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Isolation and characterization of microsatellites in albatrosses

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Albatrosses are long lived, monogamous and exhibit high levels of philopatry (Prince *et al*. 1994). In recent years, there has been some concern over population declines in six albatross species, believed to result from an increase in longline fisheries around the breeding sites (Weimerskirch *et al*. 1997). The status of four other species is unknown (Gales 1993). Grey-headed albatrosses at Bird Island have declined by 30% in the past 23 years. Similarly, wandering albatross populations at South Georgia have decreased by 28% over the same period (Croxall *et al*., 1998).

No microsatellite markers have yet been published for any of the albatross species. Here, I report on the isolation and characterization of 26 microsatellite loci cloned from two species: grey-headed albatross (*Diomedea chrysostoma*) and wandering albatross (*Diomedea exulans*). The classification of Diomedeidae, is currently under discussion. Nunn *et al*. (1996) placed extant albatrosses into four genera based on cytochrome *b* sequence (Thalassarche, Phoebetria, Diomedea and Phoebastria). The study species represent Thalassarche and Diomedea, thought to be separated by 5 Myr (Nunn *et al*. 1996). Microsatellite markers have been developed to examine intracolony and interisland variation in several albatross species and to identify the natal sites of albatrosses killed in longline fisheries.

Genomic DNA from grey-headed and wandering albatrosses was digested with *Alu*I restriction endonuclease. Size-selected fragments (300–800 bp) were ligated into *Sma*I-cut pUC19 using temperature cycle ligation (Lund *et al*. 1996) and transformed into DH5α *Escherichia coli*. Colonies were screened using $(AC)_{15}$ synthetic oligonucleotides according to Sambrook *et al*. (1989). Positive colonies were restreaked and rescreened prior to fluorescent sequencing.

A second library enriched for AC and CTTT was constructed using a hybridization-based protocol (Armour *et al*. 1994). This resulted in a 30-fold increase in the number of microsatellite-containing colonies. Approximately 0.05% of colonies from the unenriched library contained microsatellites compared with 1.7% from the enriched library. These figures are comparable with those from other avian studies (Piertney *et al*. 1998; Tarr & Fleischer 1998).

Primers were designed from 31 of the microsatellite sequences. However, only 26 amplified a visible product (Table 1). PCR reactions were carried out in 10 μL reaction volumes containing 1× thermalase reaction buffer (100 mM Tris (pH 8.3), 500 mM KCl, 1.5 mM $MgCl_2$, 0.1% Tween 20, 0.1% gelatin, 0.1% NP40), 0.1 mM dGTP, 0.1 mM dATP, 0.1 mM dTTP, 0.01 mM dCTP, 8 pmol of each primer, 1% formamide, 60 mM TMAC, 0.25 U *Taq* polymerase (Promega) and 0.1 μCi [α^{32}P]-dCTP. The only exception is the reaction mix used for locus De11 [NK buffer (67 mM Tris (pH 8), 160 mM $(NH_4)_2SO_4$, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Tween 20 and no TMAC or formamide]. All loci were amplified using a two-step annealing procedure: one cycle for 2 min at 94 °C, 45 s at T_{A1}, 50 s at 72 °C; seven cycles of 50 s at 94 °C, 45 s at T_{A1}, 50 s at 72 °C; 25 cycles of 50 s at 89 °C, 45 s at T_{A2}, 50 s at 72 °C; and one final cycle of 5 min at 72 °C. The two annealing temperatures (T_{A1} and T_{A2}) are listed in Table 1.

Cross-species screening was conducted using DNA from grey-headed (n = 50), black-browed (Thalassarche: *Diomedea melanophris*, n = 50) and wandering (n = 90) albatrosses. The wandering albatrosses surveyed represent birds from five different island groups (Adams, Auckland, Crozet, Prince Edward and South Georgia). Twenty-one loci were polymorphic in one or more species. Levels of polymorphism varied from two to 10 alleles (Table 2). Only two loci (Dc10 and Dc19) failed to amplify DNA in the wandering albatross, but did amplify successfully in the other two species. Microsatellite loci did not consistently show higher levels of variation in the focal species. Microsatellite loci also amplified polymorphic loci in distantly related Thalassarche and Diomedea. These loci should prove useful for studying population structure, kinship and mating systems in any of the four albatross genera.

