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Selecting representative microsatellite loci for genetic monitoring and analyzing genetic structure of an outbred population of orange tabby cats in China

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ABSTRACT. We optimized a panel of microsatellite markers from cat and tiger genetic data for efficient genetic monitoring and used it to analyze the genetic structure of an outbred cat stock in China. We selected a set of rich polymorphic microsatellite loci from 131 cat microsatellite loci and 3 Sumatran tiger microsatellite loci using agarose gel electrophoresis. Next, the set of optimized genetic markers was used to analyze the genetic variation in an outbred population of orange tabby cats in China by simple-tandem repeat scanning. Thirty-one loci rich in polymorphisms were selected and the highest allele number in a single locus was 8. Analysis of the orange tabby cat population illustrated that the average observed number of alleles, mean effective allele number, mean Shannon's information index, mean expected heterozygosity, and observed heterozygosity were 3.8387,

2.4027, 0.9787, 0.5565, and 0.5528, respectively. The 31 microsatellite markers used were polymorphic and suitable for analyzing the genetic structure of cats. The population of orange tabby cats was confirmed to be a well-outbred stock.

Key words: Domestic cat; Genetic structure; Microsatellite, Orange tabby cats

INTRODUCTION

Cats (*Felis silvestris catus)* are domestic animals that have had long symbiotic relationships with humans. Their inspired playfulness and independent spirit make them good pets. Cats are also important experimental animals and are used as a model animal to study various human genetic and metabolic anomalies, such as hereditary retinal blindness and degeneration (Menotti-Raymond et al., 2007). Velásquez et al. (2012) reported that cats are hosts of *Encephalitozoon intestinalis*. The domestic cat can be used as a model for nutritional management. Collison et al. (2012) found that the domestic cat is an alternative animal model of diet-induced liver disease and is ideal for nutrigenomic studies of hepatic steatosis. A study by Chew et al. (2000) showed that domestic cats readily absorb β-carotene across the intestinal mucosa and into peripheral blood leukocytes and their subcellular organelles. Experiments of β-carotene uptake kinetics showed that some aspects of β-carotene absorption and metabolism in cats are similar to those in humans. The domestic cat has also been used as a model to study assisted reproduction for endangered felid species. Mikolajewska et al. (2012) reported that the domestic cat is a good model for both wild felids and humans to study the cryopreservation of female gametes, which is a crucial component of assisted reproduction techniques. Songsasen et al. (2012) reported that both the domestic dog and cat can be used as models to examine the regulation of ovarian follicle development *in vitro*.

There are many strains of cats with different colors and strips. Most identification methods for a unique strain are related to the genes or biomarkers of the specific color and strip. Genetic testing has been used for domestic cats for the past 50 years, from the chromosome level to the sequence level, including microsatellite locus-related color and strip. Microsatellites, also referred to as short tandem repeats (STR), are useful for assessing wild and domestic cat admixtures (Driscoll et al., 2011), identifying domestic and known hybrid cats (Lecis et al., 2006), and studying the genetic differentiation between the African wild cat (*Felis lybica*) and the domestic cat (*F. catus*) in southern Africa (Wiseman et al., 2000). Microsatellite analysis has been widely used in genetic structure studies of ocelot (Janecka et al., 2011), gerbils (Du et al., 2010), and many other animals (Kim et al., 2011; Ruggeri et al., 2012). Although there have been numerous reports regarding the use of microsatellites for cat, information about microsatellite markers for monitoring the genetic structure of laboratory cat populations is limited. Genetic structure is an important biological character of an outbred laboratory animal population. Genetic monitoring is very useful to know if the population was variation or regression. In this study, we tested 134 microsatellite loci from cats and tigers to establish a set of microsatellite markers that would be suitable for genetic monitoring of cats, and then applied this panel to analyze the genetic variation in a population of orange tabby cats, representing the only experimental domestic cat population in China, bred by the North China Pharmaceutical Group New Drug Research and Development Limited Company.

MATERIAL AND METHODS

Ethics statement

All experiments and procedures involving animals were approved by the Animal Welfare Committee of Capital Medical University, Beijing, China.

Cats

Thirty-two domestic cats were selected from a pet market; these cats were chosen randomly (with no strain or sexual bias) and did not have any genetic relationships with each other. Thirty-four experimentally bred orange tabby cats were obtained from North China Pharmaceutical Group New Drug Research and Development Limited Company; these orange tabby cats were also chosen without sexual bias and were unrelated (outcrossed for three generations). The group of cats from the pet market included 11 females and 21 males of unknown age. The experimental orange tabby cats included 4 females and 30 males aged from 1-3 years old. Detailed information about the domestic cats from the pet market and the orange tabby cats is provided in **Table S1** and **Table S2**.

