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Development of novel DNA markers for genetic analysis of grey hamsters by cross-species amplification of microsatellites

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ABSTRACT. The grey hamster has been used in biomedical research for decades. However, effective molecular methods for evaluating the genetic structure of this species are lacking, which hinders its wider usage. In this study, we employed cross-amplification of microsatellite loci of species within the same genus by polymerase chain reaction. Loci screened included 107 from the Mongolian gerbil (MG) and 60 from the Chinese hamster (CH); of these, 15 polymorphic loci were identified for the grey hamster. Of the 167 loci screened, 95 (56.9%) with clear bands on agarose gel were initially identified. After sequencing, 74 (77.9%) of these matched the criteria for microsatellite characteristics, including 41 from MG and 33 from CH. Lastly, 15 (20.3%) loci with more than two alleles for each locus were identified through capillary electrophoresis scanning. To justify the applicability of the 15 grey hamster loci, genetic indexes of grey hamsters were evaluated using 46 generations of outbred stock, established 20 years ago, from Xinjiang, China. Mean effective allele numbers and expected heterozygosity of stock were as low as, respectively, 1.2 and 0.14; these were 2.8 and 4.0 times inferior, respectively,

to wild grey hamsters. This finding suggests that the genetic structure of the stock-bred population is too weak to resist artificial and natural selection, mutation and genetic drifting. In conclusion, we have developed *de novo* microsatellite markers for genetic analysis of the grey hamster, providing data and methodology for the enrichment of a genetic library for this species.

Key words: Genetic structure; Microsatellite DNA; Grey hamster; Cross-species amplification; Biomarker

INTRODUCTION

The grey hamster (*Cricetulus migratorius*) is endemic to east Europe and central Asia as well as northwestern China (Bashenina et al., 1951). Its potential value for use in biomedical research has been recognized for decades. For instance, as a diverse microbial population host (Gao et al., 2009), the grey hamster is an ideal animal model for studying zoonoses such as kala azar, plague, and hydatid diseases (Li et al., 1999; Rena et al., 2001; Liao et al., 2002). In China, Liao and colleagues developed an outbred stock (Xinjiang stock) of grey hamster from 1989 to 1992, based on a female captured in Urumqi and one male captured in Kashi city, Xinjiang, China (Liao et al., 1994). By the year 2012, this stock had been bred for over 46 generations, and microorganisms and parasites within the stock had been well controlled through repetitive bio-rederivation procedures (Liao 2002). In addition, major physiological and biochemical indexes have been systematically evaluated for the stock population, including reproduction performance, social behavior, organ coefficient characteristics, hematologic parameters, basic metabolic traits and hibernation; genetic analysis has also been performed based on biochemical methods (Liao et al., 2000; Gromov et al., 2006; Romanenko et al., 2007; Ibiş et al., 2011). These fundamental works improve the potential for the application of this animal as a novel laboratory model.

Some genetic change through multiple generations of breeding of the grey hamster is inevitable. For this reason, a developed management plan should include the application of basic genetic principles, combined with molecular genetic monitoring, to minimize harmful genetic change (Allendorf et al., 2008). However, because the genetic quality standard for grey hamsters has not been established in China, it is difficult to monitor the genetic quality of stock, presenting an obstacle for the wide usage of this animal as a laboratory species. Hence, developing genetic markers for the grey hamster may provide special benefits for the species in biomedical and genetic research.

Microsatellites are repetitive and conservative DNA sequences, composed of reiterative motifs one to six base pairs long, that are characterized by a relatively high degree of polymorphism within expressed sequence tags, protein-coding genes (and untranslated regions), or introns (De Gortari et al. 1997; Navani et al. 2002; Martin et al., 2005). As a complementary strategy to traditional phenotype-driven genetic monitoring methods, microsatellites are useful for the analysis of genetic traits, population structures and relatedness among animals (El-Ghor et al., 2010; Nidup and Moran, 2011; Song et al., 2011). Microsatellites are also used as biomarkers for monitoring genetic modifications within rodents (Du et al., 2013). Because the grey hamster genome has not been fully sequenced, it is currently difficult to directly isolate microsatellites for this species from the traditional genomic library.

