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Divergence and Phylogenetic Analysis of Myostatin Gene in Dulong Gayal

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Abstract: Using the methods of PCR amplification and sequencing, the polymorphism of myostatin (MSTN) gene were detected in 12 Dulong gayals.The results showed that, 1128 base pairs existed in the 12 samples, 1 polymorphic site was observed with 2 haplotypes, G (75%) and A (25%) and the average number of nucleotide differences (k) was 0.409 in Dulong cattle. The findings indicated that MSTN gene of Dulong gayal had a low level of gene diversity, but it did not inherit independently, with some genetic communication with yak, demonstrating a loss of genetic resource in Dulong gayal and effective measures needed to protect the gayal population.

Key words: Gayal, gene diversity, divergence, phylogenetic, MSTN

INTRODUCTION

Gayal, *Bos frontalis*, known as a rare semi-wild animal, is distributed in Dulong river basin, Nujiang river basin of Gao Li Gong Moutain in the west of Yunnan province in China and Menyu and Luoyu regions, which are still occupied by India and Assam province in India and East Bengal and Kachin province in the northern part of Burma. Gayals in China are generally called Dulong Cattle, named after the Dulong river. After the discovery of Dulong Cattle in breed resources investigation in 1978, researches on Dulong Cattle were conducted henceforth. The number, form and configuration of gayal chromosomes were all different from yellow cattle (Bos taurus) and gaur (Bos gaurus), their number of chromosomes (2n) were 58, 60 and 56, respectively. There was no submetacentric chromosome in the euchromosomes of yellow cattle, while 2 pairs in gaur and one pair in gayal (Shan *et al*., 1980), At the level of enzyme gene, Dulong Cattle lacks genetic diversity (Long *et al*., 1995). To the origin of Dulong Cattle, some researchers proposed that it originated from male gaur and female zebu (Winter *et al*., 1984). Dulong Cattle was also regarded as a species of multiple origins (Hong *et al*., 1993). Moreover, according to the local folklore, Dulong Cattle was a domesticated type of gaur with more than 100 years of domestication. As a typical population of gayal, Dulong Cattle is a unique resource of gayal in China, endangered with a population of about 1,000. It would become extinct without proper protection.

Bovine myostatin gene (MSTN), also called "double-muscling" gene, is located in the chromosome 2 of bovine. The loss of its function would lead to a feature of double muscle in cattle and the divergence of the traits restrained by the gene is under the pressure of selection. The evolutionary situation at the molecular level would help us acknowledge the divergence level of Dulong Cattle and reveal the systemic origination of bovines in China and would provide some objective proof for exploring the relation between molecular evolution and adaptive evolution. The study detected the sequences of myostatin gene in 12 Dulong cattle, analyzed the nucleotide polymorphism within population and compared with 5 other species of close kinship in bovinae and conducted a phylogenetic analysis, for the purpose of exploring the phylogenetic divergence of myostatin gene in gayal and providing some molecular biological proof for protecting the rare genetic resource.

MATERIALS AND METHODS

Animal population and genomic DNA extraction: Applying simple random sampling in typical colony methods in the central area of habitat, 12 gayal blood samples were collected from Dulong Breed Protection and Reproduction Base in Lushui county of Nujiang State, Yunnan Province. The genome DNA was extracted by hydroxy benzene/chloroform method.

Table 1: Polymerase chain reaction

P1	Forward	5'- GGCTTGGCGT TACTCAAAAGC-3'
	Reverse	5'- CTCCTCCTTACGTACAAGCCAGCA-3'
P2	Forward	5'-GTTCATAGATTGATATGGAGGTGTTCG-3'
	Reverse	5'-ATAAGCACAGGAAACTGGTAGTTATT-3'
P3	Forward	5'-GAAATGTGACATAAGCAAAATGATTAG-3'
	Reverse	5'-ATACTCTAGGCTTATAGCCTGTGGT-3'

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Polymerase chain reaction: Exon sequences and some intronic sequences were amplified from genomic DNA by the method of PCR. GenBank sequence from *Bos taurus* (GenBank Accession No: AB076403) was used to design primers (Tay *et al*., 2004) (Shanghai Genecore company). The primers were listed below. In a typical reaction, 25 uL PCR system contained 10×PCR buffer 2.5 μL, Mg^{2+} 2.5 μM, dNTP 400 μM, Taq DNA enzyme 1U (Takara company, China), Forward and Reverse primer 0.4 μM each, template DNA 1 μL. The amplification program consisted of predenaturation (94°C 3 min), followed by 34 cycles of denaturation (94°C 0.5 min), primer annealing (51°C 0.5 min) and extension (72°C 0.5 min) and a final extension (72°C 3 min). The PCR products were stored at 4°C (Table 1).

