

Microsatellite analysis of genetic diversity in the *Tupaia belangeri yaoshanensis*

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Abstract. The Chinese tree shrew (*Tupaia belangeri yaoshanensis*) has long been proposed to serve as an animal model for studying human diseases. However, its overall genetic diversity and population structure remain largely unknown. In the present study, we investigated the genetic diversity of population microsatellite DNA in wild *Tupaia belangeri yaoshanensis*. Sixteen microsatellite loci were assessed in 76 wild *Tupaia belangeri yaoshanensis*. The target microsatellite DNA fragments were amplified from the peripheral blood DNA of the animals by polymerase chain reaction (PCR), and the PCR-amplified products were verified by DNA sequencing and used for the analysis of allele, effective allele, genetic heterozygosity, polymorphism and population structure. Our results showed that of the 16 microsatellite loci examined, 5 microsatellite loci were monomorphic and 11 microsatellite loci were polymorphic. We detected 61 alleles in the polymorphic loci and found 2-10 (with an average of 5.5455) alleles per locus. Our data also showed that the observed and expected heterozygosities ranged from 0.087 to 0.8947 and 0.1368 to 0.7892 with an average of 0.3968 and 0.4796, respectively. Taken together, the results revealed a considerably high heterozygosity and high genetic diversity at the molecular level in

the population of wild *Tupaia belangeri yaoshanensis*. The identified markers from the present study may be useful for individual identification and parentage testing, as well as for the quantification of population heterogeneity in the Chinese tree shrew.

Introduction

Microsatellite markers, or short tandem repeats (STR), are polymorphic DNA loci containing repeated nucleotide sequences, typically from 2 to 7 nucleotides per unit. The length of the repeated unit is the same for the majority of the repeats within an individual microsatellite locus. By contrast, when the number of repeats for a specific locus differs, it results in alleles of varying length (1). The vast amount of data emerging for thousands of microsatellite markers across organisms makes microsatellite analysis a widely accepted tool for population genetic diversity studies, as well as for identifying individual organisms, including human and tree shrew. Microsatellite analysis includes polymerase chain reaction (PCR) amplification of the microsatellite loci using fluorescently labeled primers. The labeled PCR products are then analyzed by capillary electrophoresis or electrophoresis to separate the alleles by size (2). In the present study, we used and developed this microsatellite analysis for identifying the individual Chinese tree shrew.

Tree shrew (*Scandentia, Tupaiidae*) is a small mammal with a close affinity to primates. It belongs to the Family Tupaiidae of Scandentia and is native to tropical forests, widely distributed across Southeast Asia (3). The Chinese tree shrew (*Tupaia belangeri*) is distributed across southwest China and classified as six subspecies (*Tupaia belangeri chinensis*, *Tupaia belangeri gaoligongensis*, *Tupaia belangeri modesta*, *Tupaia belangeri tonquinia*, *Tupaia belangeri yunalis* and *Tupaia belangeri yaoshanensis*) based on morphological characteristics and geographical distribution. *Tupaia belangeri yaoshanensis* is the largest in size. It inhabits the Dayaoshan Mountain of Guangxi,

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China. Owing to its resemblance of ancestral primates (4,5), as well as their behavior and social monogamy (6), the Chinese tree shrew has been used as an experimental animal models for the study of human diseases (7).

Although some mitochondrial (mtDNA) and nuclear (nDNA) sequences and karyotype maps have been published (6,8,9) correctly, there few species or population level studies on the Chinese tree shrew. Microsatellite markers are an extremely important tool for genetic studies and for the conservation and management of genetic resources (10,11). Previous studies identified microsatellite markers of tree shrew (2,12-14); however, microsatellite markers specific to *Tupaia belangeri yaoshanensis* remain to be determined. For the conservation of genetic resources or for selecting a proper founder to establish the inbred lines, it would be worthwhile to screen the genetic diversity of these different subspecies (12).

In this study, we analyzed 16 microsatellite loci of genomic DNAs from a population of 76 individuals of wild *Tupaia belangeri yaoshanensis* by using the microsatellites developed from other subspecies of tree shrews, and evaluated the genetic diversity of this population. Our study suggests that these 16 microsatellite markers can be used to analyze the population genetics of *Tupaia belangeri yaoshanensis* and to establish animal models. Moreover, the findings of this study can be used for the protection and effective utilization of the germplasm resource of this species.

Materials and methods

Sample collection. Seventy-six wild *Tupaia belangeri yaoshanensis* animals were collected from the Dayaoshan Mountain of Guangxi, China and were used in this study. All the animals were raised at the Experimental Animal Center of Guangxi University of Chinese Medicine under specific pathogen-free conditions and were cared for in an environmentally controlled room with a 12-h light/dark cycle and a constant temperature of 22°C. The animals were provided with a standard diet and water in accordance with the guidelines for the treatment of experimental animals published by the Ministry of Science and Technology of the People's Republic of China in 2006. Genomic DNA was extracted from the blood of tail tissues of each individual *Tupaia belangeri yaoshanensis* by using the genomic DNA extraction kit (Tiangen, Beijing, China). All sampling procedures related to animal subjects were reviewed and approved by the Review Committee of Guangxi University of Chinese Medicine, in accordance with the institutional animal care and use regulations and rules. The present study was carried out in strict accordance with the recommendations established in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (eighth version, 2010). The animal use protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Guangxi University of Chinese Medicine (Nanning, China; approval nos. GXUCM2014NSF01125 and GXUCM2014NSF01136).

