Biologia **67**/5: 1013—1017, 2012
Section Zoology
DOI: 10.2478/s11756-012-0088-8

Genetic characterization of four wild species of Chinese marmots using microsatellite markers

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Abstract: Marmots are large ground squirrels, and 14 species have been reported in the world, including four species of marmots (Himalayan marmot, Tarbagan marmot, gray marmot and long-tailed marmot) living in China. Although these biological resources are abundant in China, information regarding their genetic features is lacking, hampering further study regarding them. The aims of this research were to evaluate genetic variations of four species of Chinese wild marmots, and analyzed kinship of these marmot populations. In the current study, we collected samples of four species of Chinese wild marmot and analyzed the effective allele number, gene diversity, the Shannon index, and polymorphism information to evaluate genetic variations using 13 microsatellite loci. Based on Nei's genetic distance using the unweighted pair group method, we constructed a dendrogram to analyze the population kinship. We determined that all four Chinese marmot species had high genetic polymorphisms and departure from Hardy-Weinberg equilibrium. The Chinese marmots to be divided into two large groups: Himalayan marmot was independent group. Tarbagan marmot, gray marmot and long-tailed marmot were others; Tarbagan marmot and gray marmot showed a close kinship with each other, but long-tailed marmot did not have a close relationship with the other species. The high polymorphisms and the kinship of Chinese marmot populations were correlated with geographical terrain of their habitat. Himalayan marmot was characterized as living in unique alpine meadows in Qinghai-Tibet plateau and was affected by terrain; however, Tarbagan marmot, gray marmot and long-tailed marmot were characterized as living in grassland or alpine grassland and were not affected by terrain. Genetic features of Chinese wild marmots were investigated in this study. This may give using information regarding protection of Chinese wild marmot resource and further application of biomedical research.

Key words: marmot; genetic characterization; microsatellite markers; China

Introduction

Marmots are generally classified as Mammalia, Rodentia, Sciuridae, and *Marmota*, and 14 species have been reported in the world (Thorington & Hoffman 2005). Four species of marmots inhabit China, including the Himalayan marmot (*Marmota himalayana* Hodgson, 1841), the Tarbagan marmot (*Marmota sibirica* Radde, 1862), the gray marmot (*Marmota baibacina* Kastschenko, 1899) and the long-tailed marmot (*Marmota caudate* Geoffroy, 1844) (Fan 2007). As a laboratory animal model, marmots are used to study human chronic hepatitis B virus infection, hibernation, altitude physiology and cardiovascular diseases (Huang 1996; Menne & Cote 2007). Recently, we discovered that marmots in Qinghai Province of China were resistant to high fat diet-induced atherosclerosis (Li et al. 2012). Studies regarding the ecology and histology of marmots are extensive (Brandler et al. 2010; Caridini et al. 2005; Peiffer et al. 2004). Marmots play an important role in maintenance of ecosystem and equilibrium of ecology in plateau or grassland. However, marmot is major host of plague and spread infectious diseases. Consequently, marmot is constantly hunted in China. This is reason that the number of marmot populations has reduced. Reduction of the size of marmot population directly effect ecological equilibrium of plateau or grassland. Therefore, study regarding genetic diversity of marmot population in China has great significance for controlling number of marmot, maintaining ecological equilibrium, and creating laboratory animal model. So yet, the genetic features of Chinese marmots have not been investigated using molecular genetic markers.

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Fig. 1. Geographic illustration of marmot samples collected from Northern China.

Microsatellites are DNA sequences consisting of short tandem nucleotide (< 6 base pair) repeat motifs. They are also named short tandem repeats (STRs) or simple sequence repeats (SSRs) (Tautz & Schlotterer 1994; Litt & Luty 1989; Edwards et al. 1991), and they are abundant in eukaryotic genomes and show uniform distribution (Wang et al. 1994). Major unique features of microsatellites are high variability and codominance, which makes them unique compared to other molecular markers including restriction fragment length polymorphisms, amplified fragment length polymorphisms, and random amplified polymorphic DNA. Moreover, the regions flanking microsatellites are generally conserved in the same species; therefore, using primers targeting the flanking sequences of microsatellites by a polymerase chain reaction (PCR) method can amplify SSR regions and predict genetic polymorphisms.

