Ann. Zool. Fennici 44: 401–404 ISSN 0003-455X (print), ISSN 1797-2450 (online)
Helsinki 19 December 2007

A novel method for sex determination of giant panda

Xiao Xu[1], Kun Wei[1], Wen-Ping Zhang[1], Ling Lin[1], Xiao-Fang Wang[1], Fu-Jun Shen[2], Liang Zhang[2], Zhi-He Zhang[2] & Bi-Song Yue[1,*]

[1)] *Key Laboratory of Bio-resources and Eco-environment (Ministry of Education), College of Life Sciences, Sichuan University, Sichuan 610064, P. R. China (*corresponding author's e-mail: bsyue@yahoo.com)*
[2)] *Sichuan Key Laboratory of Conservation Biology on Endangered Wildlife, Chengdu Research Base of Giant Panda Breeding, Sichuan 610081, P. R. China*

Received 26 June 2007, revised version received 7 August 2007, accepted 7 August 2007

Xu, X., Wei, K., Zhang, W. P., Lin, L., Wang, X. F., Shen, F. J., Zhang, L., Zhang, Z. H. & Yue, B. S. 2007: A novel method for sex determination of giant panda. — *Ann. Zool. Fennici* 44: 401–404.

A fast, inexpensive, and reliable method for sex determination of giant panda was developed using amplification of giant panda zinc finger alleles (ZFX/ZFY). Two sets of primers targeted a conserved region in the exon of the ZFX/ZFY genes worked in a single PCR process, and provided positive results for both males and females. Sexes of individuals were revealed by sex-specific banding patterns (females: 447 bp, 146 bp; males: 447 bp, 350 bp, 146 bp). This method was successfully tested on blood sample from pandas of known sex. Non-invasive tests revealed that this method can also be applied to non-invasive samples such as hair or feces.

Introduction

Knowledge of the sex of individuals in a population greatly facilitates the study of behaviour, breeding systems, and evolutionary ecology. Molecular sexing techniques provide a means to identify the sex of embryos and young for the study of evolution of breeding systems, and can aid in captive breeding programs and management of wild populations of species (Rosel 2003).

At present, the most common tests to sex mammals include: (1) amplification of Y-specific gene such as SRY (Woods *et al*. 1999), and (2) amplification of X and Y homologous fragments such as amelogenin (AMELX/AMELY) genes or zinc finger (ZFX/ZFY) protein genes (Ortega *et al*. 2004). The SRY system, however, produces only male-specific amplifications. Null amplification might originate either from females or PCR failures, making an equivocal result that whether non-amplification of the Y marker truly represents a female. Although co-amplification of a mitochondrial or nuclear gene fragments as an external control may address this problem (Palsbøll *et al*. 1992, Kamimura *et al*. 1997), the sensitivity and optimal condition of different primer sets are unlikely to be identical and external control may be unreliable (Fernando *et al*. 2001). Although amplification of X and Y homologous fragments with a single primer set is more reliable, discrimination of the X and Y fragments requires the presence of a size polymorphism, or a Y fragment unique restriction site to prevent false identification from incomplete digestion in RFLP analysis. Many species may not exhibit appropriate restriction sites that can be treated with regular restriction enzyme

for length polymorphisms patterns, or sex specific length polymorphisms on X/Y homologous (Wei *et al*. 2007).

ZFX/ZFY genes encode a zinc finger protein, and have been evolutionarily conserved in vertebrates (Page *et al*. 1987). The objective of this study is to develop a fast, inexpensive, and reliable method for sex determination of giant pandas based on ZFX/ZFY genes, which would be applied to single nucleotide polymorphisms (SNPs) where an appropriate restriction enzyme is not available, as well as to insertion or deletion polymorphisms.

Materials and methods

DNA extraction

Blood samples from 10 giant pandas (5 males, 5 females) and feces samples from 6 giant pandas (4 males, 2 females) were provided by Chengdu Research Base of Giant Panda Breeding. Total genomic DNA from blood was extracted using standard protocols (Sambrook *et al*. 1989). Fecal DNA extractions were performed using the QIAamp® DNA Stool Mini Kit (Qiagen Inc).

