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PRIMER NOTE

Development and multiplex PCR amplification of novel microsatellite markers in the White-tailed Sea Eagle, *Haliaeetus albicilla* (Aves: Falconiformes, Accipitridae)

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Abstract

We report the development of 14 novel polymorphic microsatellite markers cloned from the White-tailed Sea Eagle, *Haliaeetus albicilla*, a formerly threatened raptor that has received much conservation attention throughout Eurasia. We also present a protocol for multiplex polymerase chain reaction (PCR) amplification of the loci. Among 40 unrelated *H. albicilla* individuals from southern Sweden, the markers produced two to eight alleles per locus, and average observed and expected heterozygosities were 0.463 and 0.468, respectively. We further present five microsatellite markers that appeared monomorphic in *H. albicilla*, but which may be of interest for use in other raptor species.

Keywords: Accipitridae, conservation genetics, *Haliaeetus albicilla*, microsatellites, multiplex PCR

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The White-tailed Sea Eagle (*Haliaeetus albicilla*) is a large formerly threatened raptor that breeds at coastal and freshwater areas throughout most of Eurasia. Most populations of *H. albicilla* in Europe have experienced two periods of sharp decline in numbers during the last century (Helander *et al*. 2003). The main cause of the first decline was persecution by humans, while the second decline was caused by environmental pollutants such as DDT and PCB accumulating in the food chain (Helander *et al*. 2002). Since the 1980s, many populations of *H. albicilla* have recovered markedly, covered up by close monitoring activity by conservation biologists. Microsatellite markers will be useful to clarify population structure and amount of genetic differentiation between different populations of *H. albicilla*.

Blood samples were collected from *H. albicilla* nestlings belonging to the population on the Swedish Baltic coast and at inland freshwater lakes. Samples were buffered with EDTA ISSC and stored at –70 °C. Total genomic DNA was isolated using a standard phenol–chloroform protocol following treatment with proteinase K (Sambrook *et al*. 1989). An enriched library was made by Ecogenics GmbH (Zürich, Switzerland) from size-selected genomic DNA ligated into TSPAD-linker (Tenzer *et al*. 1999) and enriched by magnetic bead selection with biotin-labelled $(CA)_{13}$, $(CA)_{20}$ and $(CAAA)_9$ and $(AGG)_{10}$ oligonucleotide repeats (Gautschi *et al*. 2000). Of 960 recombinant colonies screened, 142 gave a positive signal after hybridization. Plasmids from 109 positive clones were sequenced, and primers for 21 microsatellite inserts were designed using PRIMER3 (Rozen & Skaletsky 2000). Of these markers, two were impossible to score unambiguously (Hal 11 and Hal 21; clone and primer sequences were deposited in GenBank with Accession numbers AY817050 and AY822031, respectively), and another five (Hal 16 through Hal 20; clone and primer sequences deposited in GenBank with Accession numbers AY817055, AY817056 and AY822028 to AY822030, respectively) appeared monomorphic on a panel of four to 15 unrelated *H. albicilla* individuals. The remaining 14 polymorphic primer pairs yielded interpretable and reproducible PCR (polymerase chain reaction) products, and were subsequently typed on 40 unrelated *H. albicilla* individuals.

PCR amplifications were performed using reaction volumes of 10 μL containing 10 ng of genomic DNA, 0.2 mM of each dNTP, 0.5 μM of each forward and reverse primer (one of them marked with a fluorescent dye, see Table 2),

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Table 1 Characterization of 19 *Haliaeetus albicilla* microsatellite loci: optimal annealing temperature (T_a), number of alleles per locus (n_A), observed (H_O) and Nei's (1978) unbiased expected heterozygosity (H_E)

