NOTE *Physiology*

Microsatellite loci analysis for individual identification in Shiba Inu

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ABSTRACT. Eighteen autosomal microsatellite loci were examined using 275 Shiba Inus in Japan. Eighteen dogs representing eight trios were obtained from four breeders to calculate mutation rates, and 257 dogs kept by owners were collected through veterinary clinics throughout Japan to calculate population genetic parameters and estimate discrimination power. After two loci (INU005 and AHTk253) were excluded, average expected heterozygosity (He), polymorphic information content (PIC) and fixation index (F) were 0.665, 0.623 and 0.046, respectively. The combined power of discrimination over the 16 microsatellite markers was more than 0.9999. Therefore, it is suggested that these 16 microsatellite loci recommended by the International Society for Animal Genetics (ISAG) are applicable for individual identification and parentage testing of domestic Shiba Inu in Japan.

KEY WORDS: camome, genetic diversity, genotyping, microsatellite

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Shiba Inu is a longtime popular indigenous breed of Japan, which is now becoming familiar in other countries as well. Genetic studies have shown that Shiba Inus are genetically close to wolves and different from European breeds [13]. Microsatellite markers are often used for criminal investigation, and it is reported that canine DNA can be applied as additional evidence because of close relationship and co-habituation between humans and dogs [5, 11, 12]. Additonally, maicrosatellite analysis is expected to be beneficial in canine genome wide association studies, because it can reveal the genetic relationship among randomly collected dogs and enhance the power of detection [7, 16]. As most studies on canine microsatellite have been conducted using European breeds [4, 12], we examined whether microsatelite markers recommended by ISAG are applicable for individual identification and parentage testing of Shiba Inus.

Blood samples of 275 Shiba Inus were used. Eighteen individuals (5 dams, 5 sires and 8 offsprings) representing 8 trios were obtained from 4 breeders in Japan to calculate mutation rates. Two-hundred and fifty seven dogs kept by owners were collected through veterinary clinics throughout Japan to calculate population genetic parameters and estimate discrimination power. Informed consent to use the data for academic research was obtained from breeders and owners.

DNA was extracted from anti-coagulated whole blood samples using DNeasy Blood & Tissue Kit or EZ1 DNA Blood 350 *µl* Kit (QIAGEN, Hilden, Germany), following the manufacturer's protocols. Eighteen autosomal microsatellite loci included in the Canine Genotypes Panel 1.1 (Finnzymes, Espoo, Finland) were examined. Each primer pair was designed using Primer3 (http://primer3.sourceforge.net/) with the goal of the amplicon size as shown in Table S1. Forward primers were labeled with fluorescent dyes FAM, VIC, NED, and PET, and the reverse primers were tailed with a seven base PIGtail (GTTTCTT) recommended by the manufacturer (Applied Biosystems, Foster City, CA, U.S.A.). Eighteen microsatellite loci were multiplexed into 4 sets (A–D). Multiplexed PCR amplification was performed on Program Temp Control System PC-812 (ASTEC, Fukuoka, Japan) in 25 *µl* reactions containing 0.04–0.16 *µ*M forward and reverse primers balanced for peak height, 200 *µ*M dNTPs, 2.5 *µl* of 10X *ExTaq* Buffer, 0.5 units of *ExTaq* DNA polymerase (Takara Bio Inc., Otsu, Japan), 10 *n*g of DNA extract and sterilized water to volume. The thermal cycling parameters consisted of an initial 5 min denaturation at 95°C followed by 30 cycles of: 1 min denaturation at 95°C, annealing for 1 min at 58°C (for set A and B) or 61°C (for set C and D) and extension for 1 min at 72°C. A final extension was performed for 5 min at 72°C. PCR products were purified using FastGene Gel/PCR Extraction Kit (NIPPON Genetics, Tokyo, Japan), and 1 *µl* of the purified products were mixed with 10 *µl* of HiDi formamide (Applied Biosystems) and 0.5 *µl* of GeneScan 600 LIZ Size Standard (Applied Biosystems). Samples were denatured for 3 min at 95°C. Capillary electrophoresis was carried out on an AB 3130 xl Genetic Analyzer (Applied Biosystems), and electropherograms were analyzed using GeneMapper software (Applied Biosystems). Allele calling was developed based on recommendations [1], and the number of repeat was identified by sequence determination of PCR products.

Mutation rates for each locus were calculated by counting the occurrences of discordant repeat copy number transmission from parent to offspring using the breeder samples. INU005 was not amplified successfully in this study. The mutation rate at AHTk253 was 6%, while other loci were

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Table 1. Population genetic parameters and discrimination power using 16 microsatellite loci in 257 Shiba Inus

