Parentage and Genetic Diversity Studies on Pangolin (*Manis javanica*) using Novel Microsatellite Markers

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ABSTRACT

The Malayan pangolin (*Manis javanica*) is an endangered mammal species exhibiting scales characteristics. The conservation and management of this species could benefit from a better understanding of its genetic diversity and structure. In this study, 24 novel SSRs were isolated from full-length transcriptome and they were used for assessing of parent-offspring relationship for *M. javanica*. All SSR markers were highly polymorphic with a mean of 6 alleles per locus, ranged from 2 to 10 alleles. The average polymorphism information content (PIC) was 0.62. The observed (H_O) and expected average heterozygosity (H_E) value was 0.56 and 0.67 tested in 39 individual samples. For parentage testing, the allele frequency date-based indicated that the combined exclusion probability values were over 99.99% when 9 SSRs were used. This study demonstrated that the microsatellite-based approach could be effectively utilized for parentage analysis in pangolins and that it has a significant application in selective breeding in endangered and valuable mammalian species.

INTRODUCTION

Pangolins are mammals exhibiting atypical morphological characteristics and belong to *Manis*, order Pholidota (Gaudin *et al*., 2006). These mammals have overlapped horny scales on the major parts of the body and a buccal system adapted to a myrmecophagy that makes them unique among the Old World mammalian fauna (Luo *et al*., 2007). A total of eight extant species of pangolins have been recognized and are widely distributed in Africa and Asia due to the exploitation of natural resources, as well as medicinal demand for pangolin scales and meat (Gaubert and Antunes, 2005). In addition, the illegal trade of pangolin is becoming increasingly serious, and all pangolin species are listed in the Conservation on International Trade in Endangered Species of Wild Fauna and Flora (CITES I) (Kumar *et al*., 2016; Li *et al*., 2017).

Although in situ protection is an important component of endangered species protection, in order to expand the existing population and increase genetic diversity, artificial breeding and ex situ conservation have become important measures to protect endangered animals (Wu *et al*., 2002). Under the conditions of artificial breeding, endangered animals can be guaranteed more comprehensive and adequate food and nutrition and can receive good health protection while avoiding harm of predators (Wu *et al*., 2002; Yusoff *et al*., 2016). Pedigree information can provide relevant data for breeders to make pertinent decisions, since it allows for maintenance of high levels of variability in the progeny, increase of the heterosis effect, and reduction of the levels of inbreeding (Spanoghe *et al*., 2015; Tarn *et al*., 1992). Therefore, it is particularly important to establish a stable population of pangolins that is kept artificially in order to avoid inbreeding depression and genetic drift, increase the effective population size and to select and optimize pairing for the purpose of long-term gene exchange between the individuals with the smallest relationship. In this way, not only can the domestic and foreign social needs of scientific research and public education be met, but also certain conditions for the release of captive breeding individuals to supplement wild populations can be created.

Since the 1980s, parentage analysis of animals has been widely expended not only from the development

of methods for assessing the parentage of individual progeny but also from the increase in the diversity and availability of molecular markers (Luís *et al*., 2002; Zhang *et al*., 2016). SSRs (Simple Sequences Repeats) have become the markers of choice in both animal and plant species because of their discriminatory power, codominant transmission, and reproducible properties (Ellegren, 2004; Zietkiewicz *et al*., 1994). To date, microsatellite-based parentage identification technology is the most widely used and reliable method for the identification of genetic relationships among endangered species, such as Amur tigers (Zhang *et al*., 2003a), the giant panda (Zhang *et al*., 2003b), long-snouted seahorse (*Hippocampus guttulatus*) (Pardo *et al*., 2007), *Shinisaurus crocodilurus* (Luo, 2014), chimpanzees (Zhang *et al*., 2000), *Alligator mississippiensis* (Davis *et al*., 2001), *Dermochelys coriacea* (Crim *et al*., 2002), *Acipenser sturio* (Roques et al., 2016) and *Falco cherrug* (Hou *et al*., 2018). Moreover, microsatellite analysis only requires a small amount of DNA when sampling endangered and rare species by using nondestructive methods, which can be effectively analyzed and facilitated in field investigation.