Sequencing and analysis of MSTN gene exons: Three exons are separated by 2 introns in MSTN gene. PCR products were first detected using 1.2% agarose gel or 10% PAGE gel and then sequenced (SANGON company, Shanghai, China). The sequencing results were analyzed through DnaSP software and compared with the reported GenBank sequences, *Bos taurus* (GenBank accession number: AB076403), *Bos indicus* (GenBank accession number: AY794986) and *Bos grunniens* (GenBank accession number: AY786413, AY787760, AY787761), to describe the nucleotide divergences of MSTN gene among Dulong Cattle population. Using the reported GenBank data, Bubalus bubalis (GenBank accession number: DQ159987), as the outgroup and MEGA software to construct NJ phylogenetic tree based on Kimura model, each P_B value of the nodes was detected by Bootstrap test (1000 replication).

RESULTS

PCR reaction and sequencing: The designed primers were used to amplified 12 DNA samples. The size of PCR band for each pair of primers was similar to literature (Winter *et al*., 1984). The sequences of PCR products for 12 DNA samples contained exon 1 (375bp), exon 2 (372bp) and exon 3 (381bp), which are identified with the reported exons in literature (Table 2).

Sequence analysis: the average content for each base MSTN gene in Dulong Cattle was 25.4%T, 21.4%C, 31.1%A, 22.1%G, No significant biases of base content were detected in the population. Only one nucleotide polymorphic site was observed at 639bp from ATG (nt111), with a transition from G (75%) to A (25%), while the reported normal cattle, *Bos taurus* (GenBank accession number: AB076403), zebu, *Bos indicus* (GenBank accession number: AY794986), yak, *Bos grunniens* (GenBank accession number: AY787760), shared a G haplotypes at this site, but Bubalus bubalis (GenBank accession number: DQ159987), held an A haplotypes.

DNA divergence within population and clustering analysis: The sequences of the 12 samples were analyzed by DnaSP software. The average number of nucleotide differences (k) was 0.409 in Dulong cattle. The average nucleotide diversity under the Jukes and Cantor model was 0.00036 + 0.00012, indicating a very low level of genetic divergence.

Reconstruction of phylogenetic relationship: According to the NJ phylogenetic tree (Fig. 1), the 12 gayals were

Table 2: Exon sequences of MSTN in Leiqiong and Mongolia cattle

Exon 1	1	ATGCAAAAACTGCAAATCCCTGTTTATATTTACCTATTTATGCTGATTGTTGCTGGCCCAG
	61	TGGATCTGAATGAGAACAGCGAGCAGAAGGAAAATGTGGAAAAAGAGGGGCTGTGTAAT
	121	GCATGTTTGTGGAGGGAAAACACTACATCCTCAAGACTAGAAGCCATAAAAATCCAAATC
	181	CTCAGTAAACTTCGCCTGGAAACAGCTCCTAACATCAGCAAAGATGCTATCAGACAACTT
	241	TTGCCCAAGGCTCCTCCACTCCTGGAACTGATTGATCAGTTCGATGTCCAGAGAGATGCC
	301	AGCAGTGACGGCTCCTTGGAAGACGATGACTACCACGCCAGGACGGAAACGGTCATTACC
	361	ATGCCCACGGAGTGT
Exon 2	376	GATCTTCTAACGCAAGTGGAAGGAAAACCCAAATGTTGCTTCTTTAAATTTAGCTCTAAG
	436	ATACAATACAATAAACTAGTAAAGGCCCAACTGTGGATATATCTGAGGCCTGTCAAGACT
	496	CCTGCGACAGTGTTTGTGCAAATCCTGAGACTCATCAAACCCATGAAAGACGGTACAAGG
	556	TATACTGGAATCCGATCTCTGAAACTTGACATGAACCCAGGCACTGGTATTTGGCAGAGC
	616	ATTGATGTGAAGACAGTGTTGCARAACTGGCTCAAACAACCTGAATCCAACTTAGGCATT
	676	GAAATCAAAGCTTTAGATGAGAATGGCCATGATCTTGCTGTAACCTTCCCAGAACCAGGA
	736	GAAGATGGACTG
Exon 3	748	ACTCCTTTTTTAGAAGTCAAGGGAACAGACATCCCAAAAAGATCTAGGAGAGATTTTGGG
	808	CTTGATTGTGATGAACACTCCACAGAATCTCGATGCTGTCGTTACCCTCTAACTGTGGAT
	868	TTTGAAGCTTTTGGATGGGATTGGATTATTGCACCTAAAAGATATAAGGCCAATTACTGC
	928	TCTGGAGAATGTGAATTTGTATTTTTGCAAAAGTATCCTCATACCCATCTTGTGCACCAA
	988	GCAAACCCCAGAGGTTCAGCCGGCCCCTGCTGTACTCCTACAAAGATGTCTCCAATTAAT
	1048	ATGCTATATTTTAATGGCGAAGGACAAATAATATACGGGAAGATTCCAGCCATGGTAGTA
	1108	ATCGCTGTGGGTGTTCATGA