DNA extraction and cat microsatellite marker selection

All cats were anesthetized using Zoletil, and 2-mL sterile whole blood samples were drawn into anticoagulant tubes from a foreleg vein. The anticoagulated samples were stored at low temperature until they were used in experiments. The anticoagulated samples were lysed in Red Blood Cell Lysis Buffer (HANGZHOU BIOER TECHNOLOGY Co., Ltd.) and then centrifuged to obtain white blood cells. The white blood cells were suspended in 1 mL sodium chloride Tris EDTA buffer (10 mM Tris-HCl, pH 8.0; 50 mM ethylenediaminetetraacetic acid, pH 8.0; 200 mM NaCl, and 0.5% sodium dodecyl sulfate) and digested with 0.02 mg/mL proteinase K at 55°C for 12 h. Genomic DNA was extracted from the digested samples using a standard phenol-chloroform extraction and ethanol precipitation method as previously described (Du et al., 2010). The amount of DNA in each sample was determined by measuring A_{260}/A_{280} using a microplate absorbance reader system (Bio-Rad 680, Hercules, CA, USA) and further evaluated by agarose gel electrophoresis. The DNA was diluted to a concentration of 100 ng/μL and stored at -20°C for later use as a template for polymerase chain reaction (PCR).

We obtained information for 131 cat microsatellite loci and 3 Sumatran tiger microsatellite loci from GenBank (www.ncbi.nlm.nih.gov/). Primer sequences were designed based on GenBank sequences and synthesized by Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China).

PCR procedure

DNA samples from 32 domestic cats were pooled into 6 groups (5 groups of 5 and 1 group of 7). All 134 microsatellite loci were amplified from each DNA pool. PCR was performed in 20-μL reactions containing 1 μL of each primer (10 mM), 1.2 μL genomic DNA (50 ng/μL), 2 μL PCR buffer (Mg^{2+} 1.5 mM), 1 μL dNTPs (3 mM), 0.2 μL *Taq* DNA polymerase, and 13.6 μL ddH_2O. After an initial denaturation step of 4 min at 95°C, amplification proceeded for 35

cycles of 30 s at 94°C, 30 s of annealing at a gradient temperature of 50°-60°C, and extension at 72°C for 30 s. A final elongation step of 7 min was carried out at 72°C. The annealing temperature for the amplification conditions was determined for each pair of primers.

Microsatellite analysis

PCR products (10 μL) were loaded on a 2% agarose gel, electrophoresed, and visualized under UV light after ethidium bromide staining as a primary screening. Microsatellite loci that produced distinct PCR products and showed polymorphisms among the 6 DNA pools were selected for individual sample detection in 32 domestic cats. These PCR products were amplified from individual DNA samples and analyzed by agarose gel electrophoresis to identify loci with larger numbers of polymorphisms. Next, STR scanning of these loci was performed for orange tabby cats.

Three types of 5'-fluorescently labeled primers [6-FAM (blue), HEX (green), and TAMRA (yellow)] were synthesized and used to perform PCR with genomic DNA from 34 orange tabby cats under the optimized conditions described above. After confirming successful PCR product amplification by agarose gel electrophoresis, the PCR products generated using the 3 fluorescently labeled primers were mixed at a ratio of 1:2:3 (FAM:HEX:TAMRA). A total of 1 μL of each mixture was gently mixed with 25 μL formamide and visualized using capillary electrophoresis on an ABI-3730XL DNA Analyzer system (Applied Biosystems, Foster City, CA, USA). The peak heights of the products in each sample were evaluated using the GeneMarker software (SoftGenetics, LLC, State College, PA, USA).

Genetic polymorphisms

The gene fragments identified by STR scanning were marked as a, b, c, d, and so on according to the band size from shortest to longest. The genotype at each locus was systematically recorded for each of the 34 orange tabby cats and input as aa, ab… into the Popgene3.2 program to analyze genetic polymorphisms within this cat population. The general genetic structure indices, including the observed and effective number of alleles, Shannon's information index, observed and effective heterozygosity, and Nei's genetic distance were calculated to determine the genetic variation within the stock population. Conformation to Hardy-Weinberg equilibrium on a locus-by-locus basis within the stock population was also tested using the Popgene 3.2 software.

