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Y-CHROMOSOME MICRODISSECTION AND Y-LINKED GENES IDENTIFICATION OF THE GIANT PANDA (*AILUROPODA MELANOLEUCA*)

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ABSTRACT

The Y chromosome plays an important role in the sexual reproduction of many species because it contains sex-determining genes and male reproductive factors and the Y chromosome has only been sequenced in a few mammalian species. Y chromosome has been study in several fields, such as spermatogenesis, male development and sex chromosome evolution. The giant panda (*Ailuropoda melanoleuca*) is an endangered species in China. Male giant pandas have reproductive defects such as low natural mating rate, poor sperm quality, and infertility that inhibit population growth. More information is needed on the genetics of giant panda reproduction in order to understand their biological characteristics. The Y chromosome data of the giant panda are now unavailable, though a draft genome of this species has been sequenced. Thus, in the present study, we separated single Y chromosomes of male giant panda, sequenced using high-throughput sequencing after whole genome amplification. Finally, two genes, *ZFY* and *TSPY1*, on Y chromosome and related to male fertility were annotated. This study is a supplement of giant panda genetic data.

Keywords: Giant panda, reproduction, sex chromosome, whole genome amplification, Y-linked genes

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INTRODUCTION

The giant panda (*Ailuropoda melanoleuca*) is the flagship specie of world biodiversity, also a specific and endangered species in China (Wei *et al*., 2018). The habitat of wild giant pandas is limited to certain mountains in China mainly because of human expansion and habitat fragmentation (Zhan *et al*., 2006). Giant pandas have distinct biological and behavioral traits, such as a bamboo-only diet and black-and-white fur. Compared with other mammals, the fertility of giant pandas is lower. This is more serious for captive giant pandas, which is characterized by difficulty in estrus, mating and nursing for females as well as no sperm, sparse sperm, low mating rates, and high rates of abnormal sperm in males. It is necessary to put more efforts to reduce the endangerment of the giant panda population.

The Y chromosome is a sex chromosome in male mammals. It is haploidy, specifically exists in male, and has no crossing over. Y chromosome is the smallest chromosome in mammals and has the fastest evolutionary speed. It is a special component of the genome (Sun *et al*., 2019), and a key material to study many aspects of male, such as spermatogenesis, evolution, *et al* (Jobling and Tyler-Smith 2017). Y chromosome has been used to analyze the origins and evolution of species, identify the sex of embryos, classify species, estimate genetic diversity, screen for genes related to reproduction, and study paternal inheritance. Y-linked genes usually have important functions related to sex (Han *et al*., 2018). However, since its repetitive sequences, palindrome structures, and few protein-coding genes, the Y chromosome is difficult to sequence and assemble (Wu *et al*., 2019).

Y chromosome sequence for many organisms is unavailable. To date, the Y chromosome has only been sequenced for a few mammals, including human (Skaletsky *et al*., 2003), chimpanzee (Hughes *et al*., 2010), rhesus monkey (Hughes *et al*., 2012), gorilla (Tomaszkiewicz *et al*., 2016), mouse (Soh *et al*., 2014) and pig (Skinner *et al*., 2016), and it has been partially sequenced in bull (Chang *et al*., 2013), dog, rat, marmoset and cat (Tomaszkiewicz *et al*., 2016). As far as we know, there is very little information about giant panda Y chromosome has been reported.

Single-haplotype iterative mapping and sequencing has been used to sequence the Y chromosome of human, chimpanzee, macaque, and mouse (Skaletsky *et al*., 2003). Although accurate, it is expensive and time-consuming (Tomaszkiewicz *et al*., 2016). Nowadays, whole-genome sequencing, RNA-seq and qPCR also used for studies on Y chromosome and Y-linked genes (Carvalho *et al*., 2015; Wang *et al*., 2018; Das *et al*., 2019; Xiao *et al*., 2020) . In this study, we used an alternative cost-effective method, microdissection and

high-throughput sequencing, to separate single Y chromosome and identify Y-linked genes in male giant panda.

MATERIALS AND METHODS

Sample collection: Skeletal muscle samples of male giant panda PanPan were collected from Wolong Giant Panda Nature Reserve in Sichuan province with surgical operation. Samples were cleaned with PBS immediately and took them back to the laboratory for cell culture with cryogenic container. The skeletal muscle tissues of a male giant panda were collected under permission from the Institutional Animal Care and Use Committee in the College of Animal Science and Technology, Sichuan Agricultural University under permit No. DKY-B20081003.