In China, the Guangdong province wildlife rescue center has rescued some pangolins from the illegal trad of wild animals. These individuals were raised in Dongguan Institute of Qingfengyuan Animal Medicine; however, the source and relationship between members has not been thoroughly elucidated to date. To restore this rescued population, we combined the nondestructive extraction DNA method and the microsatellite sequences in a known transcriptome of *M. javanica* (Ma *et al*., 2019) to carry out new primers and polymorphic gene loci and to establish a highly sensitive paternity identification method for one breeding population of *M. javanica*.

MATERIALS AND METHODS

One natural death *M. javanica* and 39 saliva samples (23 males and 16 females) of breeding pangolins were provided by the Dongguan Institute of Qingfengyuan Animal Medicine in 2018. Samples were stored in RNAiso reagent (Takara, Otsu, Japan) and treated with DNase I (Takara). The total RNA (Ma *et al*., 2019) for transcriptome sequencing and locating microsatellite loci. Saliva samples were stored in 95% ethanol and kept at 4℃. From the pedigree record, we know that the parents of "BB" (♀) were "A26" (♀) and "A25" (♂) in this sample population.

The genomic DNA was isolated using a HiPure Tissue DNA mini kit (Magen Inc.). Randomly selected 30 microsatellite primers for PCR amplification to screen primers that preliminarily possessed polymorphic loci. The polymerase chain reactions (PCR) were conducted in

a 10 μL reaction volume containing 5 μL 2 × EasyTaq PCR SuperMix (Trangen Inc.), 1 μL FAM-labeled M13 forward primer, 0.4 μL unlabeled M13-tailed forward primer and 1 μL reverse primer, as well as 50-100 ng of genomic DNA template in 1 μL (Boutin-Ganache et al., 2001). PCR was performed with the following protocols: one cycle of denaturation at 95 ℃ for 10 min; 35 cycles of 95℃ for 15 s with an annealing temperature 52℃ for 30 s, extension at 72℃ for 30 s, and then a final extension at 72℃ for 30 min. PCR products were analyzed with an ABI 3730XL Genetic Analyzer (Applied Biosystems). Fragment sizes were determined by comparison with the internal standard using GeneScan 500-ROX (Applied Biosystems) and GeneMarker version 1.5 (SoftGenetics, State College, Pennsylvania).

The genetic diversity parameters of SSR loci, including the number of alleles (*Na*), observed heterozygosity (*Ho*), expected heterozygosity (*He*), alleles range and polymorphism information content (PIC), were calculated using the software CERVUS 3.0 (Kalinowski *et al*., 2007). Deviation from Hardy-Weinberg equilibrium (*HWE)* and linkage disequilibrium (LD) were performed using the software POPGENE V1.31 (Yeh *et al*., 2000) and FSTAT V2.9.4 (http://www.unil.ch/izea/softwares/fstat). For paternity testing, the exclusion probabilities of each locus based on the genotype of no known parent and on the genotype of one known parent were named NE-1P, NE-2P and NE-PP, respectively. In addition, the combined exclusion probability over the 24 loci were also calculated.

To estimate the feasibility and confidence of parentage analysis, a simulation program was designed with the software CERVUS 3.0 using the following parameters: parent pair (sex known), 70 candidate parents, 200 offspring, 30% of the candidate parents were sampled, and proportion of mistyped loci was 1%. In the program, the most-likely parents of each individual were assigned a statistic delta score and LOD score, and the true parents were evaluated depending on the scores of the candidate parents. In addition, a double-blind test was performed to evaluate the capacity of these microsatellite markers.

RESULTS

Identification and genetic diversity of microsatellite markers

According to the full-length transcriptome of *M. javanica*, a total 18,693 SSR sites were detected, including 12,120 mononucleotide repeat sequences, 3,202 dinucleotide repeat sequences, 1,594 tri-nucleotide repeat sequences, 259 tetra-nucleotide repeat sequences, 52 penta-nucleotide repeat sequences and 23 hexa-nucleotide repeat sequences. According to the obtained SSR sites, 56,079 pairs of primers were designed by the software Primer Primer 5.0.

Table I. Characterization of 24 microsatellites loci and conditions of PCR in Malayan pangolin.