Recently, genomic polymorphisms of the hoary marmot, the Alpine marmot and the Chinese Himalayan marmot were analyzed by microsatellite markers (Goossens et al. 2001; Hanslik & Kruckenhauser 2000; Kyle et al. 2004; Silva et al. 2003; Xu et al. 2009). Although these four species of marmots (Himalayan marmot, Tarbagan marmot, gray marmot and long-tailed marmot) are abundant in China, information regarding their genetic features is lacking. In the current study, we collected four species of Chinese marmots from five different locations in China and investigated the genetic variations and their relationship with each other by genotyping 13 microsatellite loci.

Material and methods

Marmot samples

The marmot samples were collected from five regions in Northern China as shown in Figure 1. Himalayan marmot (*M. himalayana*) samples ($n = 38$) were obtained from Yushu County of Qinghai province; Tarbagan marmot (*M. sibirica*) samples ($n = 36$) were collected from the Chenbarhu County of Inner Mongolia Autonomous Region; gray marmot (*M. baibacina*) sample were obtained from the Turgart of Wuqia county ($n = 22$) and Hutubi County ($n = 23$) in Xinjiang Uygur Autonomous Region; and long-tailed marmot (*M. caudata*) samples were obtained from the Pamirs of Wuqia County in Xinjiang Uygur Autonomous Region ($n = 37$). Marmot tails (including hairs, muscle and coccyx) were collected from each animal and dry-stocked in the shade at room temperature until DNA extraction.

DNA extraction and PCR amplification

DNA was extracted from marmot tails using microelute genomic DNA kits (D3096–02) (Omega Bio-Tek Inc. Norcross, GA) according to the manufacturer's instructions.

In total, 13 microsatellite DNA loci were selected to amplify DNA genome, including 8 microsatellite DNA loci cloned from *M. himalayana* (SSR2, SSR3, SSR4, SSR5, SSR7, SSR9, SSR10, SSR11) (Xu et al. 2009) and 5 microsatellite loci cloned from *M. marmota* (MA001, MA002, MA018, MA066, MA091) (Silva et al. 2003). All primer sequences are shown in Table 1.

DNA amplification was performed using a thermal cycler (Eastwin, EDC-810, Beijing, China) with the following reaction conditions: initial denaturation at 94℃ for 10 min followed by 40 cycles of denaturation at 94℃ for 60 s, annealing at 55℃ for 60 s, extension at 72℃ for 90 s, followed by a final extension at 72℃ for 10 min and a hold at 4℃. The PCR products were fractionated on a 12% polyacrylamide gel followed by silver staining (Brant et al. 1991).

Data analyses

All genotypes were scored for the number of SSR bands. Genetic polymorphisms of the four species were analyzed by POPGENE32 version 1.32 (http://www.ualberta.ca/~fyeh) and Modified-Powerstate software (http://www.promega.

Table 1. Primer sequences and genetic diversity of microsatellite loci in Chinese marmot populations.