RESULTS

Primary detection of available microsatellite loci

All 134 microsatellite loci tested, including 131 cat microsatellite loci and 3 Sumatran tiger microsatellite loci, were PCR-amplified using an annealing temperature gradient. Preliminary analysis of the PCR products was performed by agarose gel electrophoresis. Of the 134 loci tested, 113 were successfully amplified. All 113 loci showed clear bands, including 2 of the 3 Sumatran tiger microsatellite loci (representative images shown in Figure 1A). The most polymorphic loci were identified by performing individual-sample PCR of 113 loci

in 32 domestic cats. Of these, 31 loci showed higher allele numbers than the others and were selected for further STR scanning experiments (Figure 1B and Table 1).

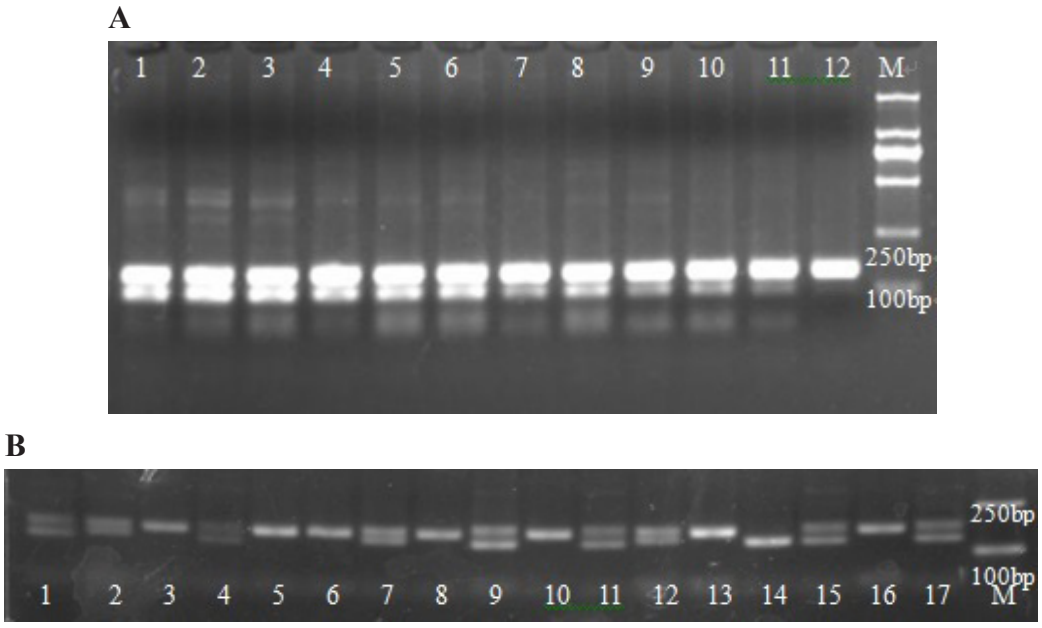

Figure 1. Gel electrophoresis of PCR products amplified from loci FCA077 (A) and FCA1056 (B) in orange tabby cats. **A.** A gradient of annealing temperatures was used to identify the best amplification conditions. *Lane M* = D2000 marker; *lanes 1* and *2* = annealing temperature at 50°C; *lanes 3* and *4* = 52°C; *lanes 5* and *6* = 54°C; *lanes 7* and *8* = 56°C; *lanes 9* and *10* = 58°C; *lanes 11* and *12* = 60°C. **B.** Individual-sample PCR result of 1 locus in 113 loci. *Lanes 1-17* = animal number.

STR scanning and genetic variation analysis

After preliminary selection by agarose gel electrophoresis, 31 loci were selected for STR scanning in orange tabby cats for genetic variation analysis. Partial STR scanning results of the selected locus FCA1056 are shown in Figure 2. These results indicated that the 31 microsatellite loci were more polymorphic than suggested by the results of the gel electrophoresis. Homozygous (Figure 2A) and heterozygous (Figure 2B) genotypes were easily distinguished for all 31 loci.

The genotypes were input into Popgene 3.2, and the mean effective allele number, mean Shannon's information, and mean effective heterozygosity were determined. Next, screening was performed using the SPSS software (SPSS, Inc., Chicago, IL, USA). The results of this screening are shown in Table 2. In the outbred orange tabby cat population, the highest expected heterozygosity and observed heterozygosity values were 0.7950 and 0.9412, respectively, both for locus FCA085. The important genetic structure parameters of the orange tabby cat stock, including the average observed number of alleles, mean effective number of alleles, mean Shannon's information index, mean expected heterozygosity, and mean observed heterozygosity, were 3.8387, 2.4027, 0.9787, 0.5565, and 0.5228, respectively. These data indicate that the cat population has comparatively high genetic diversity.

