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RESEARCH NOTE

Development of Microsatellites for the Philippine Eagle, *Pithecophaga jefferyi*, Using Next-generation Sequencing

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The Philippine Eagle, *Pithecophaga jefferyi*, is a critically endangered bird of prey that is endemic to the dipterocarp forests of the Philippines. In this study, data from next-generation sequencing (NGS) were used for the development of microsatellites for the Philippine Eagle. A total of 49,965 microsatellite loci were predicted and 20,960 primer pairs were designed. Forty (40) of these pairs were synthesized and were tested on 20 captive individuals. Twenty (20) of these markers showed high amplification success rates and exhibited polymorphism. A maximum of 24 alleles was detected across the 20 markers with an average of 13.9 alleles, which is remarkably higher than other microsatellite studies in eagles. The resulting values for observed heterozygosity (H_0) ranged from 0 to 1.00 with an average of 0.290, while expected heterozygosities ranged from 0.791 to 0.968 with an average of 0.910.

Keywords: heterozygosity, microsatellites, next-generation sequencing, Philippine Eagle, *Pithecophaga jefferyi*

The Philippine Eagle, *Pithecophaga jefferyi* Ogilvie-Grant 1896*,* occurs primarily in the steep terrains and heavy forests of the four islands in the Philippines: Luzon, Samar, Leyte, and Mindanao (Bueser *et al.* 2003). The Philippine Eagle has a small and rapidly decreasing population due to extensive deforestation and is classified as a critically endangered species. Owing to the increasing concern over the endangered status of the Philippine Eagle, studies have been initiated across the country to investigate the bird's genetic background. DNA barcodes from 43 individuals of six species of captive Accipitrids, including 31 individuals of Philippine Eagle found in the Philippine Eagle Center (PEC), were reported by Ong *et al.* (2011). Luczon *et al.* (2014) determined the genetic diversity of 22 individuals of Philippine Eagle (19 of which came from the wild and 3 were captive-bred) using mitochondrial control region. However, a more thorough knowledge of its genetic diversity is needed to provide more insights into the management and conservation of the species.

Microsatellites, variable DNA sequences comprising tandem repeats of 1–6 nucleotides, have become one of the most widely used molecular tools for conservation and population genetic studies because of their codominant mode of inheritance, hypervariability, abundance, and tolerance to variation in DNA quality and quantity (Galbusera 2000). Unlike the mitochondrial DNA control region, microsatellites are present in both coding and non-coding regions and possess a mutation rate high enough to produce and maintain extensive length polymorphism

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(Galbusera *et al.* 2000). Recently developed NGS platforms provide an innovative approach for the development of microsatellites and represent a powerful tool for population genetic studies in non-model species (Abdelkrim *et al.* 2009). In this study, NGS technology was applied to develop microsatellites for the Philippine Eagle.

Philippine Eagle blood samples were provided by the PEC – a captive breeding facility in Davao City, Mindanao Island, Philippines. Blood extraction was done via venipuncture by a veterinarian during the annual medical check-up of the raptors. Genomic DNA was extracted from blood samples with the DNeasy Blood and Tissue Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's protocol. DNA samples from four individuals of Philippine Eagle were then subjected to shotgun pyrosequencing using the Roche 454 Genome Sequencer FLX with titanium chemistry at Macrogen, Inc. (Seoul, South Korea). Two of these individuals are captive bred siblings and the other two were obtained from the wild: one from South Cotabato and the other from Zamboanga del Sur, both provinces located in Mindanao Island. Results were used to predict simple sequence repeat (SSR) loci in the Philippine Eagle genome.

Partial assembly of the Philippine Eagle genome, SSR marker search and primer design were carried out at the Philippine Genome Center (Metro Manila, Philippines). The partial assembly was completed using the Newbler software, taking as inputs the four sequence files (SFF format) from the data from Macrogen, Inc. From the partial genome assembly, SSR loci were predicted using the following search tools: MIcroSAtellite Identification Tool or MISA (Thiel *et al.* 2003), mreps (Kolpakov *et al.* 2003), and SciRoKo (Kofler *et al.* 2007). Primers were then designed for all the SSR loci using the tool Primer3 (version 2.3.6) (Untergasser *et al.* 2012). Forty (40) primers were synthesized for further testing. Twenty of these primers target microsatellite loci containing dinucleotide repeats, while the other 20 primers target microsatellite loci containing trinucleotide repeats.