Loci	Sequences primers	Number of alleles	Effective number of alleles	Polymorphism information content
SSR2	GAAATAGGCTGGTCCGTG CATACTTGATAGATGGTGGTG	9	5.3722	0.79
SSR3	GTCTGTTCAGGAGCCATC CTCATCCAGCCTTAGTGTAG	8	5.8904	0.81
SSR4	GATGGGTCAAATAATGGTAC ATGTGAAGGGTTGGGGTT	9	5.9492	0.82
SSR5	GGGAAAACCAAAATCTGAAC ACAGCAAATCTCCCACCA	9	8.2749	0.87
SSR7	ACATTGCTACCACTGCTGCTCC GACCCAGACTGAATTACATCAT	9	6.2776	0.83
SSR9	CTTCTTTCCTTCCCCATA AACTCAAGTGAGACCCTGT	9	7.3962	0.86
SSR10	GCCTGTTTGAAGACTGAT TGACCTAAAGAAATGCTAT	9	6.6994	0.84
SSR11	TCAGAAATGCAACCCAGAC GCCCCAACAGAAGGAACT	9	7.5880	0.86
MA001	AGGGGAACAGAACCAAAAGG GTTTCTTCCAGGGACAAAGCACCATC	9	5.7929	0.81
MA002	CATTTAGACGCACATTTTG GGGATGGAGAATGAGGAAG	9	8.1337	0.86
MA018	ATCCGTCCAATAAAGAAATTC GTTTCTTGTGGCTCAGTGGTCAGATG	9	5.0825	0.78
MA066	AATATGTTAAGGCAGTTCTAGC GTTTCTTCCTGATATGGAAAGATGATGT	9	4.2109	0.74
MA091	CCTGTGTGAGTCCTGGAGTC AGCCATTTAGGTTACATCTGC	9	6.3274	0.82

Explanations: SSR2, 3, 4, 5, 7, 9, 10, 11 refer to (Xu et al. 2009); MA001, 002, 018, 066, 091 refer to (Silva et al. 2003).

Table 2. Values of genetic diversity of Chinese marmot populations.

Population	Average effective allele number	Average heterozygosity	Shannon's index
Marmota himalayana	5.5206	0.8110	1.8397
Marmota sibirica	4.3813	0.7373	1.5649
Marmota baibacina	4.5034	0.7452	1.5933
Marmota caudata	3.9847	0.7293	1.5028

com/geneticidtools/powerstats/). We determined allele number, effective allele number, allele frequency, gene diversity (namely, heterozygosity), polymorphism information content, Shannon index, genetic identity and genetic distance. The Hardy-Weinberg equilibrium of species and 13 microsatellite loci were analyzed by GENEPOP version 4.1 (http://genepop.curtin.edu.au/). Finally, we constructed a dendrogram based on Nei's genetic distance by the unweighted pair group method using arithmetic averages (UPGMA) as previously reported (Nei 1972).

Results

In the current study, we collected four species of marmots from Qinghai province, Inner Mongolia Autonomous Region and Xinjiang Uygur Autonomous Region in Northern China to study their genetic diversity and genetic relationship. A total of 13 microsatellite markers were used to examine the genetic diversity of four marmot populations in China. As shown in Tables 1 and 2, a total of 116 alleles were detected by these polymorphic markers. The average number of alleles per loci was 8.9, and an effective allele number ranged from 3.9847 to 5.5206 among four populations targeting 13 microsatellite markers. The average effective allele number was 6.3843 at each locus. The heterozygosity and Shannon index ranged from 0.7293 to 0.8110 and 1.5028 to 1.8397, respectively. Among the four species, the heterozygosity and Shannon's index of *M. caudata* was lowest. The heterozygosity and Shannon's index were highest in *M. himalayana* (Table 2). Polymorphism information content of 13 microsatellite markers ranged from 0.74 to 0.87 (Table 1). By Nei's original measurements, we found that genetic identity and genetic distance ranged from 0.5067 to 0.8790 and from 0.1290 to 0.8193 among four populations (Table 3). The maximum genetic identity index was 0.8790 between *M. sibirica* and *M. baibacina,* suggesting that two populations are closely related. However, the maximum genetic distance between *M. caudata* and *M. himalayana* was 0.8193, indicating that these two populations are genetically distant (Table 3). In Hardy-Weinberg equilibrium test, 13 microsatellite loci in each population departure from Hardy- Weinberg equilibrium as well as

Table 3. Nei's original measures of genetic identity and genetic distance of four marmot populations.