Cell culturing and C-banding: Skeletal muscle cells were cultured in DMEM medium containing 10% FBS. When cells reached ~70% confluence they were collected. Cells conducted hypotonic treatment by incubating in 0.075 M KCL at 37 °C for 40 min. After this, cells were fixed three times with Carnoy's Fluid (3 volume methanol + 1 volume acetic acid).

We chose Y chromosome slides with enough mitotic chromosomes for C-banding. Slides were treated with HCL - $Ba(OH)_2$ - SSC and dyed with Giemsa, which could deeply stain the centromeres and heterochromatin. Y chromosomes are rich in heterochromatin, allowing identification.

Y chromosome microdissection and whole genome amplification: We used capillary glass needles to separate single Y chromosome as described by Scalenghe (Scalenghe *et al*., 1981). We examined slides under a microscope and anchored mitotic figures with complete chromosome numbers (2n=42), dispersive chromosomes, and Y chromosomes that were distinguishable. We touched the slide with the needle close to the Y chromosome and moved it until the Y chromosome deviated from its original place. The needle adhered with Y chromosome was slowly taken out. Chromosomes were placed in PBS and stored at -20 °C.

Single Y chromosomes are inadequate for high-throughput sequencing. Therefore, separated Y chromosomes were used as templates for whole genome amplification (WGA) using a GenomePlex WGA4 kit (Sigma, USA). WGA can be used to get 2 to 4 μg of genome DNA quickly from a small amount of DNA. This method substantially increases the amount of DNA without sequence orientation. High-throughput sequencing requires at least 3 μg of DNA that can be supplemented by WGA. We divided the 45 Y chromosomes into three tubes (15 each) and examined the quality of the WGA products using NanoDrop 2000 (concentration, Thermo Fisher Scientific, USA) and agarose gel electrophoresis (size).

High-throughput sequencing: Stranded DNA libraries were constructed using a TruSeq Library Construction Kit (Illumina，USA). Then the libraries were sequenced on an Illumina HISeq Xten with PE150. Raw reads of fastq format were firstly processed. In this step, clean reads were obtained by removing reads containing adapter and ploy-N and low-quality reads from raw data. All the downstream analyses were based on clean data with high quality. Only reads with a perfect match or one mismatch were further analyzed and annotated based on the reference genome. Hisat2 (Kim *et al*., 2015) was used to map with reference genome. Then clean reads were assembled and quantified using StringTie (Pertea *et al*., 2015) to obtain contigs and unigenes. Unigenes were then BLAST to 32 known Y-linked genes. We also identified single nucleotide polymorphisms (SNPs) using GATK (McKenna *et al*., 2010) and SAMTOOL software.

Data accession numbers: The sequencing data generated in this work have been deposited in the NCBI SRA database under BioProject Accession Numbers PRJNA545267.

RESULTS AND DISCUSSION

Y chromosome is a genetically sex-determining factor, which is the smallest acrocentric, constitutively haploid, male-specific, rich of repeat sequences and responsible for gender differentiation (Das *et al*., 2019). The repeated sequences promote frequent intrachromosomal recombination, which leads to a high degree of structural variation (Jobling and Tyler-Smith 2017). Y chromosome is poorly conserved among mammals. It was said that Y chromosome was a degenerate X chromosome and it has lost most part of its ancestral genes (Skaletsky *et al*., 2003). Transposition and retrotransposition of genes between Y chromosome and other chromosomes may cause the gene loss of Y chromosome (Hughes *et al*., 2015). Identified novel Y-linked genes usually specifically expressed in testis and play roles in spermatogenesis(Tsai *et al*., 2019) . Y chromosome may play some parts in diseases of males and females and directly influence male fertility and health.

Karyotype of giant panda: As expected, giant pandas had 42 chromosomes (2n = 42), including 20 pairs of autosomes and one pair of sex chromosomes (X and Y) (Figure 1). There were seven relatively smaller chromosomes.

C-banding: C-banding confirmed that the Y chromosome was the smallest of the giant panda chromosomes (Figure 2). After dying, the Y chromosome

was clearly marked, allowing identification and separation.