Preliminary screening of the 40 microsatellite primers was conducted using DNA extracts from 20 Philippine Eagle specimens (13 were captive bred and seven were from the wild, all of which have their origin in Mindanao). PCR amplifications were performed using the Taq DNA Polymerase dNTPack (Roche). A final volume of 20 µl of PCR mix was used which consisted of 1 µl DNA template (approximately 20 ng), 2.0 µl 10X PCR Buffer, 0.6 µl (25 mM) $MgCl_2$, 0.2 µl (10mM) dNTPs, 0.8 µl (10mM) of each primer, 14.475 µl UltraPure™ Dnase/Rnase-Free distilled water, and 0.125 µl Taq DNA Polymerase. The PCR conditions optimized for the dinucleotide primer PJ20 were as follows: 95 °C for 15 min; 35 cycles at 95 °C for 30 s, annealing temperature (T_a) for 30 s, 72 C for 30 s, and T_a for 1 min; and a final extension at 72 °C for 8 min. The PCR condition optimized for the dinucleotide primers (PJ02, PJ03, PJ04, PJ06, PJ08, PJ09, PJ14, PJ18, and PJ19) was as follows: 94 °C for 4 min; 30 cycles at 94 °C for 40 s, T_a for 40 s, and 72 °C for 40 s; and a final extension at 72 °C for 5 min. Likewise, the PCR condition optimized for the trinucleotide primers was as follows: 94 °C for 5 min; 35 cycles at 94 °C for 30 s, T_a for 30 s, and 72 °C for 30 s; and a final extension at 72 °C for 5 min.

The 40 primer pairs were screened for amplification and polymorphism among the different loci on 10% polyacrylamide agarose gels. Analysis of gel images and scoring of individual bands were accomplished using the GelAnalyzer 2010a. For all polymorphic loci providing clear electropherograms, genetic variability parameters such as the number of alleles, expected heterozygosity (H_e), and H_o were calculated using Popgene version 1.32 (Yeh *et al.* 1999). DNA sequences that showed polymorphism were submitted to GenBank and were assigned accession numbers MN427893 to MN427912.

Results from the 454 Pyrosequencing of the four specimens generated an average of 193,512 sequence reads and identified 108,171,240 bases with an average read length of 559 bases. Assembly using the reads from the four Philippine Eagle genome sequences yielded 675,348 singletons and 4,091 contigs (N50 = 1077), and the largest contig was 20,955 bases long. These contigs – including the 675,348 singletons – were used to predict SSR loci in the Philippine Eagle genome. The SSR marker search strategy using the three search tools resulted in the prediction of 49,965 microsatellite repeat loci. Among these, 32,512 (65%) were perfect repeats while 17,453 (35%) were ambiguous. SSR loci that passed the filter criteria were then used as templates for primer design and a total of 20,960 primer pairs were designed. Further classification showed that 2,755 of the loci were dinucleotide repeats, 2,810 were trinucleotides, 6,573 were tetranucleotides, 6,581 were pentanucleotides, and 1,214 were hexanucleotides.

Twenty (20) out of the 40 primer pairs showed high amplification rates and exhibited polymorphism among the different loci on 10% polyacrylamide agarose gels. These 20 primer pairs showed distinct and readable bands that are useful for population genetic analyses. Genetic variability parameters such as the number of alleles, H_e, and H_o are shown in Table 1. The allele numbers for the 20 loci ranged from six to 24. The values for observed heterozygosities ranged from 0.0000 to 1.0000 while the values for expected heterozygosities ranged from 0.7910 to 0.9679. The H_o was lower than the H_e for almost all loci with an average of 0.2900 (standard deviation, SD = 0.3824) and 0.9098 (SD = 0.0517), respectively.

