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PRIMER NOTE

Isolation and characterization of microsatellite markers in pangolins (Mammalia, Pholidota, *Manis* spp.)

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Abstract

Thirty-four polymorphic dinucleotide microsatellite loci were developed in the Malayan pangolin *Manis javanica*. Of the 34 markers, 32 and 18 were also amplified, respectively, in the Chinese pangolin (*Manis pentadactyla*) and the African tree pangolin (*Manis tricuspis*). Analysis of 24 Malayan, 12 Chinese and 2 African tree pangolins showed high levels of variability (heterozygosity ranging from 0.321 to 0.708). These are the first available microsatellite markers in Pholidota and will be an invaluable tool for evolutionary and conservation genetic studies in pangolins.

Keywords: *Manis*, microsatellite, pangolin, Pholidota

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Pangolins are mammals exhibiting atypical morphological characteristics, such as overlapping horny scales on the major part of the body and a buccal system adapted to myrmecophagy (e.g. presence of a long, sticky tongue and absence of teeth) that make them unique among the Old World mammalian fauna. Pangolins were once grouped taxonomically with armadillos and anteaters in the Edentata. However, molecular phylogenetic analysis among placental mammals showed that Pholidota were the sister group to Carnivora (Murphy *et al*. 2001), diverging from a common ancestor about 70 Ma and developing a highly specialized diet of ants and termites. Recently, a total of eight extant species of pangolins have been recognized (Gaubert & Antunes 2005), being distributed in Africa and Asia, with four species on each continent.

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The study of pangolins is important not only because of their divergent evolutionary characters, but also because of their perilous conservation status. All pangolin species are listed in Conservation on International Trade in Endangered Species of Wild Fauna and Flora (CITES II) as rare and protected wild animals. However, they are hunted indiscriminately throughout most of their range, primarily to consume their meat for food and their scales for medicinal purposes. The Chinese (*Manis pentadactyla*) and Malayan pangolins (*Manis javanica*) are considered the most-traded mammals in Asia, with thousands being illegally hunted and traded across international borders each year (TRAFFIC Southeast Asia 2004).

Microsatellite markers are useful for assessing population-level genetic diversity and have become an increasingly important genetic tool to assist in conservation decisions in endangered species. We report in this paper the isolation and characterization of 32 polymorphic microsatellite markers in the Malayan pangolin (*M. javanica*) and their

cross-species applicability in the Chinese pangolin (*M. pentadactyla*) and the African tree pangolin (*Manis tricuspis*). These are the first microsatellite markers reported in the Pholidota and they will be useful for phylogeographical, evolutionary and population genetic studies and for helping design and implement effective conservation management plans for these species.

Genomic DNA from *M. javanica* muscle tissue was isolated using a DNeasy Tissue Kit (QIAGEN Inc.). A microsatellite-enriched partial genomic library was produced according to a modified protocol for nonradioactive microsatellite detection based upon capture hybridization (Refseth *et al*. 1997) as previously described (Sarno *et al*. 2000). DNA was sequenced using both forward and reverse primers and BigDye Terminator kit (Applied Biosystems), and run on an ABI 3730 sequencing apparatus. Of the 288 clones sequenced, 48 provided sequences of sufficient quality for primer pairs to be designed. Primers were designed using PRIMER 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).

Thirty-two of 48 primer pairs produced amplifiable microsatellite amplicons in 24 Malayan pangolins and were subsequently tested in 12 Chinese pangolin and two African tree pangolin samples (Table 1). Polymerase chain reaction (PCR) products were amplified in a 10-μL system that contained 20 ng DNA, 2.0 mM $MgCl_2$, 0.4 U Ampli*Taq* Gold DNA polymerase (Applied Biosystems), 1× PCR buffer II containing 10 mM Tris-HCl (pH 8.3) and 50 mM KCl, 250 μM of each of the four dNTPs, 4 pmol reverse primer, 0.26 pmol M13-tailed forward primer and 4 pmol fluorenscently labelled M13 primer (5′-CACGACGTTG-TAAAACGAC-3′, see Boutin-Ganach *et al*. 2001). The reaction was performed in a PerkinElmer 9700 thermal cycler under the following conditions: denaturation for 10 min at 95 °C, a touchdown procedure of 95 °C for 15 s, 60–50 °C for 30 s, 72 °C for 45 s, with two cycles at each annealing temperature, then 30 amplification cycles of 95 °C for 15 s, 50 °C for 30 s, and 72 °C for 45 s, followed by an extension of 30 min at 72 °C. Microsatellite allele patterns were size-fractionated on an ABI PRISM 3100 and scored using GENESCAN 2.1 and GENOTYPER 2.5.

