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SNP DETECTION OF *GnRHR* GENE AND ITS ASSOCIATION WITH LITTER SIZE TRAITS IN GIANT PANDA

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ABSTRACT

In this study, the *GnRHR* gene polymorphism of the giant panda was detected, and its effects on litter size were analyzed. Thirty-eight giant pandas that were fed under similar conditions were randomly chosen, and polymorphism in the giant panda *GnRHR* gene coding region was detected by PCR-SSCP. The association between the *GnRHR* genotypes and litter size traits was carried out with the GLM procedure using the SAS software. The PCR-SSCP test results showed that the T516121C SNP locus existed in the *GnRHR* gene exon 1, the C504498T SNP locus existed in exon 2, but no SNP locus was found in exon 3. The T516121C and C504498T loci belong to the non-synonymous mutations. The T516121C substitution led to one amino acid mutation (Cys>Arg), as did the C504498T substitution (Ala>Val). In the group of 38 pandas, there were three genotypes at the T516121C and C504498T loci. The chi-square test showed that the T516121C and C504498T loci fit into the Hardy Weinberg equilibrium in the giant panda population ($P>0.05$). Furthermore, the correlation analysis results indicated that the different genotypes of the C504498T locus in the panda *GnRHR* gene had significant effects on the average litter size for second births ($P\leq0.05$). The average litter size for second births of giant pandas with the CC genotype was higher than that the size of the DD genotype ($P\leq0.05$). The first births of the giant pandas with the AA genotype had the maximum average litter size, and there was no significant correlation between the T516121C loci with litter size traits of giant panda. Therefore, these results suggest that the C504498T base mutation in the exon 2 of *GnRHR* could influence the litter size of giant panda. In other words, *GnRHR* could influence fertility in giant panda.

Keywords: Giant panda, *GnRHR,* SNPs, PCR-SSCP, Litter traits

INTRODUCTION

The giant panda (*Ailuropoda melanoleuca*), which is a flagship species and a typical representative for biodiversity conservation, is a rare and unique animal in China (Liu *et al*. 1999). Zhan *et al*., using fecal samples and nine microsatellite loci from our molecular census estimate, found that there may be as many as 2,500–3,000 giant pandas residing in a few small mountains of western China (Zhan *et al*., 2006). The giant panda has its own unique biological characteristics, one of which is very low fertility. As a key measurement parameter of reproduction, litter size was used to evaluate animal fertility. Analysis of giant panda calving records demonstrated that the litter size of every birth in giant panda is limited to one or two litters, with three litters occurring occasionally (Liu 1987). This finding suggests that giant panda share varied litter sizes. In other words, they share varied fertility, which is controlled by many reproductive genes. However, it is still unknown which major genes influence the fertility of the giant panda. It is well known that animal fertility is primarily affected by the hypothalamus-pituitary axis, which regulates the secretion of many hormones. The hypothalamic release of gonadotropin-releasing hormone (Gonadotropin releasing hormone, *GnRH*) plays an important role in the control of mammalian reproductive physiology and sexual gland development (Kumar *et al*. 2001; Kah *et al*. 2007; Ikemoto *et al*. 2004). *GnRHR* binds with high affinity to *GnRH* on the pituitary gonadotrope's cell surface to stimulate the biosynthesis and secretion of the gonadotropins (luteinizing hormone, *LH* and follicle-stimulating hormone, *FSH*) from the anterior pituitary and then regulates gonadal function, including both synthesizing hormones and generating gametes(Millar *et al*. 2004; Finch *et al*. 2008; Naor *et al*. 2009).The increase in *GnRH* secretion and the high concentration of pituitary *GnRHR* promotes the occurrence of ovulation in mammals, but the absence of *GnRHR* can obstruct ovulation, leading to infertility (Wise *et al*. 1984). MacColl *et al*. found that the inhibition or mutation of the protein expression of *GnRH* or *GnRHR* in some organisms can lead to individual infertility (MacColl *et al*. 2002). In addition, Kim *et al*. found that the inhibition of the *GnRH* gene and *GnRHR* gene expression limits the reproductive activity of mammals (Kim *et al*. 2003). These findings suggest that the *GnRHR* gene may be an important gene for regulating a mammal's reproductive