Note: Framed letters are polymorphic sites: R stands for G or A

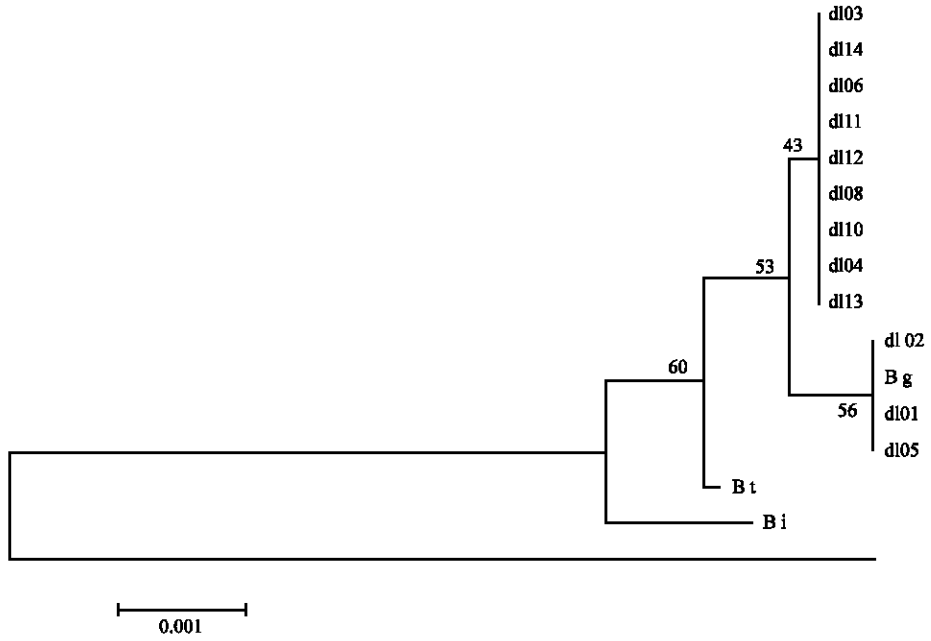

Fig. 1: NJ tree based on kimura 2-parameter model with Bootstrap test (1000 replications)

divided into 2 clusters, 9 gayals clustered into one group, 3 gayals together with yak clustered into the other group, then the 2 groups clustered with normal cattle and zebu consecutively and last, clustered with water buffalo. The above phylogenetic relations reflected basically the actual grouping situation and on the other hand, revealed that yak had some genetic relations with part of the gayal individuals.

DISCUSSION

The animal genome consists of nuclear genome and extranuclear genome, or mtDNA. Nuclear genome consists of coding DNA and noncoding DNA. The mutation of noncoding DNA is generally neutral and accumulated during the long history of evolution, researches on which would help to provide important information on the origination and divergence of species, evolution and migration. While coding DNA are similar or almost the same within species even among close related species. The tiny difference in the coding DNA region would lead to the difference of phenotypic traits. Therefore, researches on the diversity of animal nuclear genomes would be of great importance to understand the phenotypic difference and explore the history of livestock evolution. As a functional nuclear gene, the MSTN gene, especially the coding area, is relatively conservative, but in gayal, the appearance of a mutation rate of 25% was a key message, indicating that possible positive selection or gene communication with other close related species existed during the process of origination and divergence.

According to the sequence of the gayal MSTN exons, gayal population had a low level of genetic divergence, the absolute value of Pi was different from the value b ased on the mtDNA, like Cyt b gene (Guolong *et al*., 2007). However, the population size of Dulong cattle was rather small 2 decades ago, with a population of about 77. The genetic drift and environmental disturbance had long been affecting the genetic situation. Some hybrids of gayal and local normal cattle were reported with a good growth performance. The findings in our study together with the related reports demonstrated that the Dulong gayal resource was under certain destruction, requiring effective protection and avoiding hybridization (Zhao *et al*., 2003).

The morphology of gayal is similar to that of Asian gaur (*Bos gaurus*), leading to a mistake of regarding gayal as the domesticated type of Asian gaur, or the offspring of Asian gaur and normal cattle. The comparison of MSTN exon sequences among 6 Bovinae species indicated a different level of relationships. Water buffalo had most nucleotide difference from any other species and served as an outgroup. Part of gayal shared a haplotype with part of yak, indicating a very close relation between them, even the form of hybridization; while gaur had a more distant relation with yak, normal cattle and zebu than gayal, inferring that the divergence of gaur was earlier than gayal, yak, normal cattle and zebu and that gayal was not the domesticated type or the offspring of Asian gaur. The difference in MSTN exon sequences

between bovinae species demonstrated that the molecular evolution of MSTN in Bovinae was also under positive selective pressure.

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