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Table 1 Primer sequences, GenBank Accession nos and PCR conditions for 26 microsatellite loci. Loci are named according to the species from which they were isolated (Dc, grey-headed albatrosses; and De, wandering albatrosses) with the exception of D6, D24, D29 and D32

Locus	Primer sequence (5′→3′)	GenBank Accession no.	Annealing temp. (°C) T_{A1}	T_{A2}
De1	a: ACGAGATTTTCGCAGCACTT	AF096787	46	48
	b: CTCAGCTGTTGACAAATGCC			
De2	a: CCTGCCAGGATCAGAGGTT	AF096788	46	48
	b: TTGGGTTGATGGTTGGACTT			
De3	a: CTGCCAAAAAGAATTCCCTG	AF096789	50	54
	b: CCAACAAGGACTAAGCCCAA			
Dc5	a: AGGAGGGAAACTTCTCCCAG	AF096790	46	48
	b: AGCAGGGAGTGACTTGAGGAG			
D6	a: TGGTTTGCTTCCCTCACTTC	AF096791	46	48
	b: TTTGTCTCAGGGAAACAGGG			
De7	a: GAGGAAAACTCCATCTGCCA	AF096792	48	52
	b2: TCTGAACCTCTATCAAAAGG			
Dc9	a: CGTGGTATATAGCTTATGGGCA	AF096793	50	54
	b: GAGATTGTACTCCTGGGGCA			
Dc10	a: CATAGGGCACTTTTGCCAAT	AF096794	50	54
	b: GGGACATGCAGAGGTAGGAC			
De11	a: CCTGGAAAAGGCCCTTATATTC	AF096795	52	54*
	b: CACCGAGTACCATCATTCCC			
De12	a: CAAGAAGACATCTGAGTACTTTGC	AF096796	46	48
	b: CGATCTTGTAGAGGTGATGCC			
Dc16	a: TTTTCCAAAGAGATGGCACCA	AF096797	48	52
	b: GACAGCAGAGGTGGGTCTGT			
De17	a: TCTCAGGCCCTTGTCCTAAC	AF096798	48	52
	b: AAAATGGTCCCACAGAGCAC			
De18	a: TCCCTGTGTGGGTTTTTGAT	AF096799	48	52
	b: CTAAGCCCAACACCAGAAGC			
Dc19	a: GTCTCCACTGTCCAGCGTCT	AF096800	48	52
	b: CAACTCCTTACAGACAAAGGC			
Dc20	a: GGATTGCTGTGGTTTTGCTT	AF096801	48	52
	b: AACATGACACAGGAGAGTGGC			
Dc21	a: CGGGGGAACTGAAGAAGATT	AF096802	48	52†
	b: TGGACAGCCAGTGGGATAAC			
Dc22	a: AGTGGGATGGGTGTATCAGG	AF096803	48	52
	b: TGGACTAGAGGCCTTGTCTGA			
D24	a: AGCCTGCTTTTGATGGAAGC	AF096804	48	52
	b: CACTCTATTTTTCCTCCAGCTGA			
De25	a: CATGATGTCAAGGGCTCCTC	AF096805	48	52
	b: AGCCCCTTGATGTGATTTTC			
Dc26	a: GGGACAGACTTCCCTTCTCC	AF096806	48	52
	b: CTTAAGACTTGGGACTGCCC			
Dc27	a: CACCCATTTTTGCAGTTCAC	AF096807	46	48
	b: TCCCCTTGCTTGTTGATTATG			
D29	a: TTTGGGGATAGATGGGTCAC	AF096808	48	52
	b: GTTTTTCCCTTCTGTGCTGG			
De30	a: AGCCCCTTGATGTGATTTTC	AF096809	48	52
	b: CATGATGTCAAGGGCTCCTC			
Dc31	a: CTGACTCTCTGGGATGAGCC	AF096810	48	52
	b: GTACCCCTGGAAAGCCAGAC			
D32	a: GACCTCGGTACCCCTCTAGC	AF096811	48	52
	b: GTGCTGGGACCACAGGAG			
De33	a: ATCCTACCCTGATTCAGCCC	AF096812	48	52
	b: GGATCAACAACAATAGGGGTG			

*No TMAC or formamide, NK buffer.
†35 cycles.