performance. Thus, it is worth exploring *GnRHR* gene influence on the fertility of the giant panda. Given the importance of the *GnRHR* gene in mammalian reproduction, the influence of polymorphism on the reproduction of mammals has attracted a great deal of attention. Liu *et al*. reported that a G230C mutation was detected in the *GnRHR* genes of Small Tail Han sheep and Dorset sheep populations and that G230C substitution led to one amino acid mutation (Gly→Cys). This mutation may have a certain positive correlation with the reproductive capacity of Small Tail Han sheep and Dorset Sheep (Liu ZH *et al*. 2006). Chu *et al*. found a SNP locus in the *GnRHR* gene exon 1 of the Jining Grey goat, which has AA, AB and BB three genotypes, and found that the average litter size of the Jining Grey goats with the BB genotype was larger than that of goats with the AA and AB genotypes (Chu MX *et al*. 2009). In addition, Zhang *et al*. detected a SNP locus G121A in the *GnRHR* gene exon 1 of the Guizhou black goat and found that the litter size of AA genotype was significantly higher than that of the AG and GG genotypes (P≤0.05) (Zhang *et al*., 2015).

Previous studies have shown that the polymorphism of the *GnRHR* gene is closely related to the fertility of many animals. Thus, it is still necessary to confirm whether the polymorphism exists in the *GnRHR* gene and how it influences the fertility of giant panda. Thus, this study uses 38 giant pandas as experimental material to research the polymorphism of the *GnRHR* gene and the connection between the *GnRHR* genotypes and giant panda litter size traits to explore how *GnRHR* influences the fertility of the giant panda and provide a theoretical basis for the protection of high-yield litter size pandas.

MATERIALS AND METHODS

DNA samples and data collection: Blood samples were collected from 38 giant pandas (27 females) reared in the Wolong region in Sichuan Province (PR China). Records of the litter size of giant pandas were collected for statistical analysis. Using jugular vein blood, 10-ml blood samples were collected from each giant panda. With citric acid and glucose anticoagulation, they were frozen at -20°C. The samples used for the preparation of genomic DNA were subjected to kit extraction, and the remaining samples were stored at 4°C.

Primer synthesis and polymerase chain reaction (PCR): Three pairs of primers (Table 1) were designed to amplify the *GnRHR* gene coding region of the giant panda, based on the giant panda *GnRHR* gene sequence (GenBank accession no. XM_002926719.1). The primers were custom synthesized by Shenzhen Hua Da gene science and Technology Co., Ltd., China.

The 25μL volume contained 50 ng of genomic DNA, 1.0μM of each primer, 0.2 μM of dNTP, 0.5 U of Taq-DNA polymerase and 1×reaction buffer, which contained 1.5 mM $MgCl_2$.The cycling protocol was 95°C for 5 min, 40 cycles of 95°C for 30s, annealing temperature for 30s, 72°C for 40s, and a final extension at 72°C for 10 min. The products were detected by 1.5% agarose gel electrophoresis. In addition, PCR products were sent to Shanghai Yingjun Biotechnology Co. Ltd. For positive sequencing. In this study, 38 samples were analyzed by direct sequencing.

Table1.Primer sequences and information of the *GnRHR*.

Primer	Primer sequence	Amplified region	Products size/bp
P_1	F:ATCACCCAATACAGTAGTCAAG R:GAAACTCCATCCGTCAGG	exon1	618
P_2	F:GCTGCGTTGAATACTGC R:TTCGATGAAGAGCCAAA	exon2	455
P_3	F:CTTCCTTTTTGTCCACTTTG R:TTACAAAGAGAAATATCCATAGATA	exon3	317

Statistical analysis: Genotypes and allelic frequencies were calculated using the POPGENE software (ver. 1.31), and Hardy-Weinberg equilibrium for each population was analyzed using a χ^2-test. The analysis of associations between the *GnRHR* genotypes and litter traits was carried out using the GLM procedure by using SAS software (Statistical Analysis System 8.2, SAS Institute Inc.) according to the mathematical model: $Y_{ij}=\mu+G_i+E_{ij}$.