Locus	Clone ID	Primer sequences (5′–3′)	Repeat motif (based on sequenced clone)	T_a (°C)	n_A	Size range (bp)†	H_O	H_E	GenBank Accession number
Hal 01	CA1F8	F: GAATACACCCAGAACAGCAACC R: CCCAGCTGTGCTCATAACATAC	$(GT)_{17}$	60	7	128–140	0.825	0.755	AY817040
Hal 02	CA1C10	F: TGGACCACAAAGTGTAAACTTCTAA R: TGAATCTGCATGGTAAGCTCAG	$(TG)_{10}$	60	2	178–182	0.100	0.096	AY817041
Hal 03	Hali 8	F: GGGCATCCCTTCAATCTGTTAC R: ATGTTCCCAGCTAGCCCTTTC	$(CAAA)_6$	57	3	135–143	0.575	0.507	AY817042
Hal 04	CAB 1	F: TAAGGCTTTTCTTCGCGTGT R: TCAACAACCCCTCCGTAGAC	$(CA)_2AA(CA)_{12}CG(CA)_4$	57	5	155–163	0.300	0.274	AY817043
Hal 05	Hali 53	F: GCCAAAACCCTGTGAGTACC R: GTGGTCCTGTGGGACACG	$(AGG)_{10}$	59	2	109–112	0.400	0.444	AY817044
Hal 06	CAAA56.2	F: CATCCAAACTCATTCAAGCCTA R: AGAGCAGGTGTCTTTTCAGAGC	$(CAAA)_x$‡	62	3	183–191	0.475	0.456	AY817045
Hal 07	CA1F1	F: TTCAGAAGGTGCATGCAGTAG R: GGGATGTGCAAAGAAATCTACC	$(GT)_{13}$	60	2	157–161	0.550	0.425	AY817046
Hal 08	Hali 10	F: GCCGTCGGGTAAAGAGGAG R: TCTTCCTCCTCTGCTGTTGC	$(AGG)_x$‡	64	2	113–116	0.025	0.025	AY817047
Hal 09	20CA14	F: TGAGCTTTGTAGTAGCAGTGGTG R: TGCAAAAATAGAGCCAATACCC	$(AC)_{17}$	64	7	133–151	0.750	0.78	AY817048
Hal 10	CA1C9	F: CATGCACGCTGTGAATCAG R: ACCCACCAACGTTACCAGTG	$(CA)_{12}$	64	5	232–240	0.450*	0.698	AY817049
Hal 12	CA1A10	F: CACATGTTTGTGTGCACGTC R: GTGCTGCCTCTCACTGTCG	$(GT)_{10}$	64	2	236–238	0.375	0.392	AY817051
Hal 13	CAA3	F: CCACTCAGTAAGGAGCTTTGC R: CCTTGTGTTTGCTGCAGATG	$(CA)_{17}$	64	6	154–168	0.775	0.765	AY817052
Hal 14	Hali 56	F: GCTGCAGCTCTCTTGGACAC R: CAACACTTTCAGCGATGCTC	$(AGG)_x$‡	60	8	166–251	0.750	0.811	AY817053
Hal 15	Hali 54	F: CCAGTTTTATATTAAAGCTTTGGAACC R: GCAAAGAACAAAACTCCTAATAATACC	$(AGG)_x$‡	63	2	404–407	0.125	0.119	AY817054
Hal 16	Hali 45	F: TTCCCAAGAACGCAGTACATC R: TTCATACGCAACTTGATGGTTC	$(CAAA)_7(AAAACC)_4$	64	1	194	—	—	AY817055
Hal 17	Hali 41	F: AAGAATAACACCCCACACACAC R: CGCCCAGGTGAATAGGTAAG	$(CAAA)_x$‡	64	1	190	—	—	AY817056
Hal 18	Hali 2	F: GACAGGGAGCGAGTTAGTGG R: CCAGCCACAAAGGTACTAAGG	$T_9(GTTT)_x$‡	60	1	140	—	—	AY822028
Hal 19	Hali 4	F: TGTAGGCAGGTAAGGCAAAG R: TGCAGAGATTTTGCATCTGG	$(CAAA)_x$‡	60	1	212	—	—	AY822029
Hal 20	Hali 42	F: TGCCAACATAAGTCAAGTCACAG R: CCTCCCCCAAAATCCTAATG	$(CAAA)_x$‡	60	1	198	—	—	AY822030

†Allele size as estimated in GENETIC PROFILER.
*Significant heterozygote deficit.
‡Complex repeat structure.