Primers	Accession no.	Repeat type	FPr1(5'-3') / RPr1(5'-3')	Temperature (℃)	PSize (bp)	SSR locus
M13MJ-F2	MK121608	(TCCA)9	TTTCATACCGGGAAGTCCAC	59.8	169	PB676
			ATGGTCCTAACACCACGGAG	59.8		
M13MJ-F3	MK121609	(TTTA)8	CACCTGCATGTACCCCTTTT	59.9	226	PB210
			CCCCCTCAAAATACCACCTT	60.0		
M13MJ-F4	MK121610	(ATTC)8	GAGAGAAAGGGGAAAATCGG	60.0	237	PB220
			TGATAGGATGTGAGGAGGGG	59.9		
M13MJ-F5	MK121611	(AATT)8	TGGGGTCTGCTGTTTTCATT	60.5	194	PB235
			CTCCCTCTGTAGGTTGCCCT	60.6		
M13MJ-F7	MK121612	(TCAT)13	AGAAGTGATTTGCACCCCTG	60.1	228	PB272
			CAGTGGCCAGAATGGAGATT	60.1		
M13MJ-F9	MK121614	(ATGA)8	CCCTATGAGGTGGGCACTAA	59.9	276	PB387
			AACTCCATCAAAGGTGTGGC	60.0		
M13MJ-F10	MK121615	(GAAT)8	CCCAGATCCAAAATGAATGG	60.1	209	PB442
			TGCTGATGTTCACTCTTGCC	60.0		
M13MJ-F11	MK121616	(GATA)12	ATCCACCTAGGAACCTCAGC	58.2	249	PB464
			GACTCTTCGGGATTTCACACA	60.1		
M13MJ-F12	MK121617	(GATA)12	ATCCACCTAGGAACCTCAGC	58.2	242	PB4641
			GACTCTTCGGGATTTCACACA	60.1		
M13MJ-F17	MK121619	(ATAG)12	GTAATGGGGTATGTGGTGGG	59.8	213	PB537
			TCCCTGTTCAAACGGAATTT	59.4		
M13MJ-F20	MK121620	(AGAT)16	CAGTGCTCATCACATAGCAGG	59.5	177	PB652
			CATGCCTAGTGTTTCACGTTG	59.3		
M13MJ-F24	MK121621	(TTTTG)7	TTCAGCCAGGGTCTCTCAGT	60.0	188	PB714
			TGGGGTTTTTCCTCAATCTG	59.9		
M13MJ-F25	MK121622	(AAACA)9	CCAGAGAAAGGTAGGAGCCA	59.4	128	PB383
			TCCAGAAAACAGACCCAAGG	60.1		
M13MJ-F40	MK896876	(AG)10	GCAGCAGAACTCATCCAACA	60.0	191	PB221
			TACAGGGAATGGTTTCAGCC	60.0		
M13MJ-F41	MK896877	(AT)7	GCTCTGAGAATTGCCCATGT	60.2	194	PB251
			CACTGACGAGCAAAGCCATA	60.0		
M13MJ-F43	MK896879	(AT)8	TTGTCCTGGGGATCTTAGAAAA	59.9	186	PB681
			AATGGGCATTGTCTCCAAAA	60.3		
M13MJ-F44	MK896880	(AC)12	GGTTGGGGAGGGTGTAACTT	60.0	269	PB821
			TACTCCTTGGAGGCGTGACT	59.9		
M13MJ-F47	MK896881	(TC)10	AAATCTGTGGCCATCTCTCC	59.1	251	PB169
			GCCTTCCAGGTTGTTCAGAG	59.8		
M13MJ-F48	MK896882	(AC)9	AGAGCTGGGGAGGATGATTC	60.6	267	PB171
			ACCCTGCAGGAGTCTGAATG	60.3		
M13MJ-F49	MK896883	(TA)7	TCCCATCTAGAGAGCCATGC	60.3	160	PB1982
			ACCCAGGGTGTGTACCCATA	60.0		
M13MJ-F50	MK896884	(TG)10	GGGCTATTAATTCTGCCAGC	58.8	264	PB1983
			GTGCATGTTCACACACACATC	58.4		
M13MJ-F53	MK896885	(TG)8	ATGTCCTCATCAGGCAAACC	60.0	231	PB236
			CCTAGACTTACCTGCTTGAGCC	60.0		
M13MJ-F54	MK896886	(AT)6	GAGGGCCTTTAGGAAACTGC	60.2	197	PB2365
			GCTTCTAACAATTCCAGAGCTAAA	58.3		
M13MJ-F55	MK896887	(AT)6	GAGGGCCTTTAGGAAACTGC	60.2	198	PB2366
			GCTTCTAACAATTCCAGAGCTAAA	58.4		