It has been suggested that closely genetically related species, with identified microsatellites, can be borrowed for duplicating the microsatellite loci through cross amplification (De Gortari et al. 1997; De Gortari et al., 1998; Navani et al. 2002; Huang et al. 2005). Inspired by this theory, we

sought to identify microsatellites for the grey hamster from the closely related Mongolian gerbil and Chinese hamster (Li et al., 2010; Li et al., 2011). To date, not only have 130 microsatellite loci of the Mongolian gerbil been identified from 536 loci in the mouse genome (Du et al., 2010), but many loci of the Chinese hamster have also been identified from the NCBI database (http://www.ncbi.nlm.nih.gov/nuccore/?term=CGH01). Following careful selection, we have collected a pool of 167 microsatellite loci, consisting of 107 loci of the Mongolian gerbil and 60 of the Chinese hamster supplied by NCBI, and performed step-by-step screening of microsatellite loci to identify those suitable for the grey hamster.

MATERIAL AND METHODS

Animals and tissue sampling

For microsatellite loci screening, grey hamsters of Xinjiang, outbred stock were obtained from the Xinjiang Institute for Endemic Disease Control and Research, Xinjiang, China (N = 6). Animals were fed a normal diet and maintained under standard management for the duration of the study. Each hamster was euthanized by cervical dislocation, and then kidney tissue was collected and preserved in sterile tubes at -80ºC.

For stock population genetic analysis, hamsters from the Xinjiang stock with no relationship within three generations (N = 40), and wild hamsters captured in Xinjiang and Qinghai Provinces, China (N = 48) were used in order to enlarge the sampling coverage ratio and obtain representative results. Kidney samples were collected using the same method described above. Experiments and procedures performed on animals were approved by the animal welfare committee of Capital Medical University, Beijing, China.

Candidate microsatellite resources

The microsatellite loci pool was composed from GenBank and consisted of 167 loci, including 107 loci of the Mongolian gerbil and 60 demonstrated loci of the Chinese hamster (**Table S1**). Microsatellite primer sequences were downloaded from the NCBI website and synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). All chemicals and reagents used in microsatellite proliferation and detection were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA) unless otherwise indicated.

DNA extraction

Total genomic DNA was extracted from each frozen kidney tissue sample through phenol-chloroform extraction and ethanol precipitation, using a previously described method (Du et al., 2010). Extracted DNA samples were stored in 10 mMTris-HCl plus 1 mM EDTA (TE) buffer at 4°C. Samples were then quantitated by measuring the A260/A280 value using a microplate absorbance reader (Bio-Rad 680, CA, USA), and sample quality further evaluated by 0.8% agarose (w/v) gel electrophoresis. Samples were then diluted to 100 ng/μL, and stored at -20°C for later use as templates in polymerase chain reactions (PCRs).

PCR procedure

Microsatellites of grey hamsters were amplified by PCR. At the stage of microsatellite loci screening, in order to minimize variations that may have existed among individual hamsters and

to amplify as many polymorphic alleles as possible for each microsatellite locus, equal volumes of material from each of the six sampled grey hamsters was mixed gently to achieve 20 µL total DNA solution (100 ng/µL), which was used as the final template for PCR amplification. For each 15 µL PCR amplification system, the following reagents were used: 1.5 µL 10X buffer; 1.0 µL each 1.0 µM primer; 1.0 µL x 4 dNTPs (100 µM; Takara Co. Ltd., Tokyo, Japan); 1.0 µL Taq high-fidelity DNA polymerase (1.0 U; Takara); and 1.0 µL template DNA (50-100 ng). Amplifications were performed in a gradient thermal cycler (BIO-RAD Inc. ALS1296, CA USA) using the following protocol: pre-denaturation at 94°C for 5 min; 35 cycles of denaturation at 94°C for 30 s, annealing for 30 s, and extension at 72°C for 30 s; and a final extension at 72 °C for 7 min. Amplified products were stored at 4°C for further microsatellite analysis.