Table 2 Microsatellite repeat type, number of alleles and size range of alleles in black-browed (Dm) ($n = 50$), wandering (De) ($n = 90$) and grey-headed (Dc) ($n = 50$) albatrosses. For each loci, the allele size range, number of alleles, observed heterozygosity (H_O) and expected heterozygosity (H_E) are listed for each species. Loci which failed to amplify are indicated by 'np'

		Dm				De				Dc			
Locus	Repeat type	Allele size	No. of alleles	H_O	H_E	Allele size	No. of alleles	H_O	H_E	Allele size	No. of alleles	H_O	H_E
De1	$(CA)_9$	228	1	0	0	222–228	4	0.07	0.13	226	1	0	0
De2	$(AC)_{7.5}$	213	1	0	0	209–215	2	0.20	0.39	217	1	0	0
De3	$(AC)_7AT(AC)_{4.5}$	108–114	3	0.38	0.40	118–122	3	0.11	0.40	110–116	4		0.50
Dc5	$(AC)_5G(CAC)_2G(CA)_{12}$	159–167	5	0.27	0.35	163–171	5	0.14	0.28	161–167	4	0.24	0.15
D6	$(TC)_3A(TC)_2ATGCC(TC)_5$	154	1	0	0	154	1	0	0	154	1	0	0
De7	$(AC)_{11}$	204–206	2	0.17	0.26	206–208	2	0.41	0.49	204–206	2	0.13	0.17
Dc9	$(AC)_{13}$	84–102	7	0.45	0.50	86–88	2	0.05	0.05	88–104	9	0.79	0.78
Dc10	$(AN)_4(AC)_4$	113–115	2	0.17	0.15	np	np	np	np	115	1	0.09	0.08
De11	$(AC)_{9.5} + (TA)_7(CA)_4(CG)_{5.5}$	178–196	7	0.83	0.83	194–204	4	0.50	0.55	168–208	9	0.69	0.81
De12	$(AC)_{13}$	89	1	0	0	93	1	0	0	89	1	0	0
Dc16	$(AC)_{13}$	113	1	0	0	115–119	3	0.19	0.48	119	1	0	0
De17	$(CA)_2TG(CA)_{12.5}$	141–147	4	0.33	0.57	127	1	0	0	141–145	3	0.57	0.56
De18	$(AC)_7AT(AC)_{4.5}$	86–92	3	0.40	0.37	90–98	5	0.23	0.26	86–92	4	0.37	0.50
Dc19	$(CA)_{11.5}$	245–251	5	0.62	0.76	np	np	np	np	245–249	3	0.25	0.26
Dc20	$(CA)_{10.5}$	113–119	4	0.24	0.24	99–113	5	0.35	0.39	117–121	3	0.49	0.24
Dc21	$(CA)_{12}$	170–176	4	0.48	0.67	164	1	0	0	172–176	3	0.35	0.67
Dc22	$(CA)_{11.5}$	105–111	4	0.63	0.65	103–105	2	0.28	0.29	105–107	2	0.02	0.65
D24	$(CA)_{5.5}$	155	1	0	0	155	1	0	0	155	1	0	0
De25	$A_{17}GA_7$	118–128	4	0.55	0.43	116	1	0	0	118–130	10	0.55	0.94
Dc26	$(AC)_{13}$	177–181	3	0.45	0.53	181–183	2	0.31	0.38	177–181	3	0.31	0.40
Dc27	$(AC)_{11}$	94–100	4	0.47	0.49	96–98	2	0.38	0.44	90–96	4	0.39	0.49
D29	$(CT)_7(GACACA)_2$	117	1	0	0	117	1	0	0	117–119	2	0.04	0.23
De30	T_7CT_{18}	126	1	0	0	126–128	2	0.25	0.22	125–126	2	0.25	0.38
Dc31	$(CA)_4(GC)_2(AN)_3(AC)_5(AG)_2$	127	1	0	0	125	1	0	0	127	1	0	0
D32	$(AGC)_5$	104	1	0	0	104	1	0	0	104	1	0	0
De33	$(TCT)_{29}TTT(TCT)_3(TCA)_5CCA(TCA)_3$	103	1	0	0	103–202	11	0.50	0.86	103	1	0	0