Population	*Marmota himalayana*	*Marmota sibirica*	*Marmota baibacina*	*Marmota caudata*
Marmota himalayana	****	0.5481	0.5048	0.4407
Marmota sibirica	0.6013	****	0.8790	0.5067
Marmota baibacina	0.6835	0.1290	****	0.5590
Marmota caudata	0.8193	0.6797	0.5817	****

Explanations: Nei's genetic identity (above diagonal) and genetic distance (below diagonal).

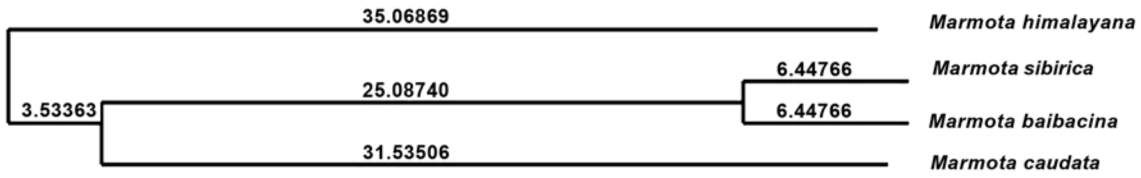

Fig. 2. Dendrogram showing the relationships among four Chinese marmot populations using UPGMA methods based on microsatellite marker data using 13 primers. Chinese marmot populations are divided into two groups.

four marmot population at each locus ($P < 0.01$).

Discussion

In general, an effective allele number and polymorphism information content are important indices for the evaluation of the locus polymorphism (Yuan et al. 2006; Heame et al. 1992). In this study, we found that both the effective allele number of 13 microsatellite loci and the polymorphism information content (>0.5) (Table 1) were relatively high in four marmot populations; therefore, these loci are highly polymorphous in the Chinese marmot population. Heterozygosity can reflect gene homogeneity and diversity among the population. In general, the population has high genetic diversity when the value of heterozygosity is higher than 0.5 in a population (Botstein et al. 1980; Nei 1978). In this study, the values of heterozygosity of the Himalayan marmot, the Tarbagan marmot, the gray marmot and the long-tailed marmot were 0.8110, 0.7373, 0.7452, and 0.7293, respectively (Table 2). Heterozygosity of Himalayan marmot inhabited in Yushu County of Qinghai province is higher than the Himalayan marmot inhabited in Tibet (0.67) (Xu et al. 2009), the heterozygosity of each of four Chinese marmot is also higher than Alpine marmot (*Marmota marmota* L., 1778) (0.53, 0.73) (Silva et al. 2003; Goossens et al. 2001), and hoary marmot (*Marmota caligata* Eschscholtz, 1829) (0.51) (Kyle et al. 2004), suggest that the four marmot populations in China have high genetic diversity. The Shannon index can be used to evaluate the compounding influence derived from selection mutation and genetic drift in a randomly mating population. Namely, a high value denotes a more complicated genetic background (Yuan et al. 2006). The Shannon indexes of the four populations are $\geq$1.5028 in this study (Table 2), suggesting that all four marmot populations possessed high genetic diversity. In addition, we found that four marmot populations departure from Hardy-Weinberg equilibrium at all loci. The samples in current study may not been enough, and the different geographical location and ecological environment in which four marmots inhabited, may promoted gene selection, and resulted in change of gene frequency. These factors might affect Hardy-Weinberg equilibrium regarding four marmot species.