Population genetic parameters were estimated with ARLEQUIN 3.01 (Excoffier *et al*. 2005). The Malayan pangolin showed a relatively high level of genetic variability, with all the 32 loci being polymorphic, from which 19 loci contained at least 10 alleles (the number of alleles per locus varied from two to 21) (Table 1). Observed heterozygosity in Malayan pangolin ranged from 0.250 to 1.000 across loci (mean of 0.708), and the expected heterozygosity varied from 0.312 to 0.942 (mean of 0.805) (Table 1). Of the 32 loci, 27 (84%) and 18 (56%) were amplified successfully in the Chinese pangolin and African tree pangolin, respectively, most likely reflecting the relative divergence time among the three species.

All loci were individually tested for deviations from Hardy–Weinberg equilibrium and linkage equilibrium with conservative Bonferroni correction in species with a sufficient sample size. We found significant heterozygote deficiency ($P < 0.001$, Fisher's exact test) for six loci (MJA06, MJA12, MJA20, MJA21 and MJA34) in *M. javanica*, which may suggest population substructure and/or the existence of null alleles. Linkage associations in *M. javanica* were detected by pairwise comparisons of loci ($P < 0.00009$, Fisher's exact test) for the following pairs/groups: (MJA19, MJA24) (MJA09, MJA22) (MJA02, MJA18) (MJA03, MJA20, MJA21, MJA34) (MJA01, MJA04, MJA14, MJA15, MJA25)

Table 1 Characterization of dinucleotide-repeat microsatellite markers isolated from the Malayan pangolin *Manis javanica* (MJA) and their cross-species amplification in the Chinese pangolin *Manis pentadactyla* (MPE) and African tree pangolin *Manis tricuspis* (MTI)

Locus (accession no.)	Repeat motif	Primer sequence (5′–3′)	No. of alleles			Size range (bp)			H_O			H_E		
			MJA	MPE	MTI	MJA	MPE	MTI	MJA	MPE	MTI	MJA	MPE	MTI
MJA01 (DQ886446)	$(GT)_{22}$	F: CAGAAGATGGCCTAGGTGGA R: CTTGGGGCAGAGCTATCTGA	13	4	0	187–219	195–201	0	1	0.778	—	0.895	0.624	—
MJA02 (DQ886447)	$(GT)_{11}$	F: GAGGGTACATCCCACAAAGG R: GGGTACTTCCGAAGGAAATG	7	4	2	223–237	229–237	229–235	0.739	0.600	1	0.780	0.700	0.500
MJA03 (DQ886448)	$(CA)_{40}$	F: TAGGTGGCAGACGATTTGCT R: CTGAGTGAGGCTGGCTTTCT	21	1	3	175–237	189	185–199	0.565	0	0.500	0.942	0	0.625
MJA05 (DQ886450)	$(GT)_{12}$	F: GTGGAAGGCAGGAAAAACAA R: CCCTTTGGGAAGAGTGTGAA	13	2	3	261–299	259–263	277–293	0.750	0	0.500	0.846	0.153	0.625
MJA06 (DQ886451)	$(CA)_{26}$	F: CTGGCAGATTCCATCTTGCT R: GGATGATGAAATACGGCTGAA	19	4	3	220–288	190–230	230–238	0.667	0.500	1	0.895	0.462	0.625
MJA07 (DQ886452)	$(GT)_{21}$	F: CAGCCCAGGTAACAGACTGG R: TTCCATCTGGGTGTCCTACAG	11	3	3	238–270	198–228	192–206	0.792	0.0833	0.500	0.862	0.226	0.625
MJA08 (DQ886453)	$(GT)_{11}$	F: CACCCACATTATTGCAAACG R: AAAGATATTGCCACCCACTTG	3	2	2	178–184	156–172	172–178	0.375	0.0833	0.500	0.312	0.219	0.375