Of the 30 primer pairs tested, 24 (80 %) produced a clear product following amplification of samples for *M. javanica*. Thirty-nine Malayan pangolins showed a relatively high level of genetic variability (Supplementary Table I), and all 24 loci were polymorphic (Table I), of which 10 loci contained at least 6 alleles. The number of alleles (*Na*) per locus varied from 2 to 10, the observed heterozygosity (*Ho*) in Malayan pangolin ranged from 0.167 to 0.795, the expected heterozygosity (*He*) ranged from 0.216 to 0.853, and the polymorphism information content (PIC) ranged from 0.202 to 0.853. The means of *Na*, *Ho*, *He* and PIC were 5.5, 0.558, 0.673 and 0.617, respectively. The Hardy-Weinberg test results indicated that 22 markers followed Hardy-Weinberg equilibrium. No significant pair-wise linkage disequilibrium was found between loci after Bonferroni correction ($P < 0.000181$) (Table II, Supplementary Table II).

Table II. Genetic diversity of 24 microsatellite markers in 35 *Manis javanica* individuals.

Locus	k	Na	Ho	He	PIC	NE-1P	NE-2P	NE-PP	HW
PB676	6	39	0.641	0.701	0.638	0.723	0.557	0.377	NS
PB210	6	38	0.632	0.746	0.692	0.672	0.497	0.314	NS
PB220	5	39	0.436	0.657	0.605	0.759	0.586	0.399	NS
PB235	7	39	0.385	0.635	0.584	0.772	0.601	0.411	NS
PB272	5	33	0.727	0.763	0.711	0.652	0.474	0.291	NS
PB387	5	38	0.474	0.511	0.477	0.859	0.692	0.509	NS
PB442	8	39	0.795	0.8	0.762	0.579	0.4	0.213	NS
PB464	6	37	0.568	0.796	0.754	0.598	0.418	0.235	NS
PB4641	6	37	0.459	0.749	0.704	0.658	0.477	0.285	NS
PB537	10	38	0.632	0.847	0.816	0.492	0.323	0.148	NS
PB652	6	35	0.6	0.802	0.759	0.59	0.412	0.23	NS
PB714	5	36	0.583	0.727	0.673	0.696	0.52	0.336	NS
PB383	5	36	0.722	0.737	0.678	0.691	0.518	0.339	NS
PB221	5	32	0.406	0.573	0.495	0.832	0.696	0.54	NS
PB251	4	37	0.757	0.697	0.629	0.741	0.578	0.409	NS
PB681	10	36	0.5	0.853	0.822	0.483	0.316	0.144	ND
PB821	4	32	0.5	0.708	0.637	0.735	0.572	0.405	NS
PB169	3	30	0.167	0.216	0.202	0.977	0.891	0.804	ND
PB171	2	31	0.419	0.508	0.375	0.875	0.813	0.719	NS
PB1982	4	33	0.727	0.597	0.533	0.815	0.66	0.491	NS
PB1983	3	32	0.75	0.538	0.419	0.86	0.775	0.661	NS
PB236	6	29	0.655	0.736	0.689	0.673	0.491	0.295	NS
PB2365	5	30	0.333	0.568	0.498	0.837	0.693	0.534	NS
PB2366	5	29	0.517	0.7	0.635	0.732	0.564	0.386	NS

Na the number of alleles, *Ho* observed heterozygosity, *He* expected heterozygosity, PIC polymorphic information content, ** or * Indicated significant deviation from HW after Bonferroni correction ($p<0.005$ or $p<0.01$).