The level of significance was defined as P≤0.05, where Y_{ij} was the record of individual j with genotype i; μ was defined as population mean; G_i was defined as the genotype effect; and E_{ij} was defined as the residual effect.

RESULT

Amplification of the coding region (CDS) of the *GnRHR* gene in giant panda: The 618, 455, and 317bp fragments of the *GnRHR* gene of giant pandas were amplified by PCR within three pairs of primers named *GnRHR* primer1, *GnRHR* primer2, and *GnRHR* primer3, respectively. The length of the amplified fragments

agreed well with the expected results, and the target bands were clear and specific, which allowed them to be used for direct DNA sequencing.

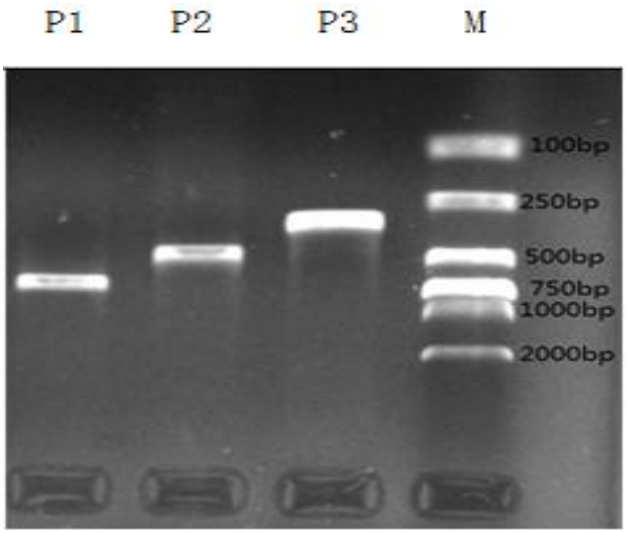

Fig.1. Partial results of the amplification of the *GnRHR* gene coding region (CDS) in the giant panda.

P1, P2 and P3, respectively, indicate 618bp and 455bp and 317bp fragments amplified; M. DNA Marker DL 2000

Polymorphisms of the *GnRHR* gene in giant panda: Comparative analysis of the giant panda *GnRHR* gene sequencing indicated two single mutation sites that were found in exons 1 and 2, respectively. According to customary nomenclature, the single mutation site in exon 1 was named T516121C, and the T516121C substitution led to one amino acids mutation (Cys>Arg). Similarly, the single mutation site in exon 2 was named C504498T, and the C504498T substitution led to one amino acids mutation (Ala>Val). A mutation site in the exon3 was not detected.

Genotypic and allelic frequencies: In the analysis of giant panda populations, three different genotypes of the T516121C locus were named AA, AB and BB, and the different genotypes of C504498T locus were named CC, CD and DD (Table 2). At the T516121C locus, the AB genotype was the predominant genotype, and allele B was the dominant allele. In the C504498T locus, the CD genotype was the predominant genotype, and allele C was the dominant allele. Furthermore, the results of the fitness of Hardy-Weinberg equilibrium were in equilibrium (P>0.05).

Table 2. Allelic and genotypic frequencies of *GnRHR*.

Mutation	site	Genotype frequency			Allele frequency		χ^2 value
T516121C	exon1	AA	AB	BB	A	B	
		2(0.05)	22(0.58)	14(0.37)	0.34	0.66	0.04
C504498T	exon2	CC	CD	DD	C	D	
		14(0.37)	15(0.39)	9(0.24)	0.57	0.43	0.23

