Conservation Genetics Resources 5(3) 2013 DOI:10.1007/s12686-013-9891-y

TECHNICAL NOTE

Isolation, characterization and predicted genome locations of Eurasian eagle-owl (*Bubo bubo*) microsatellite loci

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Running title: Eagle-owl microsatellites
Word count: 1081

Abstract We isolated 134 eagle-owl (*Bubo bubo*) microsatellite sequences. Eight of these newly isolated loci were characterized in 38 Eurasian eagle-owls in a northern European population. Sequence homology was used to assign a predicted chromosome location for the eight loci. We also redesigned primers for four previously isolated eagle-owl sequences and cross-amplified two published primer sets previously characterized in other owl species. These 14 loci were amplified in three multiplex PCR sets and displayed 2 to 9 alleles with expected and observed heterozygosities ranging from 0.33 to 0.85 and from 0.42 to 0.97, respectively. Estimated frequencies of null-alleles were low and only one locus deviated from Hardy-Weinberg equilibrium. After correcting for multiple tests, linkage disequilibrium was found for a single pair of loci. The combined probability of identity for the 14 loci was 3.5×10^{-12}. These microsatellite loci are expected to be useful for genetic monitoring, parentage analysis and population genetic studies.

Keywords *Bubo bubo* - Microsatellite loci - Multiplex PCR - Non-invasive samples - Predicted genome locations

The Eurasian eagle-owl (*Bubo bubo*) is a large owl occurring in a wide range of habitats in Asia and Europe (Cramp 1985). The global population size appears to be decreasing and the eagle-owl is listed as endangered on the Norwegian Red List (Kålås et al. 2010). To facilitate genetic monitoring of eagle-owls based on non-invasive sampling of shed feathers (e.g. Rudnick et al. 2005) we isolated new microsatellite loci, redesigned primer sets using previously isolated eagle-owl sequences and cross-amplified loci from other owl species using published primer sets.

A microsatellite-enriched genomic library was constructed. We used a confiscated captive adult female eagle-owl (CF68) assumed to be a European eagle-owl (*B. b. bubo*). The sex of the bird was confirmed using the markers M5 (Bantock et al. 2008), MP and NP (Ito et al. 2003). Genomic DNA was extracted from blood using an ammonium acetate precipitation method (Nicholls et al. 2000; Richardson et al. 2001) and the library was made using the enrichment approach of Armour et al. (1994). The library was enriched for the following di- and tetranucleotide microsatellite motifs separately: $(CA)_n$, $(GA)_n$ and, $(GATA)_n$, $(TTTC)_n$, $(GTAA)_n$ and $(CTAA)_n$, which had been denatured and bound to magnetic beads following Glenn and Schable (2005). Transformant colonies were not screened for the presence of a repeat but directly sequenced. Sanger sequencing was conducted bidirectionally using Big Dye Terminators ver. 3.1 (Applied Biosystems) and in most cases a consensus sequence created. A total of 134 unique sequences were obtained (EMBL accession numbers HF564899- HF565032). Sixteen new primer sets were designed using Primer3 (Rozen and Skaletsky 2000) and the criteria used included a maximum 0.5°C difference between the forward and reverse primers, possession of a G/C clamp and a maximum of three consecutive mononucleotide bases.

We also redesigned primers from four previously isolated eagle-owl microsatellite sequences (Isaksson and Tegelström 2002) using Primer3 (Rozen and Skaletsky 2000) to enable amplification of shorter fragments as loci with shorter fragments seem to amplify at a higher success rate when analyzing non-invasive samples (e.g. moulted feathers) characterized by low quality DNA (Broquet et al. 2007). Furthermore, we cross-amplified two microsatellite loci (*So15A6* and *Oe053*) from two other owl species (*Strix occidentalis* and *Otus elegans*) (Thode et al. 2002; Hsu et al. 2003).