Table 1 *Continued.*

Locus (accession no.)	Repeat motif	Primer sequence (5′–3′)	No. of alleles			Size range (bp)			H_O			H_E		
			MJA	MPE	MTI	MJA	MPE	MTI	MJA	MPE	MTI	MJA	MPE	MTI
MJA09 (DQ886454)	$(GT)_{21}$	F: TCTGCATAAGGTTGAAGAGCAA R: GACAAGGCAGTGTTGCTGAA	8	6	4	200–216	194–208	184–204	0.667	0.417	1	0.773	0.667	0.750
MJA10 (DQ886455)	$(CA)_{18}$	F: CTAGGGTTGGGTCCTTCCTC R: CTCAGGTGCTTTGGACTTAGG	13	7	2	211–245	233–263	205–207	0.792	0.333	0	0.828	0.427	0.500
MJA11 (DQ886456)	$(CA)_{29}$	F: CTCACCGTGACAGCAGAGAC R: GCTTATCCTGGTTTCAATCATTC	14	5	0	188–224	176–208	0	0.909	0.250	—	0.897	0.420	—
MJA12 (DQ886457)	$(CA)_{20}$	F: GGAGTGCTGAACTTGGGTGT R: TGGAGGGAAGTCTACCCAAA	5	1	2	178–186	166	180–184	0.250	0	0.500	0.631	0	0.375
MJA13 (DQ886458)	$(GT)_{16}$	F: CTGGGGATGCCCTAATTTCT R: CACAGCACAGTTGGGATTGT	10	4	3	204–226	216–222	214–264	0.652	0.700	0.500	0.701	0.715	0.625
MJA14 (DQ886459)	$(CA)_{20}$	F: CTTGGGGCAGAGCTATCTGA R: CAGAAGATGGCCTAGGTGGA	13	4	1	184–216	192–200	196	1	0.333	0	0.895	0.615	0
MJA15 (DQ886460)	$(GT)_{18}$	F: TTTCGAAGATGGCCTAGGTG R: TCTGACCCCTGTTCTCCACT	13	2	0	173–205	185–187	0	1	0.250	—	0.895	0.219	—
MJA16 (DQ886461)	$(CA)_{17}$	F: TTCCCCATCTTCTCCTTCCT R: TGAATGTTGTAAAGAGGTAAAAACCA	10	4	2	170–208	164–204	174–178	0.750	0.167	0.500	0.833	0.514	0.375
MJA17 (DQ886462)	$(GT)_{22}$	F: AAAAAGGAGGGAGCCTTCTG R: AGCCGCTGCTTTATCACACT	12	5	3	173–211	161–199	191–199	0.750	0.222	0.500	0.891	0.642	0.625
MJA18 (DQ886463)	$(CA)_{11}$	F: GATCCTCGAAACCAAGCAG R: AGGCTCTAGGCTTCGTCCTT	5	7	2	183–201	163–191	189–191	0.375	0.583	0	0.532	0.580	0.500
MJA19 (DQ886464)	$(CA)_{13}$~ $(CA)_{19}$	F: CCAAGAGCTGGAGAGTGCAT R: TCAGTTGTATTTCCACGTTCTGA	16	1	1	230–280	222	236	0.875	0	0	0.893	0	0
MJA20 (DQ886465)	$(CA)_{11}$	F: GGTTAGTGAGCCACCCTGAA R: ATCAGCGCCCCTAATACTTG	5	0	0	261–271	0	0	0.263	—	—	0.601	—	—
MJA21 (DQ886466)	$(CA)_{16}$	F: GAACCTGGGTTGGGGTAACT R: GCAGGGTTTCTCAACTTTGG	13	10	4	218–254	212–256	220–236	0.286	0.833	1	0.883	0.879	0.750
MJA22 (DQ886467)	$(CA)_{15}$	F: GGATGTGGGTATCCTTGTGG R: CCTCTCAGTGGGTGGGAGTA	13	5	4	196–226	182–198	150–176	0.870	0.400	1	0.925	0.735	0.750
MJA23 (DQ886468)	$(CA)_{30}$	F: CCACCTCACTCACACCACTG R: TGAATGTCGGGGAGAGAAAC	19	1	0	173–217	155	0	0.917	0	—	0.936	0	—
MJA24 (DQ886469)	$(GT)_{11}$	F: GAGGGGTCAGAAGTGTCCAA R: GCAGCCTGCTTCCATTTTAT	7	4	0	199–229	185–205	0	0.708	0.100	—	0.822	0.415	—
MJA26 (DQ886471)	$(GT)_{15}$	F: TGTGAAAGCAGAATGCAAAAG R: ACTTGCCTGAAGTGGACACC	9	7	0	235–251	233–253	0	0.917	0.833	—	0.882	0.813	—
MJA27 (DQ886472)	$(GT)_{16}$	F: GGTGACTTTGGGCAATTCAT R: CCCTCTTTGGAGGCATCATA	8	6	0	249–269	239–251	0	0.696	0.600	—	0.796	0.705	—
MJA28 (DQ886473)	$(GT)_{24}$	F: GCCTTCAAGTGTGCCTGTCT R: CAGGCAAAATTTGGGCTAGA	9	5	3	241–265	237–253	207–217	0.875	0.429	0.500	0.885	0.725	0.625
MJA29 (DQ886474)	$(GT)_{22}$	F: GGAGGCAAGGATGATTCTGA R: TCACACATAAGCAGAGCTCCA	10	1	0	227–251	223	0	0.833	0	—	0.842	0	—
MJA30 (DQ886475)	$(GT)_{20}$	F: GAAGCCCTAACCCCAGTCTC R: CCTGGAGCAGTAAGGGAACA	6	5	0	242–256	236–254	0	0.625	0.167	—	0.780	0.361	—
MJA31 (DQ886476)	$(GT)_{16}$	F: CCATGTGGGCCTGTATTAGG R: TCATGTGAGCCAGCACCTTA	11	0	0	192–216	0	0	0.875	—	—	0.884	—	—
MJA32 (DQ886477)	$(GT)_{8}$	F: CCCGGTCAGCTTCTTTCAAT R: ACAGGAGATGGAGTGCAGGT	2	0	0	176–178	0	0	0.250	—	—	0.330	—	—
MJA33 (DQ886478)	$(GT)_{23}$	F: GGAAGTGAGCAGCAAACACA R: CTCTCAGTCCCTTTCCGTAGA	10	0	0	192–224	0	0	0.6667	—	—	0.872	—	—
MJA34 (DQ886479)	$(GT)_{24}$	F: AGCTGCACATTCTCAGCAAA R: CCCATGTGTCCTCATTCTCA	6	0	0	217–231	0	0	0.385	—	—	0.864	—	—
Average									0.708	0.32	0.553	0.805	0.437	0.513

