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Primer Note

Polymorphic microsatellite markers for the critically endangered Balearic shearwater, *Puffinus mauretanicus*

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1 **Abstract**

2 Ten novel polymorphic microsatellite loci were isolated and characterized from the
3 Balearic shearwater (*Puffinus mauretanicus*), a critically endangered seabird. The
4 developed loci revealed a relatively low number of alleles per locus, as well as low levels of
5 polymorphism ($H_O = 0.377 \pm 0.241$). One of the loci resulted to be W-linked. All polymorphic
6 loci were successfully amplified in its closely-related species the Yelkouan shearwater (*P.*
7 *yelkouan*). These microsatellite markers would be useful for assessing population structure in
8 the Balearic shearwater and the possible hybridization process between both shearwaters
9 species.

The Balearic shearwater, *P. mauretanicus* (Lowe, 1921) is an endemic seabird of the Balearic archipelago that is considered critically endangered by the IUCN (Oro *et al.*, 2004). The current conservation status of the species is particularly fragile mainly due to very low adult survival (Oro *et al.*, 2004). An additional threat comes from potential hybridization with the closely-related Yelkouan shearwater, *P. yelkouan* (Acerbi, 1827) (Genovart *et al.*, 2005; Genovart *et al.*, 2007). Here, we present the characterization of 10 novel polymorphic microsatellites isolated from Balearic shearwater, which will provide valuable information on genetic diversity and population structuring of this species, as well as on hybridization with Yelkouan shearwater.

Two libraries enriched for CA and AAAG repeat sequences, respectively, were constructed using the protocols of Ostrander *et al.*, (1992) and Hamilton *et al.*, (1999). Genomic DNA was extracted from ethanol-preserved muscle using a standard proteinase K/ phenol-chloroform procedure, digested with *Rsa* I and *Xmn* I restriction enzymes (New England Biolabs), and ligated to universal *SNX* adaptors (Hamilton *et al.*, 1999). Several stringent washes followed by magnetic field application allowed the recovery of the biotinylated CA/ AAAG-enriched DNA that was subsequently ligated into a pGEM-T Easy vector (Promega). Approximately 850 transformed colonies (800 for the CA library and 50 for the AAAG library) were hybridized with the aforementioned probes, and positive colonies were detected using a chemiluminescent method (CDP-Star detection kit, Sigma). After a second screening process of amplification using the *SNX* adaptor and the CA and AAAG unlabelled probes, 162 recaptured clones (142 from the CA library and 20 from the AAAG library) were sequenced using universal M13 primers on an ABI PRISM 3730 automated sequencer (Applied Biosystems) with the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit (V3.0), and a total of 108 contained microsatellites. Of these, 23 were selected for primer designing (GenBank Accession N°: EU158894-EU158911, EU815884, FJ372421-FJ372424), using PRIMER3 software (Rozen, Skaletsky, 1998), and 19 were successfully optimized.

Polymorphism was evaluated genotyping 55 individuals from two localities on Balearic Islands, Cabrera ($N = 35$) and Eivissa ($N = 20$). Genomic DNA was extracted from blood preserved in ethanol, using a standard proteinase K/ phenol-chloroform procedure. PCR

amplifications were carried out on a Mastercycler EP-Gradient, and the 13 μ l reactions contained 0.2 mM of each dNTP, 2-3 mM $MgCl_2$, 1xTaq Buffer Advanced (20 mM Tris-HCl, pH 8, 100mM KCl, 0.1 EDTA, 1 mM DTT), 0.4 μ M of each primer, 0.8 U of Taq DNA polymerase (Eppendorf), and approximately 5 ng of DNA. The cycling profile was: 35 cycles of 95°C for 45 s, 50-61°C for 30 s, and 72°C for 30 s. Forward primers were fluorescently labeled (6- FAM, NED, VIC of PET, Applied Biosystems), and fragments were run on an ABI Prism 3700 DNA Analyzer (250-500 LIZ size standard). Allele scoring was performed using GeneMapper v3.7 (Applied Biosystems). Only 10 loci turned out to be polymorphic and one of them (*Puff Sma6*) resulted to be sex-linked (Table 1). The sex identification by amplification of the CDH1 gene (Fridolfsson, Ellegren, 1999), confirmed that this microsatellite is located on the W chromosome, as evidenced by its amplification only in females.

Number of alleles (N_A), observed (H_O) and expected heterozygosities (H_E) (Nei, 1987), exact test of Hardy-Weinberg (HWE), and linkage disequilibrium were calculated using Genepop v.3.3 (Raymond, Rousset, 1995) (Table 1). Number of alleles varied from two to 10 ($N_A = 5.0 \pm 2.8$), and genetic variability was very low ($H_O = 0.377 \pm 0.241$), as expected for a species that is genetically depress (Table 1). No evidence of linkage disequilibrium was found after Bonferroni correction ($P > 0.05$) (Rice, 1989). After correcting for multiple test (Rice, 1989), F_{IS} pairwise comparisons for three loci (*Puff 1.9*, *Puff PM2* and *Puff G11F*) indicated their departure from HWE due to homozygote excess. Both the low frequency of null alleles detected ($P < 0.2$, (Chapuis, Estoup, 2007), and the fact that we were able to successfully type most individuals, suggest that observed heterozygote deficiency was apparently not caused by allelic drop-outs. A probable explanation for the observed heterozygote deficiency could be an undetected genetic structure within colonies as a result of a possible non random mating, as shearwaters are extremely phylopatric and tend to breed very close to their natal nest, favoring thus genetic substructuring. However a population genetic analysis, increasing the number of individuals analyzed is needed to further assess genetic structure in this species and to understand the factors that promote the observed heterozygote deficiency.