Stepwise microsatellite analysis

Stage one: agarose gel electrophoresis-based screening of 167 cross amplified microsatellite loci

For each microsatellite amplification reaction, PCR was carried out in two steps. In the first, PCR was performed using the parameters reported above, including the concentration of $MgCl_2$ and the annealing temperature. Subsequently, 8.0 µL of each product was stained with ethidium bromide, and bands for each product were examined by electrophoresis on 1.5% agarose (w/v) gel at 140 volt for 30 min then visualization using a UV transilluminator (VilBerLouRMAT Inc., Marne la Vallee, France). In the second step, any PCR products with unclear bands were optimized by modulating the concentration of $MgCl_2$ (Takara) from 1.5 to 3.0 mM, and by optimizing the annealing temperature point, by comparing PCR product quality at four gradually fluctuated temperature points around the reported temperature. Following this, loci with clear bands within the expected size range were selected for further analysis (stage 2). For each microsatellite, experiments were repeated in triplicate.

Stage two: DNA sequencing-based screening

Microsatellite loci screened from stage one were amplified by PCR and sequenced. Briefly, each PCR amplification system (50 µL) contained the following reagents: 5 µL 10X buffer; 2.5 µL each primer (1.0 µM); 2.5 µL x 4 dNTPs (100 µM; Takara); 2.5 µL Taq high-fidelity DNA polymerase (1.0 U; Takara); and 2.5 µL template DNA (50-100 ng). Amplifications were performed using the aforementioned optimized protocols for each locus. Before products were sequenced, they were reexamined through 1.5% agarose gel (w/v) electrophoresis at 120 volt for 40 min. Based on DNA sequencing results, loci with correct core sequences were selected for stage 3. Experiments were repeated in triplicate for each microsatellite.

Stage three: capillary electrophoresis-based screening

Forty DNA samples derived from the Xinjiang stock and 48 from wild samples were used as templates for PCR amplification of the selected loci from stage two. Primers were fluorescently labeled with 6-FAM (blue), HEX (green) and TAMRA (yellow), for which the internal size standard was ROX (red). The PCR procedure was carried out as previously described (Zuo et al., 2012). Briefly, primers were first tagged to dyes at the 5' ends and PCR performed using 15 µL volume

reactions. Then, PCR products were mixed at the ratio of 1:3:5 (FAM:HEX:TAMRA), and 1 µL each mixture combined with 25 µL formamide, vortexed and sized by capillary electrophoresis on an ABI-3730XL DNA Analyzer (PE Biosystems, Foster, California, USA). Of all scanned microsatellite loci, those with at least one effective allele were confirmed as candidate loci for genetic monitoring and population studies. Experiments were repeated in triplicate for each microsatellite locus.

Population genetic structure analysis

After the genotypes of each locus were systematically recorded, the genetic structure of the hamster populations were analyzed based on the screened microsatellite combination of Xinjiang stock. Results were compared to those from wild-caught hamsters.

Statistical analysis

Genotypes of microsatellite loci were analyzed using GeneScan 3.7 software (Applied Biosystems, Foster, California, USA). The number of alleles and the mean heterozygosity within the Xinjiang stock was analyzed using Popgene software (Version 3.3; May et al., 1995). General genetic structure indexes, including the observation and effective numbers of alleles, Shannon information index, observed and effective heterozygosity, and average polymorphism information content (PIC), were calculated for genetic variation analysis within the hamster stock. In addition, the genetic distance between the Xinjiang stock and wild grey hamsters was measured according to the method of Nei's standard distance (Nei, 1972) and calculated using Popgene 3.3 software.

RESULTS

Selection of microsatellite loci

In order to find representative microsatellite loci that could reflect the general background of the grey hamster, selection was performed through careful examination of PCR products at each stage of the study. After the first round selection, 95 of 167 loci were selected, that produced clear bands on agarose gel electrophoresis. Following sequencing, 74 of the selected loci matched microsatellite qualifications; among these, 41 were identified from mice, and 33 from Chinese hamster. Of the 74 selected loci, 42 were selected that had more than one clear band on agarose gel and more than three copies of core sequences based on DNA sequencing. Finally, using capillary electrophoresis scanning, 15 microsatellite loci were selected from these 42 candidates, that had more than two identical polymorphic alleles. Detailed optimization parameters for PCR reactions of the selected loci are shown in Table 1.