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Polymorphic microsatellite loci identified in the highly clonal freshwater bryozoan *Cristatella mucedo*

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The freshwater bryozoan *Cristatella mucedo* (Class Phylactolaemata) reproduces predominantly via asexual means. Some populations undergo a short period of sexual reproduction in each season, but the sexually produced larvae are capable of only limited dispersal (Okamura 1997a). The buoyancy of asexually produced statoblasts, on the other hand, allows them to disperse widely within a site. In addition, marginal hooks on statoblasts may attach to feathers or fur, resulting in the potential for widespread dispersal via animal vectors (Okamura 1997a).

Previous work using random amplified polymorphic DNA (RAPDs) showed that *C. mucedo* populations share a high degree of genetic similarity in England, UK (reviewed in Okamura 1997b). In the Thames Valley region, *C. mucedo* conforms to a metapopulation structure with subpopulations characterized by limited sexual reproduction and low clonal diversity (Okamura 1997a,b). We are assaying levels of genetic variation within and among European populations of this highly clonal species in order to assess reproductive strategies and determine the extent of gene flow across broad spatial scales. Microsatellite loci were chosen for this study because of their relatively high mutation rates (Bruford & Wayne 1993). Here we report the first microsatellites isolated from freshwater bryozoans and only the second such markers in the phylum (Hoare *et al*. 1998).

C. mucedo specimens were collected from England, Scotland, France, Netherlands, Denmark, Sweden, and Finland. One colony from each of the seven different countries was used for the construction of a partial library. The DNA was restriction digested with *Sau*3A1, and then fragments in the 300–600 bp range were ligated into pUC18 and transformed into JM109 high-efficiency competent cells (Promega). Approximately 2200 bacterial colonies were isolated from the library, and screened with AG, AC (combined total of 57 positives), TAA, TAAA, GAAA, and CAAA (0 positives) oligonucleotide repeats. Primers were designed for 24 of the dinucleotide loci using OLIGO version 4.1 (Rychlik & Rhoads 1989). PCR reactions were performed in either Hybaid or Techne thermocyclers using 0.1–1.0 ng of DNA; 1× PCR buffer containing 500 mM KCl, 100 mM Tris-HCl, 1.0% Triton® X-100 (Promega); 1.5–2.5 mM $MgCl_2$; 200 μM each of dGTP, dATP, dTTP, and dCTP; 1–5 pmol of each primer; and 1 U *Taq* DNA polymerase (Promega).

Initial screening for polymorphic loci was performed with 12 bryozoan colonies, each representing a different population. Amplified samples from each colony were electrophoresed on 8% denaturing polyacrylamide gels, and stained with a Silver Sequence kit (Promega). Loci that appeared polymorphic without any additional nonallelic bands were kept for further screening. Subsequent screening of the variable loci 1.1, 2.2, 5.9, 6.7, and 9.4 followed an automated genotyping protocol. The PCR conditions were as outlined above, except that the forward primer was 3′-Cy5 fluorescently labelled (Pharmacia). Ten fmol of the amplified DNA from each sample was then run out on a 6% polyacrylamide gel in an ALFexpress DNA Sequencer. Internal and external size markers (Pharmacia) were included on each gel. The resulting profiles were analysed using AlleleLinks version 1.00 (Pharmacia). A total of 408 colonies from 14 populations was screened with these primers. Heterozygosity levels were calculated using POPGENE version 1.21 (Yeh *et al*. 1997).

Overall, the heterozygosity levels found in the screened loci were low (Table 1). A number of factors may contribute to the deficit of observed heterozygotes, including inbreeding (Okamura 1997b), periodic bottlenecks (Okamura 1997a), and the Wahlund effect (J. R. Freeland, L. R. Noble, B. Okamura, unpublished). Similarly low levels of heterozygosity have been found in other taxa (for example, Ayre & Dufty 1994). Despite these heterozygote deficiencies, the genetic diversity of 8–25 alleles per locus (Table 1) is high, and will provide sufficient variation to differentiate between populations.