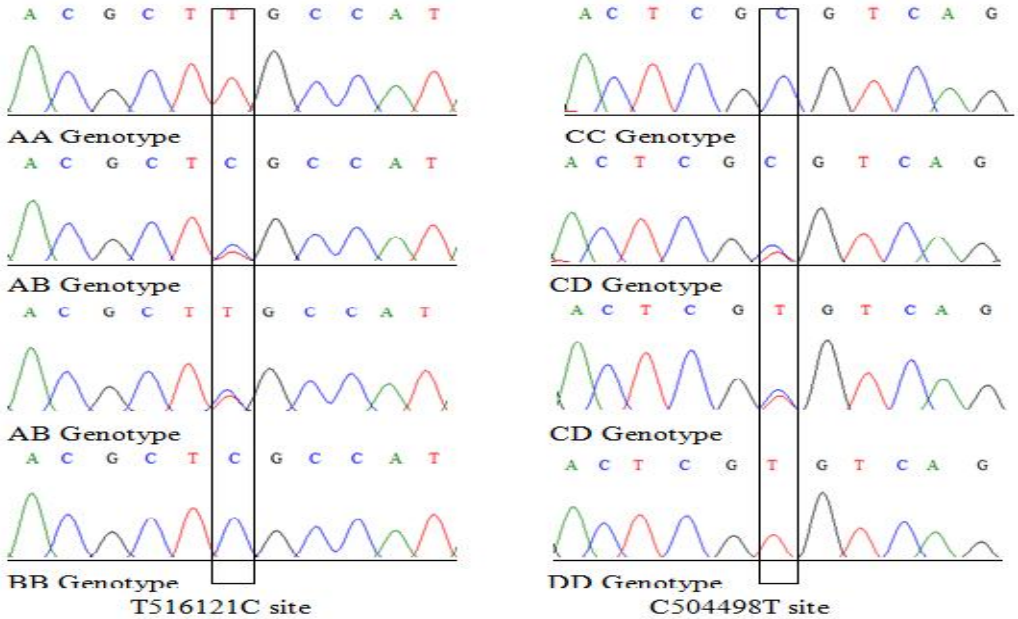

Fig. 2. Sequence comparison of different genotypes of *GnRHR* gene

The relationship between litter traits and the polymorphism of the *GnRHR* gene: The average and standard deviation for the litter sizes of different genotypes of the *GnRHR* gene in giant panda are given in Table 3. We can see from Table 3 that the first birth of the giant panda with an AA genotype had the maximum average litter size and that the first birth with the BB genotype had the minimum average litter size. The average litter size of the first birth with an AB genotype was greater than the size of the BB genotype. The giant panda with the CC genotype had a greater first birth average litter size than did the CD and DD genotypes. In addition, compared with the CD and DD genotypes, the giant panda with the CC genotype had the largest average litter size for second births (P≤0.05).

Table3. Least squares mean and standard error for litter size of different *GnRHR* genotypes.

Mutation site	Genotype	No. of samples	The average litter size of first birth	The average litter size of second birth
	AA	1	2*	1*
T516121C	AB	19	0.95±0.17^{a}	0.63±0.19^{a}
	BB	7	0.86±0.37^{a}	0.57±0.31^{a}
	CC	11	1.27±0.22^{a}	1±0.22^{a}
C504498T	CD	13	0.77±0.21^{a}	0.46±0.21^{ab}
	DD	3	0.67±0.43^{a}	0^{b}

Note: With* in the table are not included in the statistical data; Values with different superscripts within the same column differ significantly at P≤0.05.

DISCUSSION

At present, the giant panda breeding research concentrates primarily on the panda rutting characteristics, reproductive behavior, hormones related to reproduction, and artificial insemination technology. Comparatively, there are only a few studies on the molecular regulation mechanism on the fertility of the giant panda. Moreover, even fewer reports on which major genes could influence the fertility of the giant panda. *GnRHR*, which can regulate the synthesis and secretion of *LH* and *FSH*, plays a key role in mammalian reproduction. However, it is still unknown that whether *GnRHR* could also influence the reproduction of giant panda. Therefore, this study first detected giant panda *GnRHR* gene polymorphisms. Then, the association of the *GnRHR* gene polymorphism with the litter size of the giant panda was analyzed, seeking a new theoretical basis for improving the reproductive capacity of the giant panda.