Allele (repeat number)	AHTk211	CXX279	REN169O18	INU055	REN54P11	INRA21	AHT137	REN169D01	AHTh260	INU030	FH2848	AHT121	FH2054	REN162C04	AHTh171	REN247M23
9	-	-	-	-	0.012	-	-	-	-	-	-	-	0.043	-	-	-
10	-	-	-	-	0.232	-	-	-	-	-	-	-	0.142	-	-	-
11	-	-	-	-	0.049	-	-	-	-	-	-	-	0.428	-	-	-
12	-	-	-	-	0.072	-	-	-	-	-	-	-	0.148	-	-	-
13	-	-	-	0.440	-	-	-	-	-	-	-	-	0.093	-	-	-
14	-	-	-	0.191	0.039	-	0.128	0.313	0.082	-	-	-	0.119	-	-	-
15	-	-	-	0.161	0.163	-	0.230	-	0.006	-	-	-	0.027	-	-	-
16	0.181	-	-	0.130	0.245	-	-	-	-	-	-	-	-	-	-	-
17	0.173	-	-	0.002	0.189	0.142	0.002	-	0.039	-	-	-	-	-	-	-
18	0.430	0.051	0.008	0.058	-	0.790	-	0.058	0.531	-	0.035	0.004	-	-	-	-
19	0.025	0.047	0.025	0.018	-	0.021	0.095	0.216	0.165	-	0.031	0.128	-	0.039	-	0.235
20	0.191	0.195	0.438	-	-	0.039	-	-	0.072	0.025	0.006	-	-	0.014	0.788	0.490
21	-	0.008	0.101	-	-	-	0.093	0.335	0.006	0.496	0.002	-	-	0.422	-	0.091
22	-	0.331	0.232	-	-	0.008	0.002	0.078	0.099	0.041	0.317	0.004	-	0.010	-	-
23	-	0.099	0.183	-	-	-	0.158	-	-	0.021	0.014	0.282	-	0.405	0.021	0.043
24	-	-	0.014	-	-	-	0.091	-	-	0.379	0.595	0.169	-	0.111	0.138	0.138
25	-	0.076	-	-	-	-	0.198	-	-	0.035	-	0.407	-	-	0.033	0.002
26	-	0.195	-	-	-	-	0.002	-	-	0.002	-	0.004	-	-	-	-
27	-	-	-	-	-	-	-	-	-	-	-	0.002	-	-	0.002	-
28	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
29	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.018	-
Ho	0.685	0.755	0.677	0.685	0.794	0.342	0.829	0.673	0.619	0.560	0.541	0.681	0.743	0.541	0.327	0.696
He	0.715	0.795	0.710	0.724	0.815	0.354	0.840	0.734	0.667	0.606	0.543	0.710	0.749	0.644	0.358	0.675
P	0.680	0.216	0.903	0.650	0.538	0.027	0.829	0.347	0.459	0.015	1.000	0.967	0.070	0.006*	0.203	0.134
PIC	0.672	0.767	0.667	0.687	0.789	0.326	0.820	0.687	0.637	0.532	0.469	0.660	0.721	0.576	0.332	0.631
F	0.043	0.050	0.047	0.054	0.026	0.032	0.014	0.083	0.073	0.075	0.003	0.041	0.008	0.160	0.088	–0.032
PD	0.876	0.931	0.873	0.887	0.940	0.555	0.955	0.883	0.859	0.771	0.717	0.866	0.909	0.805	0.561	0.850

Values are allele frequencis, population genetic parameters (Ho, He, P, PIC and F) and discrimination power (PD). Ho: Observed Heterozygosity, He: Expected Heterozygosity, *P*: Hardy-Weinberg equilibrium exact test, PIC: polymorphic information content, F: Fixation Index, inbreeding coefficient, PD: power of discrimination. *REN162C04 showed significant deviation from Hardy-Weinberg equilibrium after Bonferroni correction.

0%. Considering the risk of misjudgment in parentage testing for Shiba Inus, we decided to exclude this locus from the panel. On the other hand, because the number of dogs used for calculating mutation rate was small in this study, the proper mutation rate at AHTk253 could be much lower, and a further study is necessary.

We finally adopted 16 microsatellite markers other than INU005 and AHTk253 in this study. GenAlEx [14] and CERVUS [9] programs were used to calculate population genetic parameters. Power of discrimination (PD) was calculated as following:

PD=1-PI; (PI is probability of identity computed by GENALEX).

Population genetic parameters (allele frequencies per locus, heterozygosity, Hardy–Weinberg test results, PIC, F and PD) are shown in Table 1. The PIC value of each marker was higher than 0.500 for 13 markers and higher than 0.700 for 4 markers, suggesting the validity and credibility of these markers [6]. The combined power of discrimination over the 16 microsatellite markers in this study was more than 0.999999, being comparable to the large-scale canine study conducted in the United States [2]. Therefore, we have indicated that these 16 markers recommended by ISAG are also applicable for individual identification of Shiba Inus.

There is a theory that the number of Shiba Inus decreased dramatically because of food shortage after World War II and the distemper epidemic in 1952 (http://www.akc.org/dog-breeds/shiba-inu/detail/#history and http://www.shibas.org/breedHistory.html), and thus, progression of inbreeding in Shiba Inus has been concerned. But, the average expected heterozygosity (He), polymorphic information content (PIC) and fixation index (F) calculated from the 16 microsatellite markers we analyzed were 0.665, 0.623 and 0.046, respectively. As the values were similar to research using different markers on Shiba Inus in Japan [8] as well as other popular breeds in other countries [3, 10, 15, 17], it is suggested that the level of inbreeding in Shiba Inus is not so high as concerned.

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