Microsatellite screening and simple sequence repeat (SSR) analysis. Sixteen previously described microsatellite loci of other species of tree shrews were used in this study (Table I). The composition of the PCR reaction mixture and procedures

of PCR were referenced as per the published methods (2,12-14), with minor modifications. Briefly, the PCR amplification was performed in 25 μ l volumes containing 1 μ l diluted DNA template, 1 μ l forward and reverse primers (the forward primer was fluorescently labeled with FAM, HEX or TAM), 2.5 μ l 10X PCR buffer [without Mg^{2+} : 100 mM Tris-HCl pH 8.8; 500 mM KCl, 0.8% (v/v) Nonidet], 0.5 μ l 10 mM dNTPs, 2 μ l 25 mM $MgCl_2$, and 0.2 μ l 5 units TaqDNA polymerase (Takara Bio Inc., Otsu, Japan), and 17.8 μ l sterilized water. Amplification was performed on an Applied Biosystems (T100; Bio-Rad, Hercules, CA, USA). Thermal cycling conditions were implemented as follows: 94°C for 3 min; followed by 30 cycles of 95°C for 30 sec, optimal annealing temperature (Table I) for 30 sec, 72°C for 30 sec, a final extension step at 72°C for 5 min and then holding at 8°C. PCR product (1 μ l) was mixed with 0.1 μ l GeneScan™ 500 LIZ® and 9.9 μ l Hi-Di™ Formamide and denatured at 95°C for 5 min, then immediately chilled on ice. After this, it was separated on an ABI PRISM 3730 XL genetic analyzer (Applied Biosystems, Foster City, CA, USA).

Statistical data analysis. For each locus in the population, the number of alleles (N_A), effective number of alleles (N_E), observed heterozygosity (H_O), expected heterozygosity (H_E), and fixation index (F_{is}), as well as tests for deviation from Hardy-Weinberg equilibrium (HWE), were analyzed using Popgene version 1.32 (15). Allele frequency data were used to calculate polymorphic information content (PIC) using the methods described by Botstein *et al* (16).

Results

The details of variability measurement across 76 individuals of the *Tupaia belangeri yaoshanensis* are summarized in Table II. Among the 16 microsatellites, seven were perfect (43.75%), four were imperfect (25%), and five turned out to be compound (31.25%).

Overall, 11 loci (68.75%) were cleanly amplified and were shown to be polymorphic. We identified 2-10 alleles (with an average of 5.5455 alleles) per locus in these polymorphic markers. TB2 with 10 alleles was the most polymorphic microsatellite, while TB3 and TB8 were the least variable with only 2 alleles each. The effective number of alleles per locus ranged from 1.16 at TB3 to 4.63 at TG1, with an average of 2.3975. The observed and expected heterozygosities were 0.087 to 0.8947 and 0.1368 to 0.7892 (with an average of 0.3968 and 0.4796), respectively. The F_{is} pronounced the most loci with heterozygote deficiencies. Seven loci (JS188, JS196, TB2, TB10, TB20, TBC10 and TBC11) significantly deviated from the HWE in the sampled population after Bonferroni's correction. The average PIC per locus ranged from 0.1266 to 0.7505 (Table II), of which, three loci showed low polymorphism ($PIC < 0.25$), four loci showed median polymorphism ($0.25 < PIC < 0.5$), while the other four loci were all high polymorphism ($PIC > 0.5$).

In this study, we detected 16 population-loci out of the 35 population-loci examined, as we tested non-specific amplification in some loci in our samples. We showed that five (31.25%) (TBC1, TBC3, TBC5, TG4 and TG19) were monomorphic, and they failed to amplify in *Tupaia belangeri yaoshanensis*, and these population-loci were proved to be

Table I. PCR primers and their sequences for the 16 microsatellite loci used in this study.