Analysis of polymorphism of *GnRHR* gene in giant panda: In recent years, the *GnRHR* gene, as a candidate gene for litter size traits, was found to be closely related to the reproductive capacity of mammals. Furthermore, a large number of researchers have detected different SNP loci in the *GnRHR* genes of various mammals, and these SNP loci were closely related to the reproductive capacity of those mammals. In this study, the SNP loci T516121C and C504498T were found in exons 1 and 2 of the giant panda *GnRHR* gene, and no SNP locus in exon 3 was detected. TheT516121C and C504498T loci belong to non-synonymous mutations, and the T516121C substitution led to one amino acid mutation (Cys>Arg). Similarly, the C504498T substitution led to another amino acid mutation (Ala>Val). Correlation analysis suggests that the C504498T mutation can affect the panda litter size trait. Because these two loci are located in the exon region that encodes protein sequences, the mutations are very important. De Roux *et al*. (1997) found that G317A and A785G, two mutations that occur in human *GnRHR* cDNA, cause the amino acid changes (Gln106Arg and Arg206Gln). Additionally, it caused the amino acid sequence to change, which weakened the binding force between *GnRHR* and ligand. This is closely related to the low function of the familial primary brain pituitary gonadal hormone, thereby affecting reproductive ability (De Roux *et al*. 1997). Jiang *et al*. (2001) reported a C1721G substitution in the 3'UTR (untranslated region) that was detected in an F2 population of Meishan×European Large White pigs, and a 1721 G allele with a greater first litter size in Meishan pigs (Jiang *et al*. 2001). Han *et al*. detected *GnRHR* gene exon 1 in Saanen Dairy Goat and *GnRHR* gene exon 2 in the Boer goat in the presence of mutation sites, and they were associated with litter size (Han D *et al*. 2009). Liu *et al*. detected the single nucleotide polymorphism of the *GnRHR* gene in the Laoshan dairy goat. These results showed that the mutation of the gene A261G for multiparous ewe GA genotype litter size was significantly higher than that of the AA genotype (Liu N*et al*. 2014). Further, Zhang *et al*. found an A/C mutation in the *GnRHR* exon 1 of Huanghuai goat and Boer goat and determined that the litter size of Huanghuai goats with the BB genotype is greater than that of the AA genotype (P≤0.05) (Zhang YJ *et al*. 2010). Sun *et al*. reported the amplification of the *GnRHR* gene exon 2 fragment and found that the Small Tail Han ewes with mutant homozygous genotype DD had 0.81 (P ≤0.01) more lambs than did those with the wild type CC

(Sun J *et al*. 2008). In addition, Dunn *et al*. used PCR-RFLP in hen groups to detect *GnRHR* gene polymorphism, showing an additive effect of the *GnRHR* gene on the number of double-yolked eggs in one generation of a commercial broiler breeder hen population (Dunn *et al*.2004). These studies show that the *GnRHR* gene is closely related to the reproductive performance. Combining the results of this study, we predicted the C504498T base mutation would impact the giant panda *GnRHR* gene function and influence the giant panda reproductive function, but the specific impact mechanism requires further research.

The relationship between litter size and the polymorphism of *GnRHR* gene in giant panda: The results of this study showed that the T516121C and C504498T loci were both in the Hardy-Weinberg equilibrium state (P>0.05). The giant panda populations from Wolong nature reserve in Sichuan Province (PR China) and the group without any artificial selection tried to make the population in their natural state. The results of this study objectively reflect the characteristics of the giant panda population. At present, the number of giant pandas in captivity shows great improvement, but the low reproduction rate problem for the giant panda has not been completely resolved. Therefore, it is very necessary to strengthen the research on the biology of giant panda reproduction and explore the related genes and mechanisms underlying giant panda reproduction. The result in this study showed that different genotypes of different SNP loci have different effects on the average litter size of the first and second births of the pandas. The different genotypes of the C504498T locus in the giant panda *GnRHR* gene had significant effects on the average litter size of the second birth (P≤0.05). We speculated that the C504498T locus may influence panda litter size related traits. Therefore, it is inferred that the *GnRHR* gene may be a major gene that controls litter size traits in giant panda. If so, the *GnRHR* gene can be considered the main candidate gene to improve giant panda litter sizes.

Conclusion: In summary, this study showed two SNP loci that were found in the *GnRHR* gene exons 1 and exon 2 of the giant panda. The C504498T locus was found in exon 2 and led to an amino acid mutation (Ala>Val). This mutation had a significant influence on the litter size of giant pandas. Therefore, these results suggest that the C504498T base mutation in the exon 2 of *GnRHR* could influence the litter size of giant pandas. In other words, *GnRHR* could influence the fertility of giant pandas. The giant panda *GnRHR* gene polymorphism was first detected in this study, and this finding provides a theoretical basis for improving the reproductive capacity of the giant pandas.

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