For genotyping, genomic DNA was extracted from the feather calamus using an automated system (Maxwell®16 Research System, Promega) and the Maxwell 16 tissue DNA purification kit following the manufacturer's protocol. Loci were PCR amplified with fluorescently labeled forward primers (Applied Biosystems DS-33 dye set). Initially, single PCRs were performed on four wild eagle-owls from Norway. Those loci identified as polymorphic in four individuals were typed in 38 presumably unrelated eagle-owls (23 females and 15 males; sex determined with the primers M5 (Bantock et al. 2008), MP and NP (Ito et al. 2003)) from Luroy municipality (66°21'N, 12°36'E) in northern Norway using multiplex PCR. Multiplexing was performed with Qiagen multiplex PCR Plus kit (Applied Biosystems) following the manufacturer's protocol, but using a 10-µl reaction volume. PCR products (0.8 µl) were mixed with Genescan 500 LIZ (Applied Biosystems) size standard (0.25 µl) and Hi-Di formamide (9.75 µl) following capillary electrophoresis on an ABI 3130xl Genetic Analyzer (Applied Biosystems). Alleles were scored using Genemapper ver. 4.0 software (Applied Biosystems). To avoid problems with allelic drop-out (cf. Andreassen et al. 2012), homozygous genotypes were only included when the peak height was greater than 300 relative fluorescence units (RFU).

The mean number of alleles, observed and expected heterozygosities, and deviation from Hardy-Weinberg equilibrium were estimated using Arlequin ver. 3.5.1.3 (Excoffier and Lischer 2010). Linkage disequilibrium was evaluated using Genepop ver. 4.2 (Rousset 2008) and null-allele frequencies estimated with Micro-Checker ver. 2.2.3 (van Oosterhout et al. 2004). A Bonferroni correction for multiple statistical tests was (Rice 1989) applied to linkage disequilibrium p-values. The probability of identity was calculated using GenAlEx ver. 6.5 (Peakall and Smouse 2012).

Of the 22 loci initially tested in four individual eagle owls, 14 were polymorphic. Twelve of the polymorphic loci were assigned an autosomal location in the zebra finch (*Taeniopygia guttata*) genome based on sequence homology (following Dawson et al. 2006; Figure 1). Two loci (*BbuS116* and *BbuS132*) could not be assigned a chromosomal location in either the zebra finch, chicken (*Gallus gallus*) or turkey (*Meleagris gallopavo*) genome.

The 14 polymorphic loci showed a mean of 4.9 alleles per locus (range 2 to 9; Table 1). Heterozygotes were present in both sexes for all loci indicating none are sex-linked. The mean expected heterozygosity was 0.66 (range 0.33 to 0.85) and mean observed heterozygosity was 0.70 (range 0.42 to 0.97; Table 1). There was no indication of null-alleles in any loci, however one locus (*BbuS132*) deviated significantly ($p < 0.05$) from Hardy-Weinberg equilibrium (Table 1). After correcting for multiple tests, a single pairwise locus combination (*Bb100–Bb126*) displayed significant linkage disequilibrium. The combined probability of identity for the 14 loci was 3.5×10^{-12}. Cross-species application revealed that all 14 loci amplified successfully and many were polymorphic in the spotted eagle-owl (*B. africanus*) and Indian eagle-owl (*B. bengalensis*; Table 2). In conclusion, these eagle-owl microsatellite loci and their multiplex-PCR assays will be useful for family analysis, monitoring and population genetic analyses.

Acknowledgements We thank Hollie Marshall and Mike Wheeler at the University of Worcester, UK and Cotswold Falconry, UK for kindly providing the eagle-owl blood sample used to create the library, James McKay for providing the spotted eagle-owl and Indian eagle-owl samples, Espen R. Dahl for field assistance in Norway, and Terry Burke for project support. Marker development was performed at the NERC Biomolecular Analysis Facility at the University of Sheffield (supported by the Natural Environment Research Council, UK)

and the molecular lab at Norwegian Institute for Nature Research in Trondheim, Norway. Financial support was provided by the Norwegian Directorate for Nature Management, Research Council of Norway, Norwegian Institute for Nature Research, Hedmark University College and County Governor in Nordland.

