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A Y-linked SNP in *SRY* Gene Differentiates Chinese Indigenous Swamp Buffalo and Introduced River Buffalo*

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ABSTRACT : The complete coding region sequence of the *SRY* gene in Chinese swamp buffalo was determined by PCR product sequencing. Comparison of swamp and river buffalo *SRY* gene sequences revealed a single nucleotide polymorphism (SNP, C/G) at the 202 bp site of the coding region. Further, a total of 124 male domestic buffaloes were genotyped at this SNP site using the PCR-SSCP method, and it was found that all Chinese indigenous swamp buffaloes had a guanine (G) at this site, while introduced river buffaloes and crossbred buffaloes showed a cytosine (C). Our findings suggested that this Y-linked SNP displayed type-specific alleles differentiating swamp and river buffaloes, and could be used as an effective marker to detect crossbreeding of swamp buffaloes with introduced river buffaloes in native buffalo populations, and thereby assess genetic diversity status and make proper conservation decisions for indigenous swamp buffaloes. In addition, this SNP can be potentially applied in the study of Asian water buffalo phylogeny from a male perspective. (**Key Words :** Swamp buffalo, River buffalo, *SRY*, SNP, Genetic diversity, Conservation)

INTRODUCTION

China is rich in domestic animal genetic resources. In recent years, growing numbers of studies focused on genetic diversity of the indigenous livestock species, such as goose (Tu et al., 2006), cattle (Jin et al., 2005), sheep (Sun et al., 2003), pig (Li et al., 2000; Wang et al., 2004). However, the domestic buffalo, as an important livestock species closely related to crop production, is often neglected due to its smallholder farming system, low reproduction rate and low efficiency. According to FAO statistics (2005), there are approximately 22.3 million water buffaloes in China, which is the third largest water buffalo population in the world. Chinese indigenous buffaloes are of swamp type and classified into twenty-four geographically defined populations in terms of body conformation, adaptation to environment and distribution (Chen and Xu, 2004). However, rapid mechanization of farming system and widespread crossbreeding of native swamp buffalo with introduced river buffalo have resulted in dramatically decline of some local buffalo populations and consequently threatened the indigenous swamp buffalo genetic resources (Ma et al., 2002). In recent years, therefore, more conservation efforts have been initiated in order to maintain water buffalo genetic diversity and native swamp buffalo resources with potentially economic, cultural, and ecological values for the sake of future market demand and research needs.

Many previous studies have been reported on the water buffalo genetic diversity. A variety of genetic markers, including allozyme (Amano 1983; Barker et al., 1997a), mitochondria DNA (Amano et al. 1994; Tanaka et al., 1995; 1996; Kikkawa et al., 1997) and autosomal microsatellites (Barker et al., 1997b), all demonstrated the huge genetic variations between swamp buffalo and river buffalo. Since crossbreeding of two types of buffalo is predominantly practised by indigenous female swamp buffaloes with introduced male river buffaloes in China, detection of genetic markers on Y chromosome of river buffalo will benefit for effectively assessing the extent to which crossbreeding occurred in a swamp buffalo population. However, as for genetic variations of buffalo revealed by molecular Y-linked markers, up to now only one polymorphic Y-specific microsatellite has been reported in swamp buffalo (Edwards et al., 2000). *SRY* gene, locating in non-recombining region of Y chromosome, is a male-specific gene and has received considerable attentions in

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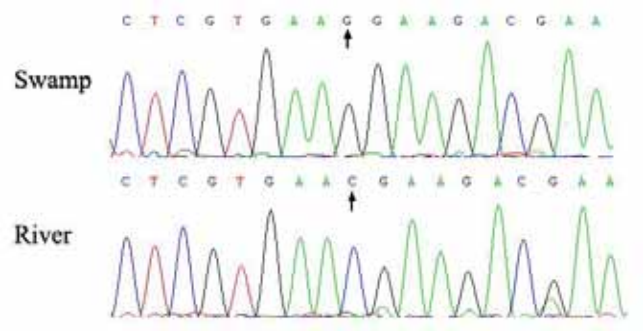

Figure 1. A SNP in swamp buffalo *SRY* gene in comparison with river buffalo detected by sequencing.

genetic diversity and evolution studies of animals in recent years (Kikkawa et al., 2003). In the present study, the sequence and polymorphism of *SRY* gene was determined in Chinese swamp buffalo in comparison with river buffalo, and its genetic variation in various buffalo populations was further analyzed.