Table 1. Information for the 31 loci, including names, primer sequences, size of 31 microsatellite loci, optimal annealing temperature, and optimal concentration of Mg^{2+}.

Locus	Chr.	Primer sequence (5'-3')	Size (bp)	Annealing temperature (°C)	Concentration of Mg^{2+} (mM)
FCA077	C2	GGCACCTATAACTACCAGTGTGA ATCTCTGGGGAAATAAATTTTGG	139-155	58-60	1.5
FCA954	D2	ATGTTTTAAGTGCCAACGCC CTTGACCGAGGTCAGAATTACC	189	54-60	1.5
FCA823	B1	AGGGTGTGCTAGAACTAGCTGG CATTTAGAGGTTCAGGACTGGG	213	56-60	1.5
FCA770	A1	TCAAGAGTCTTTGCTCAAGGG TTTACTTAGGACTGACAGGGCA	103	54-58	1.5
FCA043	C2	GAGCCACCCTAGCACATATACC AGACGGGATTGCATGAAAAG	122-128	60	1.5
FCA044	B4	AGGGCCTGAACCAAGAGAAT TATTTACAGAGTGCACAGAGGAGG	160	54-60	1.5
FCA69	B4	AATCACTCATGCACGAATGCAATTTAACGTTAGGCTTTTTGCC	100	50-56	1.5
FCA559	B1	GCCAAAATGTTCAAGAGTGGTTTTGGCTTGATGAGCATCA	150	54-56	1.5
FCA976	D4	TCCATTTACCTGGGAAATTCCACCCTCATGTCTTGGCAATC	226	50-60	1.5
FCA090	A1	ATCAAAAGTCTTGAAGAGCATGG TGTTAGCTCATGTTCATGTGTCC	109-120	60	1.5
FCA1240	D2	TCCTGATGTGGCAGTTAAACCGCATGCCTTGAACCTTTCAT	137	56-60	1.5
FCA176	A1	GGAAACTTGGAAAGCAAAACC TCCACAGTTGGAGTTCTTAAGG	220	56-60	1.5
FCA045	D4	TGAAGAAAAGAATCAGGCTGTGGTATGAGCATCTCTGTGTTCGTG	185	56-60	1.5
FCA723	A1	TGAAGGCTAAGGCACGATAGACGGAAAGATACAGGAAGGGTA	221	56-60	1.5
FCA085	E2	GGTCCTCACGTTTTCCTATGTCTGTATGAGATGCGGT	270	56-60	1.5
FCA084	A1	TAGGTGAATGTTGGGATTTATGGAACTGAAGACCAAATTGATGAG	150	50-60	1.5
FCA210	B4	TGAGCCACCTAGGCACTCTTAGAAGCATCCAGTGACAATGG	215	58-60	1.5
FCA1344	B1	ACAGTTCCCTTACACACACACGGGCTTCGAAACCAAATTCAA	237	56-60	1.5
FCA453	A1	AATTCTGAGAACAAGCTGAGGGATCCTCTATGGCAGGACTTTG	200-220	60	1.5
CSPG2	A1	GATCCAACTCCAGCCTACCAACTCCTAGGGGGCAAATGAC	240	58-60	1.5
FCA1242	D2	CACCACGGGTAAAGATGCTT AGAAAATCCACACACAGCACC	280	56-60	1.5
FCA672	F2	AAGTTGCTTGCACACACTGCTCCAAGAGCCTTTTCAGTTAGG	110	60	1.5
FCA1056	B4	GGTGTGAGGGCCTATTCTGAGGATGTCTCCCTTGACTGGT	142	54-60	1.5
FCA764	A1	TGAGGGTGAGATGGAAGGAGACTAACCAATCCTGGTGGTCC	219	58-60	1.5
FCA1239	D2	AAAAGCCCTGACACCCAAGCTTGACCTTAATTGCTCATTGG	145	56-60	1.5
FCA700	B1	CCCTTAAAATCGCAGCTCTGAATCCAAGGAAAACAGGCCT	239	56-60	1.5
FCA742	D4	TCAATGTCTTGACAACGCATAAAGGATTGCATGACCAGGAAC	149	52-60	1.5
FCA678	A1	TCCCTCAGCAATCTCCAGAAGAGGGAGCTAGCTGAAATTGTT	133-234	54-60	1.5
FCA391	B3	GCCTTCTAACTTCCTTGCAGATTTAGGTAGCCCATTTTCATCA	129-273	58-60	1.5
FCA480	C1	GATCTCACACTTCATGGGACTGACTGCATCCCAAAGGTTTTG	260-270	60	1.5
FCA096	E2	CACGCCAAACTCTATGCTGACAATGTGCCGTCCAAGAAC	200-224	54-60	1.5

