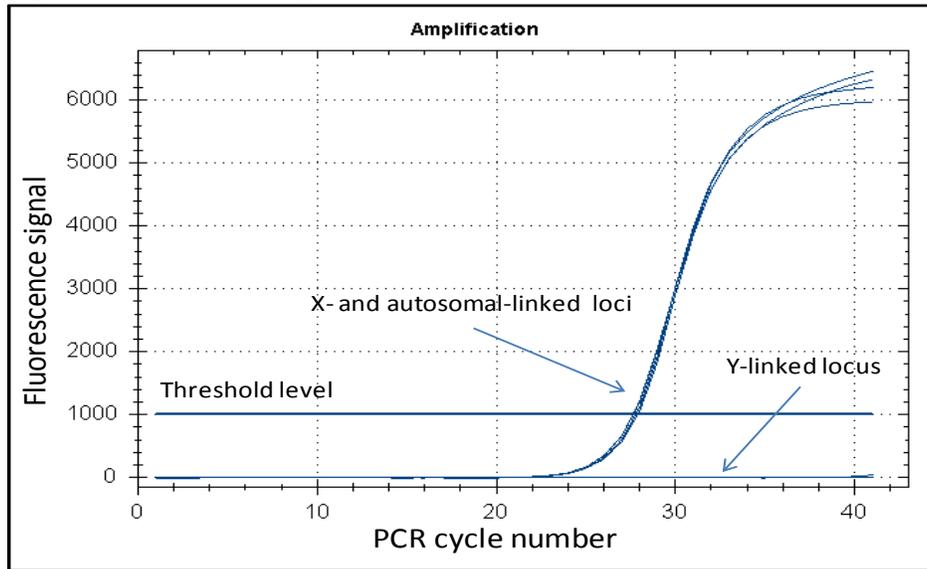
replicated to ensure that results are accurate, which is standard practice in studies based on RT qPCR (Nolan et al., 2006; Alasaad et al., 2011a).

The future of RT qPCR is bright as technology becomes ever more rapid, cost-effective, easier to use and capable of processing higher throughputs. RT qPCR is a robust and much used methodology for biological investigation (Ghasemian et al., 2012; Yang et al., 2012) and hence we expect our novel technique for animal sexing to be widely applicable. Our study used samples of the threatened Siberian tiger whose sex was known; further studies, nevertheless, are still needed to standardize this method for other animal species based on specific primers.

ACKNOWLEDGEMENTS

The authors would like to thank Mike Lockwood for

(A) Female Siberian tiger



(B) Male Siberian tiger

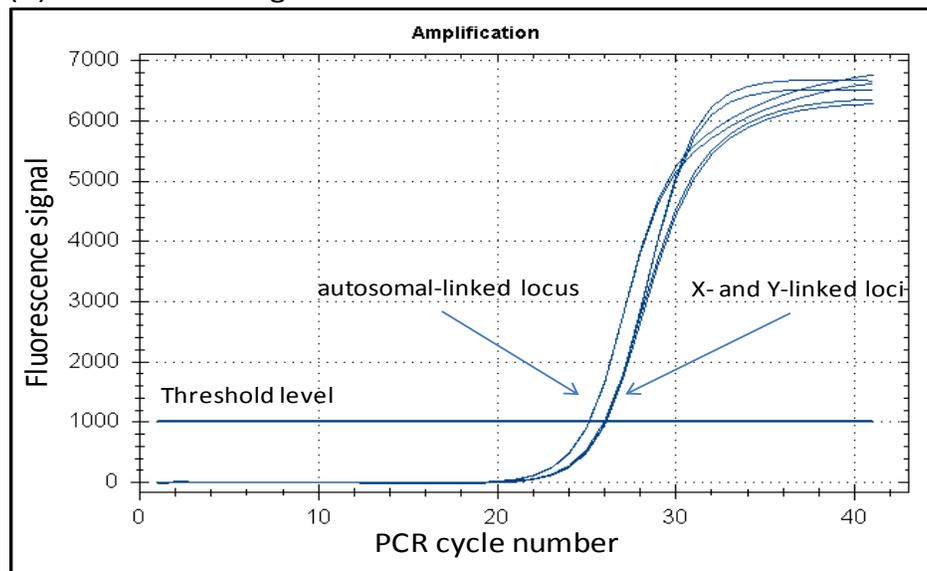


Figure 2. Representative amplification plots of X-/Y- and autosomal-linked loci from female and male Siberian tiger samples. (A) Female Siberian tiger amplification plot showing the failure of the Y chromosome-linked locus to amplify. (B) Male Siberian tiger amplification plot, with one C_T difference between X-/Y- and autosomal-linked loci.

correcting the English of the paper. The research was supported by Proyecto de Excelencia RNM 06400 (Junta de Andalucía, Spain) and Juan de la Cierva Grant (Ministerio Innovación y Ciencia, Spain).