MATERIALS AND METHODS

Animals and DNA extraction

A total of 116 skin samples of male swamp buffaloes were collected from eight populations in China: Enshi (10) in Hubei province; Dechang (10) in Sichuan province; Dehong (10) in Yunnan province; Fuzhong (30) in Guangxi province; Xinfeng (10) in Jiangxi province; Fuan (24) in Fujian province; and Xinglong (10) in Hainan province; Shannan (12) in Shanxi province. The blood samples of eight male river buffalo individuals were obtained from two river buffalo breeds, Indian Murrah (2) and Pakistani Nili Ravi (6), which are reared in Guangxi Buffalo Research Institute. Genomic DNA was isolated from tissue or blood cells using a standard proteinase K digestion followed by phenol/chloroform extraction (Sambrook et al., 1989).

PCR amplification and sequencing

According to the available *SRY* gene sequences of river buffalo (Genbank accession no. AY341337) and cattle (AF148462), a pair of primers (BSRY, 5′-TTGCA CTAAATCAGTCTCTG-3′ and 5′-TGTTAGCGAGAGTA AGGAAG-3′) was designed to amplify a segment of 948 bp including complete coding region and partial flanking sequences of domestic buffalo *SRY* gene. PCR reaction was carried out in a final volume of 20 μl containing 50 ng of genomic DNA, 2 mM MgCl2, 200 μM each dNTP, 10 μM of each primer, 1 unit of Taq DNA polymerase. The mixture was incubated at 95°C for 10 min followed by 30 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 30 s, and a final incubation at 72°C for 5 min. The PCR products were excised from 1.0% agarose gel and purified with the BioGene Geneclean III Kit (Carlsbad, CA, USA), and then were sequenced on the ABI 3730XL DNA Sequencer using the BigDye™ Terminator Cycle Sequencing Kit (Applied Biosystems, Foster, CA, USA). Both forward and reverse primers were applied to sequence.

PCR-SSCP analysis

Based on the sequencing results, a single strand conformation polymorphism (PCR-SSCP) method was developed to test the genotype of *SRY* gene of all individuals. Specific primers flanking the SNP (BSRYS, 5′-ATGTGAAAGGGGAGAAAATG-3′ and 5′- AGGTCGATA TTTATAGCCCG -3′) were designed to amplify a fragment of 258 bp. The mixture including 2 μl of PCR products and 5 μl denaturing buffer (98% formamide, 0.09% xylene cyanole FF and 0.09% bromophenol blue) of each individual was denatured at 94°C for 10 min, followed by a rapid chill on ice for 10 min, and then electrophoresed for 14 h at 7 V/cm on 12% polyacrylamide gel. The DNA bands were visualized by silver staining (Qu et al., 2005).

Analysis of crossbred buffaloes by mitochondrial cytochrome b gene

The cytochrome b gene was amplified by a pair of specific primers (5'- ACCACGACCAATGATATGAAAAA CC -3' and 5'- GAGGTTGGTTGTTCTCCTTTTCTGG -3') and sequenced in the 12 male Shannan buffaloes with the same genotype of river buffalo *SRY* gene. The experimental condition was same as described above.

RESULTS

Sequence analysis and SNP identification of swamp buffalo *SRY* gene

Taking one genomic DNA sample selected from the Enshi population as a PCR template to amplify and sequence *SRY* gene, the sequence in length of 843 bp of Chinese swamp buffalo *SRY* gene was firstly identified (GenBank accession no: DQ119747). Then, we searched for its coding region using the ORF finder software (Tatusov T. and R. Tatusov, http://www.ncbi.nlm.nih.gov/gorf/gorf.html). In complete agreement with those of river buffalo and cattle, the coding region of *SRY* gene of swamp buffalo consists of 690 nucleotides, encoding 229 amino acids. Through comparing the *SRY* gene coding region sequences of swamp with river buffalo, it showed a very high identity, by 99.9%, except one nucleotide locating at the site of 202 bp of the *SRY* gene coding region was a cytosine (C) for river buffalo but a guanine (G) for swamp buffalo, which led to a missense replacement of glycine in stead of arginine (Figure 1).