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196 **Figure 1** Predicted chromosome locations in the zebra finch (*Taeniopygia guttata*) genome of 12 microsatellite loci polymorphic in the Eurasian

197 eagle-owl (*Bubo bubo*)

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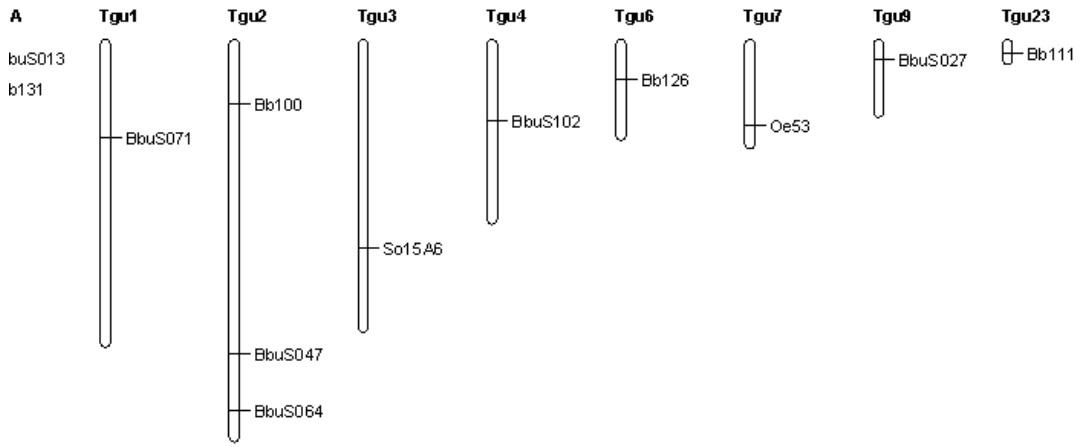

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Table 1 Characterization of 14 microsatellite loci in a northern European eagle-owl (*Bubo bubo*) population[a]

Locus	Clone name and EMBL accession number	Primer sequences (5'-3')	Repeat motif	PCR multiplex set	*n*	A	Expected allele size[b]	Observed allele size range	H_O	H_E	P_{HWE}	F_{NULL}
Characterization of eight novel eagle-owl (Bubo bubo) microsatellite loci												
BbuS013	BB12_C09	F: VIC-TTTCATAGAAGTCTCTCTCCACTACG	$(GT)_{18}$	A	38	3	150	145–151	0.74	0.67	0.840	-0.044
	HF564911	R: TTGAGGCTTATCATTTCTTCTGC										
BbuS027	BB12_F07	F: NED-TCATGAGGAACTTTCAGTGCTC	$(TATC)_{10}$	A	38	6	247	251–271	0.82	0.78	0.929	-0.028
	HF564925	R: GAAGAAAGGCAGCTCTCACC										
BbuS047	BB13_C01	F: 6FAM-GCACTGTTTGGATGTGTGGA	$(GT)_{13}$	A	38	3	134	138–142	0.50	0.55	0.681	0.028
	HF564945	R: CCTTTACTGCAGCCCTGTGT										
BbuS064	BB13_F10	F: NED-TGTAGTAGTAGCGCTCATTGCAG	$(CA)_{14}$	B	38	2	190	184–188	0.42	0.34	0.170	-0.067
	HF564962	R: CCATTTACTTACTGACTGCTTTGG										
BbuS071	BB13_H05	F: VIC-GATCCATCTCTTAGGGAAACACC	$(GA)_{12}$	B	38	2	121	117–119	0.42	0.51	0.343	0.052
	HF564969	R: TTCTGCATAGTTTGTTCACATTCAC										
BbuS102	BB14_F05	F: 6FAM-AACTGATTTGGAAACCACCATC	$(GATA)_8$	A	38	5	221	222–238	0.66	0.74	0.748	0.045
	HF565000	R: CTGGAACACCCAGTGTTTGTC										
BbuS116	BB16_B03	F: PET-GTTTCTGCAGCTGGGTCAG	$(TATC)_8$	B	38	5	202	198–218	0.66	0.65	0.383	-0.009
	HF565014	R: AAACAGTTTCCATGCCTTACG										
BbuS132	BB18_D10	F: VIC-TCATTGTAGGTCCCATCCAAC	$(TTCTA)_{22}$	B	38	8	177	159–204	0.87	0.78	0.012	-0.056
	HF565030	R: CCATATCTATCAAGCAACCTTGG										
Characterization of six previously isolated microsatellite loci												
Bb100[c]	Bb100	F: NED-TGTACCGCAAATCAAGGACA	$(TG)_8$ TA $(TG)_4$	C	38	9	163	162–188	0.87	0.85	0.720	-0.016
	AF432094	R: AGTATGCCCAGTGAACACCA										
Bb111[c]	Bb111	F: PET-GTTTTCCCTGTAGCCGACAA	$(AG)_{10}$	C	38	3	185	187–191	0.67	0.63	0.436	-0.026
	AF432096	R: TCAAGTCATCACCAATATCTAAGCA										
Bb126[c]	Bb126	F: VIC-CCAGAAGGGTTGTCATCTCC	$(GA)_{15}$ A_7 $(GA)_2$	C	38	5	179	165–181	0.76	0.72	0.899	-0.033
	AF432097	R: CAGCTTCTTTCAAGATTTCCAGA										
Bb131[c]	Bb131	F: 6FAM-TCTAGGAGGTGAAGGGGCTA	(CA) A_2 $(CA)_3$ A $(CA)_4$ CG $(CA)_{11}$	C	38	3	124	119–125	0.45	0.44	0.895	-0.008
	AF432098	R: CAGATGCTGTAGCACTGTTCCT										
Oe053[d]	Oe053	F: PET-CTCTGCATCTTAACGCACAGGAC	$(CTAT)_{12}$	C	38	6	203	218–238	0.97	0.79	0.166	-0.108
	AY312424	R: CCTCCAAGTGGACAGGAAAAGC										
So15A6[e]	15A6	F: VIC-ACCTCAGAAGCAGACAGAACC	$(GATA)_{13}$	C	38	8	149	119–155	0.95	0.84	0.732	-0.068
	AF510325	R: CCTTTGCGATTGCTGTAAC										