REFERENCES

- Aasen E, Medrano JF (1990). Amplification of the ZFY and ZFX genes for sex identification in human, cattle, sheep and goats. *Biotechnology* 8:1279-1281.
- Alasaad S, Soriguer RC, Abu-Madi M, El-Behairy A, Díez Baños P, Píriz A, Fickel J, Zhu ZQ (2011a). A real time qPCR-based assay for the identification of *Fasciola* spp. *Vet. Parasitol.* 179:266-271.
- Alasaad S, Soriguer RC, Chelomina G, Sushitsky YP, Fickel J (2011b). Siberian tiger's recent population bottleneck in the Russian Far East revealed by microsatellite markers. *Mamm. Biol.* 76:722-726.
- Cenariu M, Eموke P, Groza I (2012). Sexing bovine pre-implantation embryos using the polymerase chain reaction: A model for human embryo sexing. *Afr. J. Biotechnol.* 11:4455-4458.
- Dubiec A, Zagalska-Neubauer M (2006). Molecular techniques for sex identification in birds. *Biol. Lett.* 43:3-12.
- Ellegren H (1996). First gene on the avian W chromosome (CHD) provides a tag for universal sexing of non-ratite birds. *Proc. R. Soc.*

- Lond. 263:1635-1641.
- Ghasemian S, Najafzadeh H, Reza Seifi M, Jalali A, Galehdari H (2012). Developing high performance and cost effective real time (PCR) for P-glycoprotein gene expression analysis in rat. *Afr. J. Biotechnol.* 11:11501-11508.
- Griffiths R, Orr K (1999). The use of amplified fragment length polymorphism (AFLP) in the isolation of sex-specific markers. *Mol. Ecol.* 8:671-674.
- Kalz B, Jewgenow K, Fickel J (2006). Structure of an otter population in Germany – results of DNA and hormone analyses from faecal samples. *Mamm. Biol.* 71:321-335.
- Livak KJ, Schmittgen TD (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2^{-ΔΔC_T} Method. *Methods* 25:402-408.
- Longmire JL, Maltbie M, Pavelka RW, Smith LM, Witte SM, Ryder OA, Ellsworth DL, Baker RJ (1993). Gender identification in birds using microsatellite DNA fingerprint analysis. *Auk* 110:378-381.
- Menotti-Raymond M, David VA, Chen ZQ, Menotti KA, Sun S, Schäffer AA, Tomlin JF, Agarwala R, O'Brien SJ, Murphy WJ (2003). Second-generation integrated genetic linkage/radiation hybrid maps of the domestic cat (*Felis catus*). *J. Hered.* 94:95-106.
- Nesje M, Roed KH (2000). Sex identification in falcons using microsatellite DNA markers. *Hereditas* 132:261-263.
- Nolan T, Hands RE, Bustin SA (2006). Quantification of mRNA using real-time RT-PCR. *Nat. Protoc.* 1:1559-1582.
- Putze M, Nuernberg S, Fickel J (2007). Y-chromosomal markers for the European brown hare (*Lepus europaeus*, Pallas 1778). *Eur. J. Wildl. Res.* 53:257-264.
- Ramos PS, Bastos E, Mannan RW, Guedes-Pinto H (2009). Polymerase chain reaction-single strand conformation polymorphism applied to sex identification of *Accipiter cooperii*. *Mol. Cell. Probes* 23:115-118.
- Rozhnov VV, Sorokin PA, Naidenko SV, Lukarevskiy VSH, Hernandez-Blanco A, Litvinov MN, Kotlyar AK, Yudin VG (2009). Noninvasive individual identification of the Amur tiger (*Panthera tigris altaica*) by molecular-genetic methods. *Boklady Biol. Sci.* 429:518-522.
- Sacchi P, Soglia D, Maione S, Meneguz G, Campora M, Rasero R (2004). A non-invasive test for sex identification in short-toed Eagle (*Circaetus gallicus*). *Mol. Cell. Probes* 18:193-196.
- Sambrook J, Fritsch EF, Maniatis T (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Sullivan KM, Mannucci A, Kimpton CP, Gill P (1993). A rapid and quantitative DNA sex test: fluorescence-based PCR analysis of X-Y homologous gene amelogenin. *Biotechniques* 15:636-638.
- Vali N, Doosti A (2011). Molecular study for the sex identification in Japanese quails (*Coturnix Japonica*). *Afr. J. Biotechnol.* 10:18593-18596.
- Wang LC, Severinghaus LL, Chen CT, Liu YL, Pan CH, Huang D, Lee HY, Lir JT, Chin SC, Pu CE, Wang CH (2008). Sex identification of owls (Family Strigidae) using oligonucleotide microarrays. *J. Hered.* 99:187-192.
- Yang X, Li F, Liu C, Zhang X, Liu K, Fang W, Wu Z, Xie D, Zhang C, Wang Q, Zhao F (2012). Analysis of the copy number of exogenous genes in transgenic cotton using real-time quantitative PCR and the 2^{-ΔΔC_T} method. *Afr. J. Biotechnol.* 11:6226-6233.