Locus	Primer sequences (5'→3')	T _m (°C)	Repeat motif	Size range (bp)	Accession no./ (Refs.)
JS188	F: ACACACACAAAACCTCATTTTATCC R: TCTACACGAATGTGCCAACC	57	(CA) _n	186-196	DQ-334279
JS196	F: ACCTCCTGGTGGCTTGC R: TAATTGCAGGATGCTTCAGG	57	(GT) _n	304-344	DQ-334280
TB2	F: AATATGCTGGTAGGACCAGAAAC R: ATTGGGTTGTTGGGAAAGTC	60	(AAG) _n (GAGAA) _n	189-275	(11)
TB3	F: TCTGGACCTAGCAACTTGCTTTC R: TTCTTCACCAAGTGTGAGCATAA	58	(TAAA) _n	254-259	(11)
TB8	F: CTCTGACTTCAAGACCTGGAAC R: CTTGAAAGCAAAGGCAGTACAA	55	((A/G)TC(C/T)) _n	453-465	(11)
TB10	F: ATTGGAGACCACTCTACCCTTTA R: TGGATTCCACATAGTCTACCG	60.4	(ATTT) _n	456-461	(11)
TB16	F: TCCTTAGTATGGACTTGTTC R: AGGATCACAGGGCAGGGAGACT	58	((A/G)AAG) _n	212-228	(11)
TB20	F: TGATCTCCAAAGGCTTCTCCAT R: GAACCTTCCCGTATGGGTCAGT	60	(AAAAC) _n	221-237	(11)
TBC1	F: TGGCAATTGGGAGAATTAGG R: GCTGCCTTCATTCACCTCTC	48	(TG) _n	177	(12)
TBC3	F: TGGCAGGATTTCTTCATTC R: TCATTGCACGAGAATTCCA	44	(GT) _n GC (GT) _n	160	(12)
TBC5	F: GCTGCCTTCATTCACCTCTC R: TGGCAATTGGGAGAATTAGG	48	(AC) _n GCAG(AC) _n	173	(12)
TBC10	F: CCTTACTGACCAGTGCTGAGG R: GCCCAGACACAGAGAGGTTC	52	(TGG) _n CTGTA(G) _n A(GCTGGG) _n	113-182	(12)
TBC11	F: GACCTGGTGGCATGAAAAC R: TCGGGGAAATATGGAGAGAA	51.5	(GT) _n GC(CT) _n	157-167	(12)
TG1	F: TTCCTGTTACTCTGTTTTTTTCAGG R: ACATGGCTAACTGTGTGCTTTG	52	(CA) _n	152-183	AY-064160
TG4	F: TGAAAACCTGGCAATTCATATGC R: CAATCCTTTTTCGTTAGTTTTGTG	52	(CA) _n TA(CA) _n TA(CA) _n	152	AY-064161
TG19	F: ACCCCTCCCTAAAGGAACT R: CGCCCTATAGAAACCTCTCC	55	(CA) _n TAAA(CA) _n	171	AY-064163

PCR, polymerase chain reaction; F, forward; R, reverse.

polymorphic microsatellite loci in *Tupaia belangeri chinensis*, *Tupaia glis* and *Tupaia minor* (2,12-14).

Discussion

A high level of genetic diversity is essential for the long-term survival of populations, and the extent of variation determines their ability to adapt to changing environments. The provision of the primers for these microsatellite markers facilitates the studies of population structure, gene flow, and mapping of the tree shrew genome. In the present study, we analyzed

16 microsatellite loci in 76 animals of wild *Tupaia belangeri yaoshanensis* and showed that these *Tupaia belangeri yaoshanensis* had a considerably high heterozygosity, suggesting that the 16 markers may be useful for individual discrimination and parentage testing, as well as for the quantification of population heterogeneity.

We also compared our results with the same microsatellite markers reported in other subspecies including *T. glis*, *T. minor* and *Tupaia belangeri chinensis*, and found some genetic differences between *Tupaia belangeri yaoshanensis* and other subspecies (12,12-14). Since there are six subspecies

Table II. Polymorphic microsatellite markers identified in *Tupaia belangeri yaoshanensis* in this study.

Locus	N_A	N_E	H_O	H_E	F_{is}	PIC
JS188 ^a	9.000	3.6098	0.6892	0.7279	0.0467	0.6884
JS196 ^a	4.000	1.4777	0.1714	0.3256	0.4697	0.3052
TB2 ^a	10.000	3.6554	0.7714	0.7317	-0.0619	0.6849
TB3	2.000	1.1573	0.1200	0.1368	0.1171	0.1266
TB8	2.000	1.2227	0.1486	0.1834	0.1840	0.1656
TB10 ^a	6.000	2.3112	0.0870	0.5715	0.8467	0.4739
TB16	4.000	1.6284	0.3194	0.3886	0.1722	0.3500
TB20 ^a	5.000	2.1457	0.4583	0.5277	0.1416	0.4417
TBC10 ^a	6.000	1.2344	0.1781	0.1912	0.0623	0.1817
TBC11 ^a	5.000	3.2996	0.5263	0.7016	0.2448	0.6468
TG1	8.000	4.6301	0.8947	0.7892	-0.1412	0.7505
Mean	5.5455	2.3975	0.3968	0.4796	0.1893	0.4378

N_A , observed number of alleles; N_E , effective number of alleles; H_O , observed heterozygosity; H_E , expected heterozygosity; F_{is} , fixation index; PIC, polymorphism information content. ^aSignificant deviations from Hardy-Weinberg equilibrium.

in Chinese tree shrews, further study is necessary to determine the genetic diversity of other subspecies, as well as the genetic relationship between the subspecies.

Recent studies have shown that microsatellite markers are useful as a tool in the investigation of population genetics, ecology, and conservation of the *Tupaia belangeri yaoshanensis*. Our data reported here provide further scientific basis for the identification, breeding, and laboratory animalization of *Tupaia belangeri yaoshanensis*, and should pave the way for making use of the *Tupaia belangeri yaoshanensis* in Guangxi with both scientific and economic value.

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