Number of individuals genotyped (*n*); Number of different alleles observed (A); Observed heterozygosity (H_O); Expected heterozygosity (H_E); Probability of deviation from Hardy-Weinberg equilibrium (P_{HWE}), Estimated frequency of null-alleles according to the Brookfield1 method implemented in Micro-Checker (van Oosterhout et al. 2004) (F_{NULL})

[a]An additional eight primer sets were tested of which three were monomorphic (*BbuS105*, *BbuS080*, *BbuS099*), three amplified non-specific fragments (*BbuS029*, *BbuS094*, *BbuS108*) and two failed to amplify (*BbuS093*, *BbuS123*)

[b]The expected allele size was based on the sequence of the cloned *Bubo bubo* individual (CF68) for the newly isolated *BbuS* loci

[c]For these loci, the eagle-owl sequences were isolated by Isaksson and Tegelström (2002) but new primer sets redesigned specifically for this study to enable amplification of shorter fragments

[d]Sequence isolated from the Lanyu scops-owl *Otus elegans botelensis* and the published primer set used (Hsu et al. 2003)

[e]Sequence isolated from the Mexican spotted owl *Strix occidentalis lucida* and the published primer set used (Thode et al. 2002). 'So' added to original locus name (15A6) to indicate species identity

213 **Table 2** Allele sizes in the spotted eagle-owl (*Bubo africanus*) and Indian eagle-owl (*B.*
214 *bengalensis*).

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Locus	*Bubo africanus* (n = 2)	*Bubo bengalensis* (n = 1)
BbuS013	139, 141	153
BbuS027	251, 255	255, 259
BbuS047	127, 133, 135	149
BbuS064	184	183, 189
BbuS071	117, 121	119
BbuS102	238, 242, 246	226
BbuS116	198, 202	210
BbuS132	149	144, 159
Bb100[a]	150, 154	164, 168
Bb111[a]	193	191
Bb126[a]	160, 168	169
Bb131[a]	112, 114, 122, 128	125
Oe053[b]	210, 228, 236	222
So15A6[c]	135, 139	123, 143

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217 n, number of individuals genotyped

218 [a]For these loci, the eagle-owl sequences were isolated by Isaksson and Tegelström (2002) but new primer sets redesigned specifically for this
219 study to enable amplification of shorter fragments

220 [b]Sequence isolated from the Lanyu scops-owl *Otus elegans botelensis* and the published primer set used (Hsu et al. 2003)

221 [c]Sequence isolated from the Mexican spotted owl *Strix occidentalis lucida* and the published primer set used (Thode et al. 2002). 'So' added
222 to original locus name (15A6) to indicate species identity