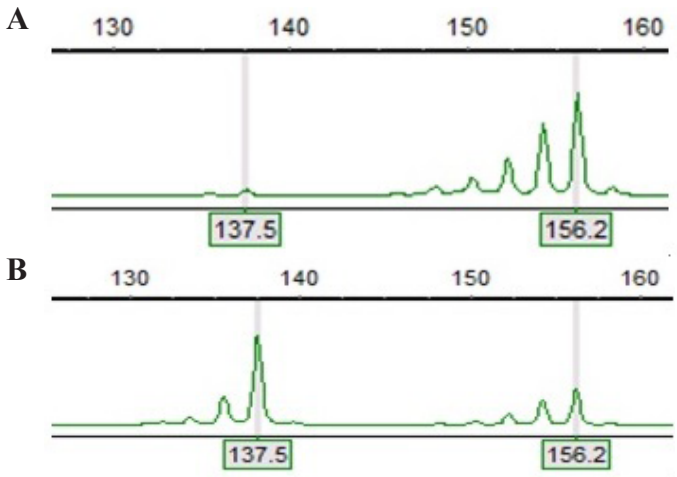

Figure 2. STR scanning result for locus FCA1056 (A: homozygosity; B: heterozygosity). **A.** The sequence size was 156 bp. In the figure, the highest value of the 3 continuous peaks was the size of this microsatellite locus. If the locus was homozygous, only a wave of 3 continuous peaks was observed; if the locus is heterozygous, 2 waves of 3 continuous peaks were observed, and the highest value of the first is the actual value. **B.** The first peak size was 138 bp, while the second was 156 bp.

Table 2. Observed number of alleles, effective number of alleles, Shannon's information index value, and expected and observed heterozygosity values for each of the 31 microsatellite markers in 34 orange tabby cats.

Loci	Observed number of alleles	Effective number of alleles	Shannon's information index	Expected heterozygosity	Observed heterozygosity
FCA077	3	1.9610	0.7347	0.4974	0.5000
FCA954	3	2.7426	1.0501	0.6449	0.6471
FCA823	6	3.8988	1.5274	0.7546	0.6765
FCA770	6	2.5158	1.2277	0.6115	0.7059
FCA043	4	2.0533	0.8097	0.5206	0.5294
FCA044	4	2.6453	1.0966	0.6313	0.2647
FCA069	3	1.3040	0.4370	0.2366	0.2647
FCA559	4	2.7232	1.1637	0.6422	0.5882
FCA976	3	2.0735	0.8524	0.5255	0.6176
FCA090	3	2.8649	1.0751	0.6607	0.4118
FCA1240	4	2.5890	1.0572	0.6229	0.5588
FCA176	4	2.5103	1.1181	0.6106	0.6176
FCA045	3	2.1114	0.8037	0.5342	0.4412
FCA723	6	3.9320	1.4597	0.7568	0.7353
FCA085	8	4.6148	1.7621	0.7950	0.9412
FCA084	4	2.7822	1.1673	0.6501	0.5588
FCA210	3	2.2381	0.8756	0.5615	0.4412
FCA1344	3	2.2468	0.9402	0.5632	0.3235
FCA453	5	2.7458	1.2289	0.6453	0.5882
CSPG2	2	1.6374	0.5779	0.3951	0.0000
FCA1242	3	1.2304	0.3737	0.1901	0.2059
FCA672	4	2.0885	0.9368	0.5290	0.5294
FCA1056	3	2.1811	0.9200	0.5496	0.6176
FCA764	3	1.8233	0.7855	0.4583	0.4706
FCA1239	3	1.8721	0.7398	0.4728	0.5588
FCA700	4	2.4388	1.0660	0.5988	0.5588
FCA742	4	1.8721	0.8849	0.4728	0.4706
FCA678	4	2.5604	1.0770	0.6185	0.6765
FCA391	3	1.5696	0.6556	0.3683	0.3824
FCA480	3	1.9461	0.8416	0.4934	0.5588
FCA096	4	2.7104	1.0939	0.6405	0.7647
Orange tabby cat population (average value)	3.8387	2.4027	0.9787	0.5565	0.5228