With a multiple alignment of the *SRY* gene sequences of different species within Bovidae family, including taurine

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                    11111111 1112222222 2333333334 4444444444 4555555555 5666666666 66666
               1355666777 8901112345 5680123346 7334588990 0122344779 9022344458 9011222444 67778
               8969146067 6851789304 9072465897 0463234055 9019726796 8405501657 8246179136 31297
Zebu cattle    CTCCCGTAGA TGCTGTACGG CGGCGAAACA TGCCTGAGCG CCTACCTTGT CAAGGCACCG AACAGGCTAA AGTTT
Taurine cattle ..........  ..........  ..........  ..........  ..........  ..........  .......G..  .....
Banteng cattle ..........  ..........  ..........  ....T.....  ..........  ..........  ..........  .....
Yak            ..........  ..........  .....T....  ..........  ..........  ..........  ..........  .....
American bison .....T....  ..........  T.........  ..........  ...T......  ..........  ..........  .....
European bison ..........  ..........  T.........  ..........  ..........  .G........  ..........  .A...
Swamp buffalo  .GAT......  C...A..A..  .ATGT...GC  .T.A.A.AT.  ..........  T.........  .G....A..  .....
River buffalo  .GAT......  C...A..A..  .AT.T...GC  .T.A.A.AT.  ..........  T.........  .G....A..  .....
Sheep          AGA.T.CGAC  .A.C.CGA.A  ......CCG.  C.T..AG..C  G...AGCCAC  ...C.T.GTA  GG.TAC..TC  ..CG.
Goat           AGA.T.CGAC  .AT...CAC.  ......TCG.  C.T..A....  GAC.AG.CAC  ..TCCTC.TA  GGA..C..TC  G.CGC
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Figure 2. Alignment of the coding region of *SRY* gene of several species within Bovidae family. Multiple sequence alignment was conducted using CLUSTAL W integrated in MAGE3 software (Kumar et al., 2004).

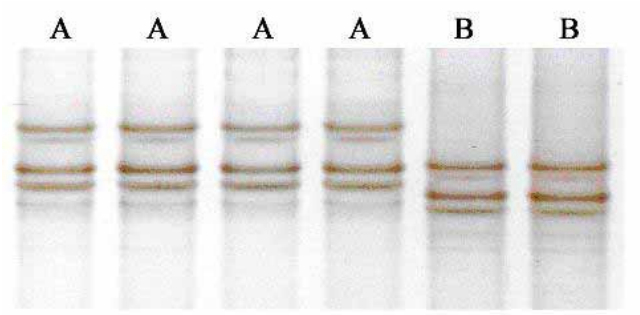

Figure 3. Partial results of PCR-SSCP analysis. Lanes 1-4: A (202G); Lanes 5, 6: B (202C).

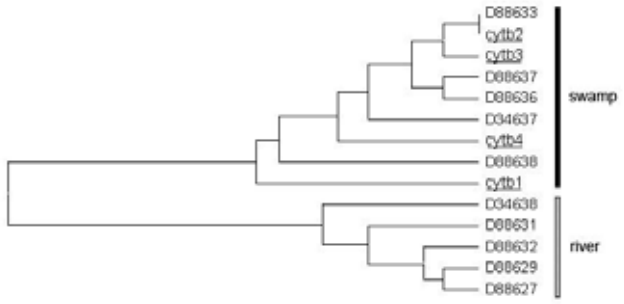

Figure 4. Phylogenetic relationships among cytochrome b gene sequences of domestic buffalo constructed in MAGE3 software adopting Kimura 2-parameter distance and UPGMA method. In which cytb1, cytb2, cytb3 and cytb4 are four haplotypes found in Shannan buffaloes in this study, and the others are from Kikkawa et al. (1997).

cattle (AF148462), zebu cattle (AY079145), banteng cattle (AY079146), American bison (AY079141), European bison (Z30321), yak (AF148463), goat (Z30646), and sheep (Z30265), we found no mutation at this site in these species except in domestic buffalo (Figure 2). So this SNP (G202C) in *SRY* gene appeared to be unique to the water buffalo (*Bubalus bubalis*).

PCR-SSCP analysis

Taking the sequenced sample as a control, we clearly genotyped all the samples on this SNP (C/G) at the site of 202 bp of *SRY* gene coding region by PCR-SSCP method. The results indicated that all the male swamp buffalo individuals from various populations, with the exception of 12 Shannan buffaloes, presented identical DNA bands pattern, defined as A, while eight river buffalo individuals showed another genotype, called B, with altered mobility in the gels (Figure 3). It should be noted that 12 individuals from Shannan population showed the same genotype as that of river buffalo.