Glass needle-based chromosome microdissection is a standard approach developed in the 1980s (Scalenghe *et al*., 1981) and remains frequently applied. It is an efficient way to isolate DNA from specific chromosomes and/or specific chromosome sections by using chromosome microdissection and microcloning technique (Zhang *et al*., 2016). The combination of those two methods can also be applied to establish chromosome specific probes of different species (Kosyakova *et al*., 2013). It has been used for chromosome isolation and analysis of Drosophila (Scalenghe *et al*., 1981; Wesley *et al*., 1990), human (Senger *et al*., 1990; Al-Rikabi *et al*., 2019) , spinach (Deng *et al*., 2013), corn and potato (Soares *et al*., 2020), but has not been used for research on giant panda chromosomes. In this study, we cultured muscle cells of male giant panda, then used C-banding and glass needle-based microdissection to separate single Y chromosome of giant panda.

Whole genome amplification: Since a single Y chromosome could not meet the sample size requirements of high-throughput sequencing, whole genome amplification (WGA) was performed on Y chromosome before sequencing.

The WGA products were approximately 300 bp in size (Figure 3, Table 1). The bands were clear, sole and thick, which means the WGA products were highly concentrated. The 260/280 values ranged from 1.71 to 1.78 and the concentrations were all >900 ng/μl.

High-throughput sequencing and gene annotation: DNA libraries of the Y chromosome were generated from giant panda muscle cells and sequenced using a HISeq Xten (Illumina). The raw data were 29.26 Gb, and after filtering the clean data were 29.24 Gb, including 21,182,741 reads. These reads were mapped to available giant panda data and assembled into 12,791 scaffolds (2.74 Mb), which mostly ranged from 100 to 400 bp (12,169, about 95.14% of the total), and N50 was 202 bp. Only four scaffolds were longer than 1000 bp.

We identified 16,949 SNPs, including 1,696 deletions and 1,315 insertions, by comparing our data to that of the available giant panda genome. There are limited data for comparison, and there is even less information about these SNPs, including their sequences, annotations, and analysis.

We compared our data with 32 Y-linked genes of 18 other mammals (Bidon *et al*., 2015), and identified two Y-linked genes in the giant panda: *ZFY* and *TSPY1*.

The zinc finger protein, Y-linked (*ZFY*) gene, located on the short arm of the Y chromosome (Yp11.3), X-degeneration region of the Y chromosome (Skaletsky *et al*., 2003). It is highly expressed in sperm cells (Hansen *et al*., 2006), and initially considered to be one of the most important sex-determining genes (Page *et al*., 1987). *ZFY* is highly conserved throughout eutherian mammals (Koopman *et al*., 1991) and regulate other genes through “zinc-finger” domains that bind to DNA. The “zinc-finger” domains of *ZFY* are related to nucleic acid-binding proteins and affect early-embryo sex determination and sperm formation (Page *et al*., 1987). At present, it can skew the sex ratio of offspring towards females by interrupting the mRNA expression of *ZFY* on the Y chromosome (Zhang *et al*., 2018) .

The *TSPY* gene (testis-specific protein y-encoded) was initially found in human testicular tissue and is a member of the *DYZ5* tandem repeating unit (Manz *et al*., 1993), which plays a crucial part in the testosterone physiology of male animals (Vogel *et al*., 1997). Copy number variability of *TSPY* gene is related to animal age (Oluwole *et al*., 2017).The *TSPY* gene functions through phosphorylation in the early stage of spermatogenesis and male preponderant tumors (Oram *et al*., 2006; Kido and Lau 2019) .

TSPY1 is a testicular-specific expression gene in the short arm of the Y-chromosome (Ratti *et al*., 2000). It is one member of the *TSPY* gene family, is a multi-copy gene, and is considered to be a functional *TSPY* gene because it possesses a promoter region. *TSPY1* is a member of the TSPY/TSPYY_L/SET/NAP-1 superfamily and contains a highly conservative SET/NAP domain. *TSPY1* can be combined with multiple transcription factors and is mainly involved in gene expression regulation, chromatin remodeling, protein translation, cell cycle processes, and other important cell life activities. Initially, human *TSPY1* gene was mainly expressed on prespermatogonia, prespermatocytes and spermatogonia, spermatocytes and some round spermatids of embryo testes (Kido and Lau 2005). Mouse *TSPY1* can bind to histones in the sperm cell cytoplasm, so it participates in chromatin modification and / or composition during spermatogenesis (Kido and Lau 2006). Usually, the *TSPY1* protein is only expressed in male testicle tissues, and it participates in sperm formation (Yang *et al*., 2018). However, recent studies have found abnormally high expression of *TSPY1* in tumors, such as gonadal cell tumors, testicular germ cell tumors and melanoma, prostate cancer and hepatocellular carcinomas (Li *et al*., 2007), suggesting that *TSPY1* may be essential for the development of tumors.