Parentage analysis

The exclusion probability of individual locus ranged from 0.144 to 0.804 (Table II) when both parents information were not known (NE-PP), and its combined exclusion probability values were higher than 99% and 99.99% when 4 and 9 SSRs were used separately (Fig. 1); The exclusion probability of individual locus ranged from 0.316 to 0.891 (Table II) when one parent information was known (NE-2P), and its combined exclusion probability values were higher than 99% and 99.99% when 3 and 5 SSRs were used separately (Fig. 1); The exclusion probability of individual locus ranged from 0.483 to 0.977 (Table II) when information for both parents were known (NE-1P), and its combined exclusion probability values was higher than 99.99% when 3 SSRs were used (Fig. 1). This test confirms that the parents of "BB" (♀) were "A26" (♀) and "A25" (♂), which was consistent with the pedigree record from Qingfengyuan medicine institute. In addition, the results also supported that "C9" and "72" were parent-offspring relationships (Supplementary Table III).

Fig. 1. Combined exclusion probabilities of 24 polymorphic loci analyzed for parent known (E-1P) and one parent known (E-2P).

DISCUSSION

In this study, we firstly used 24 newly isolated SSR loci to identify the paternity of a breeding population of *M. javanica*. Although Luo *et al.* (2007) had screened 32 polymorphic microsatellite markers for *M. javanica* in 2007, the usefulness of these loci for paternity testing was not further validated due to the rarity of species and fewer samples.

The level of population genetic diversity directly reflects the individual's ability to adapt to the environment. The greater the abundance in variation of genetic diversity within a species is, the greater the adaptability of the species to environmental changes is (Michaux *et al.*, 2005). In our study, the genetic polymorphism of the artificially raised *M. javanica* population was relatively high, 20 loci of 24 SSRs

showed high polymorphism (PIC >0.5), and the average observed heterozygosity (*Ho)* and expected heterozygosity (*He*) in Malayan pangolin were 0.557 and 0.673, respectively. This finding indicated that the population of artificially raised pangolin can avoid inbreeding under correct guidance, which is conductive to the recovery of the pangolin population (Botstein *et al*., 1980).

Compared with *He* of other rare and protected animals, such as the giant panda (*He* = 0.637) (Zhang *et al*., 2003b), American alligator (*He* = 0.76) (Davis *et al*., 2001), Chinese crocodile (*He* = 0.54) (Luo, 2014), Rhesus macaques (*He* = 0.62) (Morin *et al*., 1997) and Lynx (*He* = 0.53) (Carmichael *et al*., 2000), the genetic diversity of *M. javanica* was not significantly higher than that of other species. The low genetic diversity in *M. javanica* observed in this preliminary microsatellite survey suggests an urgent need for population management and conservation of this species, since the loss of genetic diversity in endangered species is often associated with inbreeding and a reduction in reproductive fitness (Reed and Frankham, 2001).

In our study, the combined exclusion probability for NE-1P and NE-2P were higher than 99.99% when 4 SSRs were used. Moreover, when 9 SSRs were used, the values of three type parents information was higher than 99.99%. In previous reports on paternity testing, usually 6 SSRs markers may make the exclusion probability of pedigree confirmation more than 99% (Zajc, 1999). 10 and 18 polymorphic SSR loci can be used to accurately identify the relationship between individuals of Amur tiger (Zhang *et al*., 2003a) and *Acipenser sturio* (Roques et al., 2016) separately. These results indicated that the selected SSRs had a very high power and were suitable for parentage analysis. The more loci that we selected, the higher the accuracy was that could be reached in parentage analysis.

In conclusion, the parentage analysis investigated in this study, which was based on kinship testing and inferential methods, was demonstrated to be particularly hopeful regarding the development of pedigree information validation or parental assignment programs. As a genetic marker, microsatellites can objectively reflect the genetic diversity of pangolin and can be used in a paternity test. Therefore, the combination of microsatellite markers and traditional ecological research for carrying out genetic diversity analysis and the paternity test of pangolin will promote the protection, as well as rational development and utilization, of rare species resources and scientific resource management.

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Supplementary material

There is supplementary material associated with this article. Access the material online at: https://dx.doi.org/10.17582/journal.pjz/20190816080848

Statement of conflict of interest

The authors have declared no conflict of interest.

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