Furthermore, we confirmed this SNP by sequencing the *SRY* gene of another one swamp buffalo from Dechang population, one from Shannan population, one from Murrah, and one from Nili Ravi. Results demonstrated that guanine was present at this SNP in the individual with A genotype, but cytosine in the B individuals.

Cytochrome b gene sequences of crossbred buffaloes

In order to further identify the origin of the 12 Shannan buffaloes with the same *SRY* genotype as that of river buffalo, a 574 bp DNA fragment of cytochrome b gene, which is exclusively maternally inherited, was determined (GenBank accession nos. DQ480075-DQ480078) and compared to the published cytochrome b sequences of domestic buffaloes (Kikkawa et al., 1997). A total of four haplotypes, named cytb1, cytb2, cytb3 and cytb4, were revealed. Sequences analysis showed that all the four haplotypes in the 12 Shannan buffalo were of swamp mitochondrial type (Figure 4).

DISCUSSION

Samples of Shannan buffalo population

According to previous reports (Chen and Xu, 2004), native Shannan buffalo is belonging to swamp type with typical swamp buffalo morphology and body conformation. In our study, however, twelve Shannan buffalo samples were detected to show the same genotype of *SRY* gene as that of river buffalo. After checking the figures of sampled

individuals, we found that these samples have some characters of river buffalo, such as long tail, black skin, and short curled horns. Besides, our study on mitochondria DNA also showed that they had mtDNA haplotypes of indigenous swamp buffalo. Hence, it is confirmed that these samples are crossbred offspring of swamp buffalo×river buffalo crossbreeding.

Is this Y-linked SNP type-specific?

Domestic buffaloes have been classified into two types, swamp and river buffaloes, with distinct morphology and separated distribution (Mason, 1974). Cytogenetic (Fischer and Ulbrich, 1968), biochemical (Amano, 1983) and molecular genetic (Amano et al., 1994; Tanaka et al., 1995; 1996; Kikkawa et al., 1997) studies also demonstrated their marked divergence. In this study, we found that these two types of domestic buffaloes showed different alleles of the SNP at the site of 202 bp of coding region of *SRY* gene, G in swamp buffalo and C in river buffalo. Moreover, it has been reported that cytosine was presented at the same site of *SRY* gene in Mediterranean river buffalo (Parmar et al., 2004). Therefore, it seems that this Y-linked SNP shows type-specific alleles in domestic buffalo differentiating swamp buffalo and river buffalo. However, further investigations based on larger samples from various regions, such as Indian subcontinent and Southeast Asian, are required to confirm this hypothesis.

Application of this type-specific SNP

Two imported river buffalo breeds, Murrah and Nili Ravi, had been used in Chinese native buffalo genetic improvement since 1970's, but the magnitude of crossbreeding in different local swamp buffalo populations remained unclear. Since the Y-linked SNP of *SRY* gene found in this study showed distinct alleles in Chinese swamp buffalo and imported river buffalo breeds, it can be used as an effective marker for sensitively detection of the hybrid individuals paternally derived from river buffalo in Chinese indigenous buffalo populations. In this respect, analysis of individual genotypes of this type-specific marker and its genetic variations of various local buffalo populations would provide supports to objectively assess genetic diversity status of indigenous buffaloes, subsequently, further make proper conservation decisions.

In addition, although previous genetic diversity studies using microsatellite (Barker et al., 1997b) and mitochondria DNA (Kikkawa et al., 1997; Lau et al., 1998; Kierstein et al., 2004) revealed the obviously differentiation between swamp and river buffalo, their phylogenetic relationships are still largely undetermined. Y-linked DNA polymorphisms are of great value for the study of evolutionary relationships between different populations from paternal origin (Bradley et al., 1994; Hanotte et al., 2000; Kikkawa et al., 2003). So it is expected that this Y-specific SNP marker would act as an useful marker for investigating domestic buffalo phylogeny from a male perspective. Studies on distribution and frequencies of two alleles of this SNP in different domestic and wild buffalo populations will gain an insight into the domestication and dispersion of Asian water buffalo (*Bubalus bubalis*).

In summary, a type-specific SNP of *SRY* gene was identified in Chinese swamp buffalo and introduced river buffalo in this study, which could be used to assess genetic diversity, make conservation decisions and investigate the evolutionary history of the domestic buffaloes. Besides, the simple and effective PCR-SSCP method established here can directly be applied to genotype the SNP in other buffalo populations.

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