DISCUSSION

Domestic cat was originally bred as a type of pet, but is also an important experimental animal. Some breeds of cat are genetically distinct and have several inherited diseases (Lyons et al., 2006), which can be used as models of hereditary and infectious diseases (Dow et al., 1987). There is only 1 outbred stock of orange tabby cats in China, which has been maintained by the North China Pharmaceutical Group New Drug Research and Development Limited Company. The group of orange tabby cats originated from a mutated male fox tabby cat of an outbred stock. The coat color and pattern of the orange tabby is clearly distinct from that of the fox tabby but is similar to that of the tiger tabby. Thus, researchers bred these cats using hybridization and backcrossing, and successfully established an outbred orange tabby population stock for more than 30 years. The total number of breedings has reached more than 1000. This stock can be used as companion animal for its beautiful color and strip. It is also used as a model in human disease research. However, the genetic background of this population is unclear. In the present study, genetic variation of this outbred stock was evaluated using microsatellite DNA markers. Generally, the key genetic parameters obtained in the present study indicate that this population of cats exhibits high genetic diversity. A precise estimate of genetic diversity is a function of the number of loci analyzed, the heterozygosity of these loci, and the number of animals sampled in each population (Barker, 1994). The Food and Agricultural Organization recommends that at least 25 randomly selected animals be used in population studies, and Barker et al. (1994) suggested that loci with at least 4 different alleles should be used in diversity studies to reduce the standard error of the estimated distance. In our study, the average observed number of alleles was 3.8387, which met this standard. The mean effective allele number was 2.4027, which was lower than that in other reports examining domestic laboratory animals, such as gerbils (Du et al., 2010) or hamsters (Li et al., 2010). Most of the 31 microsatellite markers deviated significantly from Hardy-Weinberg equilibrium. This result was also supported by differences between expected and observed heterozygosities (Table 2), indicating the occurrence of non-random mating. Of the 31 loci studied, most (77.42%, 24/31) showed observed heterozygosity values lower than or equal to the expected values (Table 2). This result, combined with the lower effective allele number, indicated that although random mating occurred in this orange tabby cat population, there is an additional degree of inbreeding in this group. The mean Shannon's information index in this cat population was 0.9787, reflecting the polymorphisms in these microsatellite loci. Mean expected heterozygosity is an important genetic parameter that reflects genetic heterozygosity. This value in our study was 0.5565, indicating that the orange tabby cat stock exhibited the characteristics of a good outbred stock. These data showed that the panel of 31 microsatellite loci was efficient for genetic monitoring in cats.

Our 31 microsatellite loci included 2 of the 3 loci (including FCA700 and FCA559, except locus FCA254), as reported by Lyons et al. (2006) to be linked with the tabby gene. Schmidt-Kuentzel et al. (2009) found significant linkage to the O locus for the markers FCA1466 and FCA1494. Although these 2 loci were included in our original set of 131 cat loci, they were not efficiently amplified and showed low polymorphism. Thus, they were not included in the genetic monitoring group. Lipinski et al. (2007) performed a parentage comparison test among 17 worldwide commercial and research laboratories to develop a microsatellite-based panel for the domestic cat. In their study, 19 microsatellite markers were included

in the comparison test and genotyped across samples; based on robustness and efficiency, 9 autosomal microsatellite markers were ultimately selected to be included in a single-multiplex "core" panel for cat identification and parentage testing. Our 31 loci included only 2 (FCA069 and FCA678) of these 9 loci, which exhibited quite high levels of polymorphism within the 31 loci. These 9 loci, in addition to 2 gender markers, were found to be sufficient for parentage, gender determination, and identification testing in random-bred and purebred domestic cats, as well as several wild felid species (Pilgrim et al., 2005). However, an additional panel of markers should be developed to evaluate the tabby orange cat population in China because it is a new breed of outbred stock. Our results provide another panel of microsatellites that can be used to analyze genetic structure.

In our study, PCR of pooled DNA samples was used to facilitate the selection of loci that could be effectively amplified by PCR. Although this method may lead to bias in the allele frequency, it is a cost-effective method for any study related to microsatellite markers (Megens et al., 2008). Additionally, our results from 5 pooled DNA samples of cats from pet market also showed that it is helpful to minimize the variations that may exist among individual animals and to amplify as many polymorphic alleles as possible for each microsatellite locus.

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Supplementary material

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