0.4 U of HotStarTaq DNA polymerase (QIAGEN) and 1 μL of 10× HotStarTaq (QIAGEN) reaction buffer containing Tris-HCl, KCl, $(NH_4)_2SO_4$ at a final concentration of 1.5 mM $MgCl_2$. We used the following PCR programme on a PTC-225 machine (MJ Research): 35 cycles at 95 °C for 30 s, a locus-specific annealing temperature (Table 1) for 30 s, and 72 °C for 30 s. Before the first cycle, a prolonged denaturation step (95 °C for 15 min) was included and the last cycle was followed by an additional annealing step at the corresponding annealing temperature for one minute and a final extension step for 8 min at 72 °C. The amplified products were diluted with water, mixed with internal size standard ET-ROX 400 (Amersham Biosciences), run on a MegaBACE 1000 instrument (Amersham Biosciences) and analysed using GENETIC PROFILER 2.0 (Amersham Biosciences). Furthermore, we developed a multiplexing protocol (Table 2) for all polymorphic markers, with special care taken to compare results from single marker amplification versus

Table 2 Multiplexing protocol for *Haliaeetus albicilla* microsatellite markers

Multiplex	Marker	Fluorescent dye	Amount of each primer (F&R) (μM)*	T_a (°C)*
1	Hal 01	HEX	0.35	59
	Hal 02	FAM	0.30	
	Hal 05	TET	0.12	
	Hal 07	TET	0.10	
2	Hal 03	FAM	0.35	57
	Hal 04	HEX	0.65	
	Hal 06	TET	0.20	
	Hal 14	HEX	0.75	
3	Hal 08	FAM	0.10	63
	Hal 13	HEX	0.45	
	Hal 12	FAM	0.20	
	Hal 15	FAM	0.60	
4	Hal 09	FAM	0.16	64
	Hal 10	TET	0.15	

*Other PCR conditions (reagent mix and temperature profile) are identical to the conditions described for single marker runs.

multiplexing runs. Using this multiplexing protocol, 14 polymorphic microsatellite loci could be amplified in four PCRs, significantly reducing laboratory costs and time.

Using GENEPOP, web version 3.4 (Raymond & Rousset 1995), expected and observed heterozygosities were determined and exact tests for departure from Hardy–Weinberg equilibrium were performed. Only one locus showed significant heterozygote deficit (Hal 10, $p < 0.0001$), possibly due to the presence of one or more null alleles. Using Fisher's exact test for linkage disequilibrium (LD) implemented in the same software, LD significant at $0.01 < p < 0.05$ was found for three pairs of loci (Hal 01 & Hal 04, Hal 06 & Hal 08, Hal 10 & Hal 12). However, none of these values remained significant after sequential Bonferroni correction (Rice 1989), which we employed to account for multiple testing. Except physical linkage, demographic factors such as recent population bottlenecks or admixture can lead to LD between loci (Frankham *et al*. 2002). Based on the data presented here, we cannot rule out either hypothesis.

The results (Table 1) show that these loci are polymorphic and thus will be a valuable resource to study genetic variability and gene flow among different *H. albicilla* populations. Since the White-tailed Sea Eagle is listed under the CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora) and is legally protected in almost its entire distribution range, the microsatellite markers presented here can also be useful to determine the origin of feathers and skins from confiscated material. Finally, we believe that our markers also will prove applicable in other species of the Accipitridae family, which contains many species of high conservation interest.

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