Table 1. Description of 20 microsatellite loci developed for *Pithecophaga jefferyi*.

Primer set	Repeat motif	Locus	Primer sequence (5'–3')	Melting temperature (°C)	Size range (bp)	Allele number	Observed heterozygosity (H_o)	Expected heterozygosity (H_e)
1	$(AT)_8$	PJ02	F: cctgctctcccttaacaccc R: tcaagtcaaagcctcccgtc	56	422–468	24	1.00	0.9679
2	$(CG)_8$	PJ03	F: gtcagggtcggtggagc R: tgatctgaggtcgcaaaccc	50	483–529	13	0.00	0.9282
3	$(AC)_8$	PJ04	F: ttgccaaaggctgtgcattc R: cccacacttggatggacctc	55	402–460	18	0.20	0.9551
4	$(TC)_9$	PJ06	F: cccttgtctaacgtgcctct R: gatggctctcctttgtgcct	56	354–428	14	0.00	0.9385
5	$(AT)_8$	PJ08	F: ctcctgcccagaaagactgg R: ctgtgccaacttcttgtgcc	55	498–582	21	0.50	0.9526
6	$(AT)_8$	PJ09	F: gacgtcatgctcagttccca R: ctcctgcccagaaagactgg	53	237–291	17	0.95	0.9551
7	$(TG)_9$	PJ14	F: ttggacctgtgtggtttggg R: agcccaacagaaaccaggag	57	147–161	8	0.05	0.8577
8	$(TG)_9$	PJ18	F: tgatggtacttggtggtggc R: aggtacaggctttctttggact	55.	356–480	16	0.00	0.9385
9	$(GT)_{14}$	PJ19	F: gcggctcctagacttagctg R: atgtcatcgcagaggcacaa	59	323–407	22	1.00	0.9628
10	$(TA)_9$	PJ20	F: ttgccaaggtgatattatgtgt R: gttccctccttccagtgctc	49	158–170	8	0.05	0.8705
11	$(CCG)_7$	PJ21	F: gagaggagagggtcctccg R: gggtgagggaaacgacctg	57	516–570	15	0.00	0.9487
12	$(AGG)_6$	PJ23	F: aggctcagggaaatgcagac R: tcctaggagtccctggcatc	57	232–256	8	0.00	0.8615
13	$(CCT)_8$	PJ24	F: catcgttggccaagaagcac R: atgaggatcaggaccgacca	57	166–187	11	0.15	0.8872
14	$(AAC)_6$	PJ27	F: actcctgctcctcttcctgt R: ggaagagctggagggaaatga	57	411–519	8	0.15	0.8628
15	$(CCT)_7$	PJ29	F: cttcagggacaacctgctcc R: ttcagaaagggccaaaggca	57	274–283	14	0.00	0.9333
16	$(GCC)_6$	PJ30	F: ctcgcagggtcttactgcaa R: ggctcggaatgttggtcagt	57	441–489	10	0.50	0.8436
17	$(GCA)_9$	PJ31	F: agatgttgagagcagtgggc R: tgcattgctgcatcatcacg	57	356–404	18	0.25	0.9462
18	$(CCT)_6$	PJ32	F: tcctcttcctcctctccagc R: gagagagcgaagcagatccc	57	163–178	9	0.00	0.8462
19	$(AAT)_9$	PJ38	F: agccagcaactaagcaccat R: ggttggcgagtagggaacaa	57	255–282	6	0.05	0.7910
20	$(AGG)_{11}$	PJ40	F: gggatcgggatgtgagagga R: cccttcaccaacttccctgg	59	253–277	17	0.95	0.9487

Owing to their polymorphic quality, a large number of alleles, and high values of H_e, these microsatellite loci are useful for population genetic studies of the Philippine Eagle – studies that are important for the conservation and management of the species. Moreover, the microsatellites developed in this study for the Philippine Eagle can be tested for cross-species amplification in other species of birds belonging to family Accipitridae.

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