Based on the genetic distance, we constructed a cluster dendrogram (Fig. 2) that illustrates genetic kinship among the four populations. We can divide Chinese marmot populations into two large groups: *M. himalayana* is an independent group that was affected by the terrain of Qinghai-Tibet plateau, and *M. sibirica, M. baibacina* and *M. caudate* were not affected by the terrain. Furthermore, a close kinship exists between *M. sibirica* and *M. baibacina* but not with *M. caudata.* This relationship feature is compatible with geographic distribution and the habitat of the marmots. For example, *M. himalayana, M. sibirica, M. baibacina* and *M. caudata* inhabit the Qinghai-Tibet plateau, the eastern prairie of Inner Mongolia Autonomous Region and the western region of Daxinganling, Altai and Tianshan Mountain, and Pamirs, respectively. *Marmota himalayana* is a unique species because the marmots live in alpine meadows in Qinghai-Tibet plateau and are affected by the terrain of Qinghai-Tibet plateau. Genetic interactions caused by crossbreeding between *M. himalayana* and other marmot populations in China may be geographically blocked by the Kunlun Mountains, the Taklimakan Desert, and the Kumtag Desert, which are located in the middle of Pamirs, Tianshan Mountain, Altai and Qinghai-Tibet plateau. Consequently, compared with the other three populations, the genetic features of *M. himalayana* are unique and different from other species. Altai and the North slopes of Tianshan Mountains in Xinjiang Uygur Autonomous Region borders Mongolia and the Inner Mongolia eastern prairie. Genes derived from *M. baibacina* and *M. sibirica* may be transferred via the vast grassland in Mongolia; therefore, kinship is relatively close between *M. sibirica* and *M. baibacina.* Pamirs is the most western region in China, and the distance between Pamirs and the south slope of Tianshan Mountain is small; therefore, genetic interactions or crossbreeding between *M. baibacina* and

M. caudata may occur frequently. *Marmota caudate, M. baibacina* and *M. sibirica* can be classified into the same group. Moreover, many rivers are distributed in the middle of Pamirs and the south slope of Tianshan Mountain. This geography may affect the transfer of genetic information between *M. baibacina* and *M. caudata.* Compared to the kinship of *M. sibirica* and *M. baibacina,* the *M. caudata* is more distant (Fig. 2).

Recently, we succeeded in breeding *M. himalayana* in our laboratory, and we have attempted to investigate their usefulness for the study of human chronic hepatitis B virus infection and lipid metabolism (unpublished data).

In conclusion, genetic features of four Chinese wild marmots were investigated in this study. This study may be useful for protection of Chinese wild marmot resource and further application of biomedical research.

Acknowledgements

This work was supported by Qinghai Province Grant of Science and Technology Capacity-building (2009-TY-05).

References

Botstein D., White R.L., Skolnick M. & Davis R.W. 1980. Construction of a genetic linkage map in man using restriction fragment length polymorphisms. Am. J. Hum. Genet. **32 (3):** 314–331. PMID: 6247908

Brandler O.V., Nikolsky A.A. & Kolesnikov V.V. 2010. Spatial distribution of *Marmota baibacin* and *M. sibirica* (*Marmota,* Sciuridae, Rodentia) in a zone of sympatry in Mongolian Altai: Bioacoustic analysis. Izv. Akad. Nauk. Ser. Biol. **3:** 380–384. PMID: 20583623

Brant J.B., Gustavo C.A. & Peter M.G. 1991. Fast and sensitive silver staining of DNA in polyacrylamide gels. Anal. Biochem. **196 (1):** 80–83. DOI: 10.1016/0003-2697(91)90120-I

Caridini A., Hoffmann R.S. & Thorington R.W. 2005. Morphological evolution in marmot (Rodentia, Sciuridae): size and shape of the dorsal and lateral surfaces of the cranium. J. Zool. Sys. Evol. Res. **43:** 258–268. DOI: 10.1111/j.1439-0469.2005.00316.258-268

Edwards A., Civitello H., Hammond H.A. & Caskey C.T. 1991. DNA typing and genetic mapping will a trimeric and tetrameric tandem repeats. Am. J. Hum. Genet. **49 (4):** 746–756. PMID: 1683171

Fan W. 2007. Effect of ketamine hydrochloride on braking anesthesia of Himalayan marmot. Chin. J. Zoonoses **23 (4):** 418.