It has reported that *TSPY1* may regulate human spermatogonial proliferation via the *USP7*-mediated p53 signaling pathway (Shen *et al*., 2018). *TSPY1* also has other biological functions. The SET/NAP domain of *TSPY1* is a molecular companion of histones that interact with many transcription factors, playing a key role in some biological processes, including transcription, translation, DNA replication, and gene expression (Svacinova *et al*., 2011). *TSPY1* can also be combined with translation extension factors, such as *EEF1A1* and

EEF1A2, to participate in protein synthesis, cell proliferation, and gene transcription of germ cell tumors (Kido and Lau 2008; Lau *et al*., 2011). Oram *et al*. (Oram *et al*., 2006) found that overexpression of *TSPY1* could lead to increased expression of the oncogene epidermal growth factor receptor (*ERBB*), the WNT pathway member *WNT5A*, the RAS family member (*RAP1A*) and some growth factors (such as *CCND2*). It also reduced the expression of MAP kinase inhibitor (*DYSP5*) and transforming growth factor β3 (*TGF-β3*). This suggests that *TSPY1* may be combined with transcription factors or proteins through the SET/NAP domain, or by activating signaling pathways such as WNT and MAPK, to promote the proliferation and cycle conversion of hepatocellular carcinoma cells, and to participate in cell differentiation, signal transduction, and gene expression regulation.*TSPY1* has a copy number variant that does not depend on a single-fold group of Y chromosomes, so its copy dose is positively correlated with sperm motility and count but unrelated to sperm yield (Krausz *et al*., 2010). It is a candidate gene for weak sperm, which may inform genetic counseling for male sterility.

Testicular development, spermatogenesis, sperm quality and gonadal diseases in male mammals are important factors that affect population numbers. Understanding the molecular mechanisms of animal spermatogenesis and the pathogenesis of gonadal diseases will help us improve the reproductive ability and prognosis of male mammals.

The study of Y chromosome is of great significance to solve the reproductive problem of male giant pandas. Unfortunately, we did not assemble the Y chromosome sequence of the giant panda, nor did we analyze its structure, which was our original research goal. Up to now, we just know littler information about giant panda Y chromosome (Han *et al*., 2018). This study used high-throughput sequencing and homologous alignment of giant panda Y chromosomes to identify two Y-chromosome genes, *ZFY* and *TSPY1*, which may play important roles in reproduction and gonad tumors of male giant panda. Continuing to explore the Y-chromosome genes of male giant pandas will provide insight into the reproductive issues in male giant pandas and related diseases. In addition, this study provided an economy and time-effective method on Y chromosome research, which could provide a new sight for related studies. It would be further improved to better provide methodological support for chromosome research.