Genetic structures of Xinjiang stock compared to wild grey hamster

Based on analysis of PCR results of the 15 identified microsatellites in 40 outbred (Xinjiang) grey hamsters and 48 wild grey hamsters, a general static analysis of the genetic structure was performed. As shown in Table 2, the mean observation number of alleles in wild grey hamsters was 4 times higher than that in the outbred, and the mean effective number of alleles was 2.8 times higher in wild than in outbred hamsters. The Shannon information index in wild hamsters was 5.4 times higher than in the Xinjiang stock. Not surprisingly, the mean observational and expected

heterozygosity in wild type hamsters was 1.6 and 4 times higher, respectively, than those of the Xinjiang stock. Lastly, Nei's standard distance of the wild population was 4 times higher than that of the Xinjiang stock. Taken together, these results demonstrated a much lower level of genetic variation within the outbred Xinjiang grey hamster population than in wild grey hamsters.

Table 1. PCR amplification parameters for 15 microsatellite loci of the grey hamster.

Microsatellite locus	Primer sequences (forward one the first line and reverse on the second for each locus)	Mg^{2+} (mM)	Annealing temperature (ºC)	No. alle	Rang. alle (bp)
DQ459500	GTTTTTATGCCAATAACATCCTGT CAATCCCGAATAACCAAAGAAC	2	50.6	4	181-184
DQ459487-1	TTCTGGGACAGGTATCCAAGGT TGATGCTCAGTGGGTAGAGTGC	1.5	60	3	209-240
DQ459495	TACTCTCAACAAACATCTTCAGGCT AGACAAGTGCTCTACCAGTGACCTA	1.5	48.1-60	2	209-215
D11Mit4	CAGTGGGTCATCAGTACAGCA AAGCCAGCCCAGTCTTCATA	1.5	48.1-60	2	249-262
DQ459502-1	TGATGGGATTAAAGCATGTACCAC TCCAAGGGCACTGGCACTCAT	1.5	56.6-60	6	105-119
DQ459502-2	CCACCACCACCAAGCACAATC TCCAAGGGCACTGGCACTCAT	1.5	48.1-60	6	106-119
DQ342059-2	CCAGAAGTTGAAGGCAGAAAGA CTGTTCAGGAAGGATTAGGAGG	1.5	58	2	122-124
DQ459488-1	CAGGCATATATACAGGCAGAAT GGGAACTTAGGAGAAACTGGA	1.5	56.6	4	136-146
DQ342060	CCAGGATAGGCTCCAAAGCTAC ACCCCACTCAGAAATCAAAGAC	1.5	54.7-58	2	98-99
DQ459489-2	TTGGTGAAATGACCCATTCGTG TGTCTTGGTCCTTTCTTGATGCT	2	48.1-58	22	215-261
DQ459488-2	TTCTGTTTCCTCTTCCGCAAT CTAAGAATAAGTTACTGTGTGTGCG	2	48.1	5	316-328
D3Mit215	TAAACATCTAGAAGATGCTGCAGG CTGCATGGCCAGGACTAGTT	2	54.7	10	315-338
D9Mit323F	AAATCCTGGAACCCTTCACC TGTGTGCACCTACACTCAGTTC	2	50.1-54.7	2	363-367
D3Mit130	AACACATGAAACGTGTGCGT TGATAGGCATGCTTAAGCCC	2	48.1	20	446-476
D7Mit227	GAGTCCTCAGCAGATATTACTCAGC CTGATGTCTCATCATTTGGGG	2	48.1-50.6	2	305-315

Chrom, Chromosome; No. allele, number of alleles; Rang. alle, range of alleles.

Table 2. Genetic structure indexes of outbred compared to wild grey hamsters.