H_O, observed heterozygosity; H_E, expected heterozygosity.

and (MJA27, MJA31). However, further linkage mapping or additional testing in more individuals will be necessary to confirm the linkage among these loci.

These polymorphic microsatellites represent the first set of intraspecies genetic markers available for the Pholidota. Their high variability across species endemic to different continents confirm the value of these markers for evolutionary and conservation genetic studies in pangolins.

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Erratum

The authors (Luo S-J et al.) of the paper 'Isolation and Characterization of Microsatellite Markers in Pangolins (Mammalia, Pholidota, *Manis* spp.)' have brought to our attention that a requested correction was not made in the abstract of this article. This paper appeared in *Molecular Ecology Notes*, Volume 7, Issue 2, pp269-272.

The revised abstract follows:

Thirty-two polymorphic dinucleotide microsatellite loci were developed in the Malayan pangolin *Manis javanica*. Of the 32 markers, 27 and 18 were also amplified respectively in the Chinese pangolin (*M. pentadactyla*) and the African tree pangolin (*M. tricuspis*). Analysis of 24 Malayan, 12 Chinese and 2 African tree pangolins showed high levels of variability (heterozygosity ranging from 0.321 to 0.708). These are the first available microsatellite markers in Pholidota and will be an invaluable tool for evolutionary and conservation genetic studies in pangolins.