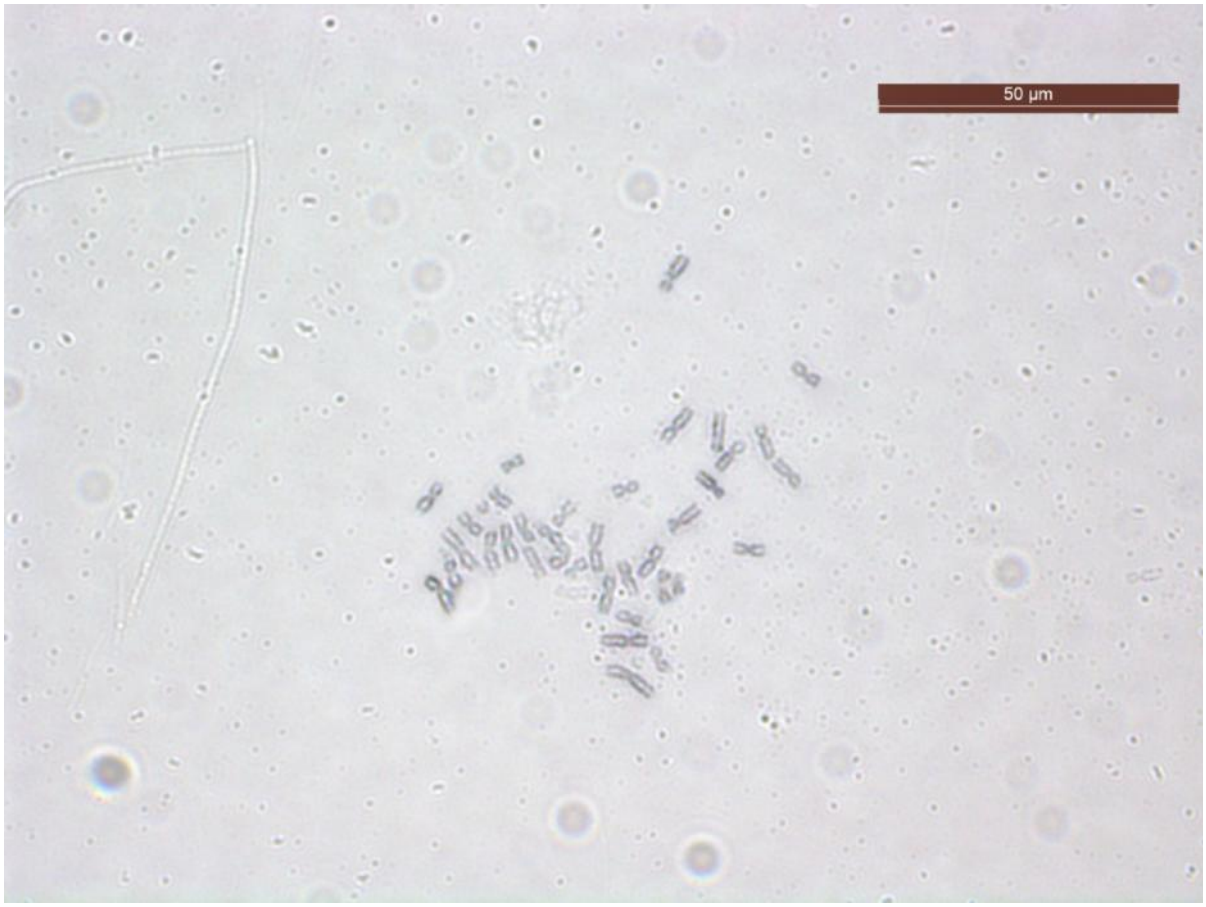

Figure 1 Karyotype of giant panda. Giant pandas have 42 chromosomes (2n=42), with seven relatively smaller chromosomes, including the Y chromosome.

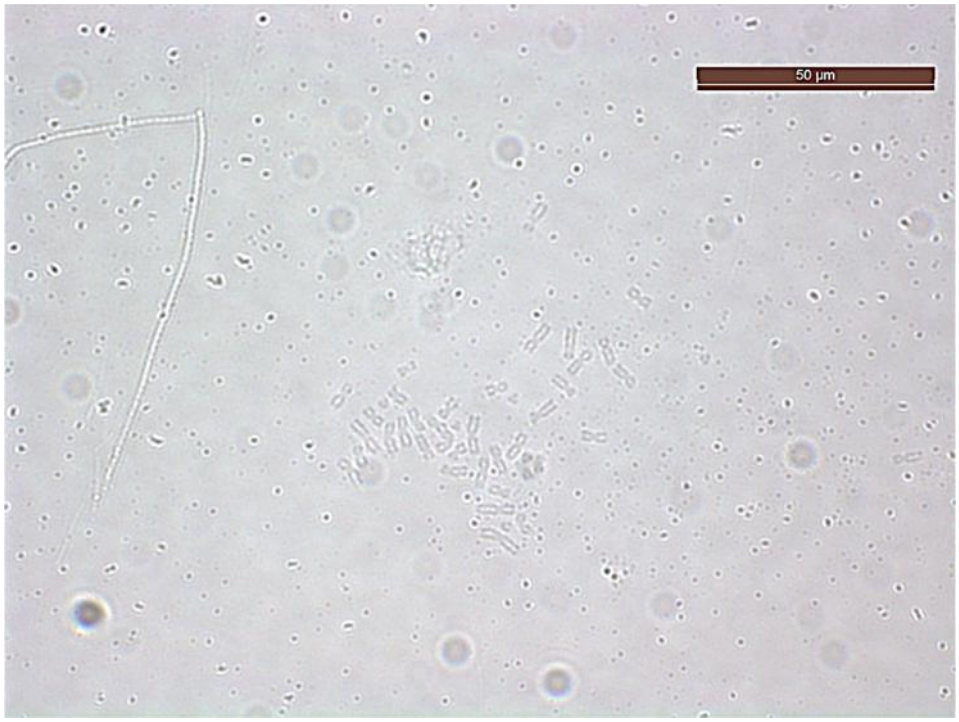

Figure 2 C-banding of giant panda chromosomes. After dying with Giemsa, the heterochromatin is a deeper color than the euchromatin. Almost the entire Y chromosome was dyed, allowing it to be discriminated from the other small chromosomes.