Sequencing of ZFX/ZFY

Partial, giant panda ZFX/ZFY exons were amplified using P1-5EZ: 5´-ATAATCACATGGAGAGCCACAAGCT-3´ and P2-3EZ: 5´-GCACTTCTTTGGTATCTGAGAAAGT-3´ (Aasen *et al*. 1990) for cloning and sequencing. We used genomic DNA extracted from blood samples from 3 males and 3 females as templates. PCR was performed in a 50 μl total volume reaction mix containing 10–30 ng of template DNA, 2.5 mM $MgCl_2$, 1.5 μM of each primer, 200 μM of dNTPs, 1× reaction buffer, and 2.5 U Taq polymerase (Applied Biosystems, Inc). The PCR profile was 95 °C/5 min, [95 °C/45 s, 55°C/1 min, 72 °C/1 min] × 30 cycles, 72 °C/10 min, 10 °C/∞. PCR products were electrophoresed on a 2% agarose gel and stained with ethidium bromide.

ZFX fragments (447 bp) from females and both ZFX and ZFY fragments (447 bp) from males were excised from the gel, and purified using the Agarose Gel Extraction Kit (OMEGA Inc.) according to the manufacturer's instructions. Each purified product was ligated onto PMD18-T vector (TaKaRa Janpan) and transformed into JM109 component cells. To confirm sequence integrity, four independent clones from each female and eight independent clones from each male were sequenced with universal primers on an ABI 3730 DNA analyser (Applied Bisystems, Inc.). The ZFX sequences were determined directly from the homozygosity (ZFX/ZFX) in female samples. The ZFY sequences were obtained by deducting the ZFX sequence from the heterozygous (ZFX/ZFY) sequences in male samples. Sequences have been deposited in GenBank with accession numbers DQ916308 for ZFX and DQ916309 for ZFY.

Sex determination

Based on sequences of ZFX/ZFY obtained, a site of A/G substitution at position 122 that presents the differences between ZFX and ZFY was selected for designing the allele specific primers. In detail, base "G" was found at position 122 of ZFX sequence while base "A" at the same position of ZFY sequence. The allele specific primers FR1: 5´-CTTCTGTATTTTGTTGGGTCCTTTC-3´ and FR2: 5´-TACTCAAAAAATGGTGGATAAGGAA-3´ confronting each other at 3´ end with base specific to the alleles were designed as follows: FR1 for the anti-sense primer of ZFX with the anti-sense base of polymorphism site at 3´ end; FR2 for the sense primer of ZFY with sense base of polymorphism site at 3´ end (Fig. 1).

Sex determination was performed by using the two primers sets (P1-5EZ/P2-3EZ and FR1/FR2) in a single PCR process. We tested this method using DNA extracted from blood and feces as templates in PCR. Amplifications were carried out in 20 μl reaction volumes containing 10–30 ng template DNA, 2.5 mM $MgCl_2$, 1.5 μM of each primer, 200 μM of dNTPs, 1× reaction buffer, and 1.5 U *Taq* polymerase (TaKaRa, Japan). A touch-down program for reaction optimization was used, which consisted of an initial 5 min at 95 °C, 10 cycles of 45 sec at 95 °C,

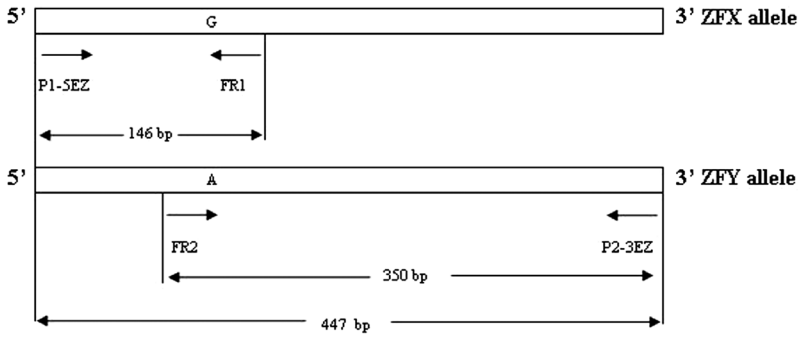

Fig. 1. Principle of the sex determination of giant panda. The polymorphism site used here is a A/G substitution at position 122 of ZFX/ZFY. At the 3´ end of the internal confronting primers FR1 and FR2, the base specific to each allele is designed. 146 bp: size of amplicon representing the ZFX allele; 350 bp: size of amplicon representing the ZFY allele; 447 bp: size of amplicons from both ZFX and ZFY.