Origin	O-A	E-A	Shan	PIC	O-H	E-H	Nei's
Wild	6.0667	3.3043	0.9647	100.00%	0.2236	0.4564	0.4517
Outbred	1.5333	1.2435	0.1775	26.67%	0.1433	0.1141	0.1127

O-A, mean observation number of alleles; E-A, mean effective number of alleles; Shan, Shannon's information index; PIC, polymorphism information content; O-H, mean observation number of heterozygosity; E-H, mean expected heterozygosity; Nei's, Nei's genetic distance.

DISCUSSION

In order to obtain representative loci with reliable polymorphisms, we adopted three methods for step-by-step selection. We demonstrated that each of the selected microsatellite loci could be amplified successfully, and had multiple alleles.

Over the past decade, a wide range of molecular techniques has been used to determine

genetic variations of *Cricetulus*, including DNA sequencing (Xie and Zhang, 2005; Neumann et al., 2006; Partridge et al., 2007); Random Amplified Polymorphic DNA (RAPD) (Wang et al., 2002); and allozyme analysis (Kartavtsev et al., 1984a, b; Mezhzhzerin, 2001). Employing microsatellite biomarkers to study the genetic structure of wild animals has proved to be successful (Schmidt et al., 2008; Oishi et al., 2009). For example, Song et al (2011) reported on the molecular genetic profiles of Shanyi A and E inbred strains of Chinese hamster with 16 loci screened from a microsatellite-enriched genomic DNA library. Although conservation of microsatellites among species within the same genus has low efficiency, and often yields null alleles (Hardy et al. 2003; Dutech et al. 2007), we have previously obtained microsatellite loci of the Mongolian gerbil from the mice genome, by enlarging the candidate microsatellite loci pool (Du et al., 2010). In the current study, 15 microsatellites were identified for the grey hamster, based on those found in the Mongolian gerbil and Chinese hamster, both related species (Li et al., 2010; Li et al., 2011).

Reasons that may account for the poor heterozygosity observed within the Xinjiang hamster stock in the current study are outlined below. Firstly, the stock population was established from a single male and female hamster, captured in different cities, meaning that initial breeding was based on inbred, rather than outbred strategies, with a resulting restriction in population genetic diversity. Secondly, an insufficient number of microsatellite with multiple alleles been selected may accounts for the poorheterozygosity. Previously, based on the same screen strategy, we had successfully identified 32 microsatellite loci to monitor the genetic structure within three outbred Chinese miniature swine populations (Wang et al., 2014). According to that study, microsatellite loci used for population genetic studies need to satisfy certain qualifications, such as employing at least 25 loci (four alleles for each locus) and having no linkage relations among the loci (Barker, 1994; Benavides et al., 2000). Unfortunately, in the current study, the selected microsatellite number with multiple alleles was not sufficient for a standard selection, although they were selected from a pool of 167 candidate loci.

A high mean effective number of alleles indicates a population is stable enough to evade new variation when facing pressure of genetic drift, mutations, and natural and artificial selection (Takezaki and Nei, 1996). Since all genetic structure indexes examined here were lower than those of wild grey hamsters, which implies that the Xinjiang stock has high genetic structural consistency, low genetic variation capacity, and that its genetic structure may be too weak to resist selection, mutation and genetic drifting.

The grey hamster is a popular animal model not only for infectious diseases studies, but for studying human hunger and thirst resistance, the assessment of its resistance to anticoagulants like warfarin and male infertility (Dong et al., 1991). In this study, we developed a microsatellite-based genetic analysis of the first outbred stock of the grey hamster. However, owing to the limitations of the method, it is difficult to provide an overview of the genetic structure for the stock so far. Thus, the development of further genetic measurement strategies for interpreting the genetic structure of this hamster population is warranted.

In conclusion, this study provides informative data and a practical method for enrichment of the genetic data library of the grey hamster, and the identification of 15 microsatellite loci as a means to monitor the genetic structure of outbred stocks of grey hamster.

Conflicts of interest

The authors declare no conflicts of interest.

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Supplementary material

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