In addition, 23 Yelkouan shearwaters were analyzed, and all newly-developed markers were able to cross amplify, showing one monomorphic locus, and similar values of genetic polymorphism, despite the lower number of individual assayed (Table 2).

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Table 1. Summary data for microsatellite developed from the Balearic shearwater (*Puffinus mauretanicus*)*

Locus	Repeat motif	T ^a (°C)	Dye	Primers sequence 5'-3'	N	N _A	Allele range	H _o	H _E	F _{IS}	GenBank No.
<i>Puff 1.5</i>	(GT) ₁₀	53	NED	F: AGGCAGCAACATAGTCAC R: GCACTGATAAGGGAACCTT	54	2	172-174	0.037	0.037	-0.010	EU158894
<i>Puff 1.9</i>	(GT) ₁₅	52	VIC	F: ATCTAAATAAGGTGTCTGC R: CCATATACATAACAGTAAA	55	5	129-145	0.327	0.690	0.528	EU158895
<i>Puff 4B</i>	(GT) ₂₆	61	6-FAM	F: TGGTTTTGATGAACCGTATCTC R: TGGCTGAGAGTAAAGTCAATGG	53	6	177-296	0.509	0.509	-0.001	FJ372423
<i>Puff C5A</i>	(GT) ₁₂	58	NED	F: GCTGGTTGCTTCTTGAGACC R: CTTTCCCCCTTCCTCCCTAC	54	5	180-188	0.577	0.632	0.088	EU158896
<i>Puff C5D</i>	(GT) ₁₅	58	VIC	F: GCCCCTTGCTACATTTTC R: TCACCATTTACTGCCATGTATCA	55	9	173-187	0.709	0.750	0.054	EU158897
<i>Puff G2C</i>	(GT) ₁₄	50	NED	F: CTGAAATCAGACATTATTTTC R: ACAATATGCAAACCTTTTG	50	7	153-165	0.580	0.674	0.140	EU158898
<i>Puff G2F</i>	(GT) ₉	60	VIC	F: GCTCTCCCAGTTTGCACG R: TCCCATGCTTTAACCCTG	51	2	198-200	0.020	0.058	0.662	EU158899
<i>Puff G11F</i>	(GT) ₂₅	60	6-FAM	F: CCCTGACTTGGGACCAAG R: TGTGACGTGCCACTGTATTG	45	9	168-200	0.289	0.695	0.587	EU158900
<i>Puff PM2</i>	(GT) ₁₆	55	6-FAM	F: AAAGATGCTTAAGGATACATAACTGC R: TTGTTGGCAAAAGTGATAAGGTT	55	5	170-178	0.345	0.574	0.401	EU158901
<i>Puff Sma6</i>	(GTCTTTCT) ₂₂	53	6-FAM	F: GGGCTGTGGGAAAGGAGTTA R: TGTTTCCAGCAGCAATCAGT	32 ⁼	10	307-405	NA	NA	NA	EU815884

* N= number of individuals assayed, N_A= number of alleles per locus, expected (H_E) and observed (H_o) heterozygosities, F_{IS}= Wright's statistics. Bold F_{IS} values are significant probability estimates.

⁼ *Puff Sma6* locus is W-linked and amplified only in females. The sex ratio obtained by amplifying the CHD1 gen was 33 females, 21 males, (N=54).

Table 2. Estimated variability at ten microsatellite loci on the Yelkouan shearwater (*Puffinus yelkouan*)*

Locus	N	N _A	Allele range	H _O	H _E	F _{IS}
<i>Puff 1.5</i>	23	2	172-174	0.044	0.044	-0.000
<i>Puff 1.9</i>	23	5	129-143	0.217	0.616	0.652
<i>Puff 4B</i>	18	4	177-206	0.444	0.589	0.251
<i>Puff C5A</i>	23	5	180-188	0.652	0.654	+0.003
<i>Puff C5D</i>	23	7	173-187	0.870	0.821	-0.060
<i>Puff G2C</i>	23	6	153-163	0.739	0.688	0.076
<i>Puff G2F</i>	23	1	200	/	/	/
<i>Puff G11F</i>	23	9	168-200	0.217	0.815	+0.737
<i>Puff PM2</i>	23	4	170-176	0.304	0.545	+0.447
<i>Puff Sma6</i> ⁻	12	7	308-422	NA	NA	NA

* N= number of individuals assayed, N_A= number of alleles per locus, expected (H_E) and observed (H_O) heterozygosities, F_{IS}= Wright's statistics. Bold F_{IS} values are significant probability estimates. ⁻*Puff Sma6* locus is W-linked and amplified only in females. The sex ratio obtained by amplifying the CHD1 gen was 12 females, 9 males, (N=21)..