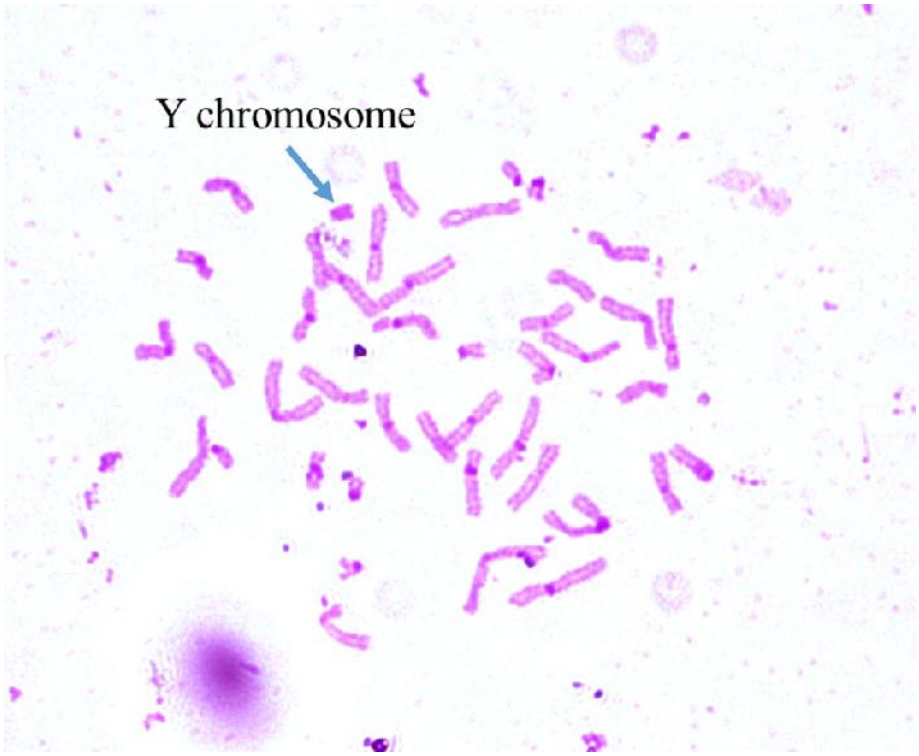

Figure 3 Gel electrophoresis result of Y chromosome after whole genome amplification. Microdissected Y chromosome was small, and the size was about 200 bp.

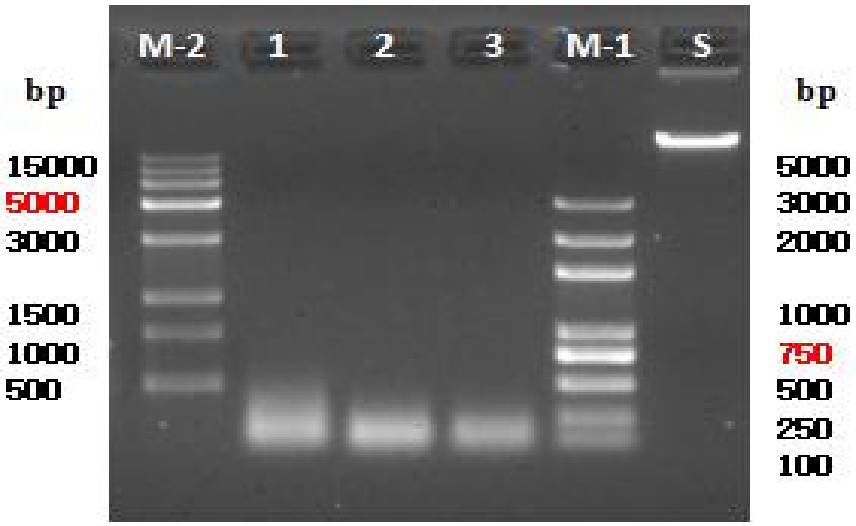

Table1 Concentration and purity of Y chromosome WGA products.

Index	Sample ID	Concentration (ng/ul)	OD 260/280
1	Y chromosomes-1	1104.3	1.72
2	Y chromosomes-2	967	1.75
3	Y chromosomes-3	984.3	1.74

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Conflict of interest: The authors declare there is no competing interests exist.

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