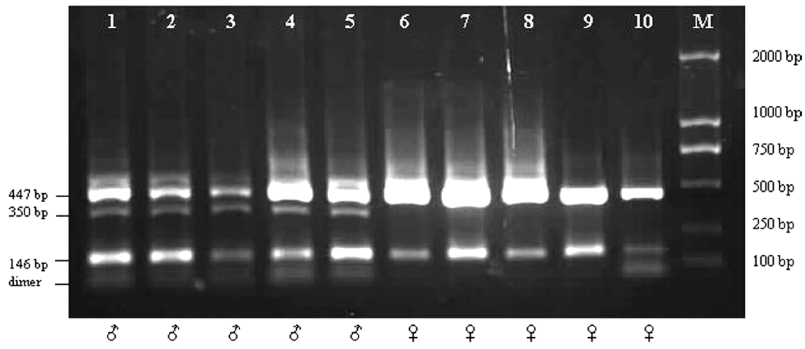

Fig. 2. Agarose gel displaying results of sex determination from giant pandas' blood samples. Sizes of amplicons are indicated on the left, and gender of each sample is given at the bottom. Lane M: DL2000 DNA Marker (TaKaRa, Japan).

1 min annealing starting at 60 °C and decreasing with 1 °C per cycle and 1 min at 72 °C, 30 cycles of 45 sec at 95 °C, 1 min at 50 °C and 1 min at 72 °C, and a final extension at 72 °C for 10 min. PCR products were separated by electrophoresis through a 1.5% agarose gel at 100 volts for 50 min and stained with ethidium bromide.

Results and discussion

All expected segments were successfully amplified from both blood and feces samples of giant panda (Figs. 2 and 3). Three target bands (447 bp, 350 bp and 146 bp) were yielded in males and two (447 bp, 146 bp) in females. Although an additional non-specific band was observed in both males and females, this did not affect the sexing result based on the different banding patterns. Results obtained by this method were in complete agreement with the anatomically proven sex of all the pandas tested.

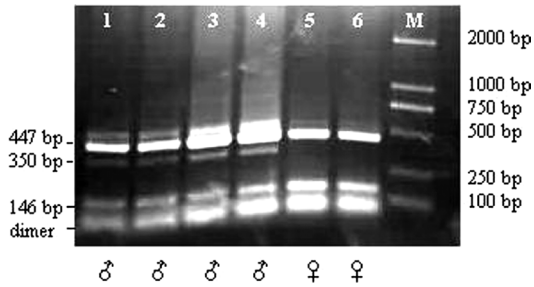

Fig. 3. Agarose gel displaying results of sex determination from giant pandas' feces samples. Sizes of amplicons are indicated on the left, and gender of each sample is given at the bottom. Lane M: DL2000 DNA Marker (TaKaRa, Japan).

High amplification success rates (100%) from tests of blood and feces samples indicated that our method for sex determination of giant panda was reliable. Successful amplification in non-invasive samples confirmed the efficiency of this test, revealing that this method can be applied to non-invasively collected samples. The

size discriminations among these three target bands (447 bp, 350 bp, 146 bp) were big enough to allow a quick detection by a simple agarose gel electrophoresis, and excluded the need of the time-consuming polyacrykamide gel electrophoresis or the expensive capillary electrophoresis for the genotyping when the discrimination of amplicons was not significant.

Indeed, besides the site of position 122, we identified two additional sites of A/G substitution at position 89 and 277 that also represented the differences between ZFX and ZFY. However, we chose the site at position 122 in out test, since the sequences surrounding this site allowed for four primers (including P1-5EZ and P2-3EZ) with a similar melting temperature, which is often considered to be vital (Tamakoshi *et al*. 2003).

It should be noted, however, that due to the limited number of samples described here, more samples of known-sex giant pandas from both captivity and wild should be sexed using our method to confirm the robustness of our primer sets. In this study, we developed a fast and reliable method for sex determination of the giant panda. This method will provide an opportunity for sex determination of juvenile or newborn pandas whose sex dimorphisms are not obvious, and with non-invasive samples such as hairs or feces for detecting the sex ratio of wild panda population.

Acknowledgments

This work was funded by Research Fund of Giant Panda Breeding of Chengdu.

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