Goossens B., Chikhi C., Taberlet P., Waits L.D. & Allaine D. 2001. Microsatellite analysis of genetic variation among and within Alpine marmot populations in the French Alps. Mol. Ecol. **10 (1):** 41–52. DOI: 10.1046/j.1365-294X.2001.01192.x

Hanslik S. & Kruckenhauser L. 2000. Microsatellite loci for two European sciurid species (*Marmota marmota, Spermophilus citellus*). Mol. Ecol. Notes **9 (12):** 2163–2168. DOI: 10.1046/j.1365-294X.2000.10535.x

Heame C.M., Ghosh S.T. & Yodd J.A. 1992. Microsatellite for linkage analysis of genetic traits. Trends Genet. **8 (8):** 288–294. DOI: 10.1016/0168-9525(92)90256-4

Huang X.L. 1996. Study of *Marmota himalayana* as laboratory animal. Chin. J. Lab. Anim. Sci. **6:** 70–72.

Kyle C.J., Karels T.J., Clark B., Strobeck C., Hik D.S. & Davis C.S. 2004. Isolation and characterization of microsatellites markers in hoary marmots (*Martmota caligata*). Mol. Ecol. Notes. **4 (4):** 749–751. DOI: 10.1111/j.1471-8286.2004.00810.x

Li Y., Wang Z., Tao Y., Fan W., Li M., Huang B., Zhao S., Fan J. & Liu E. 2012. Characteristics of Himalayan marmots and their response to an atherogenic diet. Exp. Anim. **61(4):** 461–466.

Litt M. & Luty J.A. 1989. A hypervariable microsatellite revealed by in vitro amplication of a dinucleotide repeat within the cardiac muscle actin gene. Am. J. Hum. Genet. **44:** 397–401.

Menne S. & Cote P.J. 2007. The woodchuck as an animal model for pathogenesis and therapy of chronic hepatitis B virus infection. World J. Gastroenterol. **13 (1):** 104–124. PMID: 17206759

Nei M. 1972. Genetic distance between populations. Am Nat. **106 (949):** 283–292.

Nei M. 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics **89 (3):** 583–590. PMID: 17248844

Peiffer A.K., Florant G.L. & Summers S.A. 2004. Seasonal changes in Akt/PKB phosphorylation and fat cell size in Marmots. FASEB J. **18 (4, Suppl. S):** A314–A314.

Silva A.D., Luikart G., Allaine D., Gautier P., Taberlet P. & Pompanon F. 2003. Isolation and characterization of microsatellites in European alpine marmots (*Marmota marmota*). Mol. Ecol. Notes. **3 (2):** 189–190. DOI: 10.1046/j.1471-8286.2003.00392.x

Tautz, D; Schlötterer C. 1994. Simple sequences. Curr. Opin. Genet. Dev. **4 (6):** 832–839. DOI: 10.1016/0959-437X(94)90067-1

Thorington R.W. Jr. & Hoffman R.S. 2005. Family Sciuridae, pp. 754–818. In: Wilson D.E. & Reeder D.M. (eds), Mammal Species of the World. A Taxonomic and Geographic Reference, Johns Hopkins University Press, Baltimore, 2000 pp. ISBN: 0801882214, 978-0801882210

Wang Z., Weber J.L., Zhong G. & Tanksley S.D. 1994. Survey of plant short tandem DNA repeats. Theor. Appl. Genet. **88 (1):** 1–6.

Xu J.H., Wang L.L., Xue H.L., Wang Y.S. & Xu L.X. 2009. Genetic structure of Himalayan marmot (*Marmota himalayana*) population alongside the Qinghai-Tibet Railway. Acta Ecol. Sin. **29 (5):** 314–319. DOI: 10.1016/j.chnaes.2009.09.010

Yuan C.Z., Wang J.M., Ma Y.H., Qu X.X., Shang Y.G. & Zhang N.B. 2006. Genetic diversity of indigenous sheep breeds in Shandong province based on microsatellite markers study. Chin. J. Appl. Ecol. **17 (8):** 1459–1464.

Received November 21, 2011
Accepted March 156, 2012