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PERMANENT GENETIC RESOURCES

Microsatellite primers for relatedness and population structure in great frigatebirds (Pelecaniformes: Fregatidae)

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Abstract

Eighteen moderately polymorphic microsatellite loci were isolated and characterized for great frigatebirds *Fregata minor*. Polymorphism for the 12 dinucleotide and six tetranucleotide markers was assessed in 23 birds from a Hawaii population. Allelic diversity ranged from two to 12 (mean 5.9), with observed heterozygosity from 0.304 to 0.956 (mean 0.637). Three loci showed homozygote excess, possibly due to null alleles. One additional pair of loci exhibited strong gametic disequilibrium. Thus, at least 14 loci will be useful for studies of relatedness and population structure.

Keywords: *Fregata*, frigatebirds, Hawaii, microsatellites, primers, seabirds

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The decreased heterozygosity associated with inbreeding can compromise immune function (Whiteman *et al*. 2006). This could have dire consequences for the viability of natural populations, as human activity tends to promote both inbreeding (Andersen *et al*. 2004) and the spread of emerging pathogens (Daszak *et al*. 2000). Testing for a relationship between inbreeding and immune function requires a measure of relatedness between mates, but pedigree data tend to be available only for short-lived organisms. Here we develop microsatellite markers to measure genetic similarity of mates in great frigatebirds *Fregata minor* — seabirds that can live more than 40 years (Juola *et al*. 2006) and that inbreed regularly in at least one population (Cohen & Dearborn 2004).

Following Hamilton *et al*. (1999), we constructed microsatellite-enriched genomic libraries from one male and one female great frigatebird from Tern Island, Hawaii. Genomic DNA was extracted from blood using phenol–chloroform (Sambrook & Russell 2001) and digested with *Hae*III, *Xmn*I and *Nhe*I (New England Biolabs). The resulting fragments, mainly 200–1200 bp, were ligated to SNX linkers, hybridized to biotinylated oligos $(CA)_{10}$, $(AAAG)_5$ and $(AAGG)_5$, and isolated from unhybridized fragments with magnetic beads (Dynabeads M-280 Streptavidin, Invitrogen). Fragments were amplified with SNX primers and ligated into pBluescript II SK + plasmids, then transformed into *Escherichia coli* XL-10 Gold (Stratagene).

We polymerase chain reaction (PCR) screened the clones with primers T7 (5′-GTAATACGACTCACTATAGGGC-3′) and the enrichment oligos, looking on agarose gels for smears rather than a distinct band or no band. Promising clones were PCR amplified with T7 and T3 (5′-AATTAAC-CCTCACTAAAGGG-3′). Amplicons of 350–900 bp were cycle sequenced with BigDye 3.1. on an ABI PRISM 371 or 3130XL Genetic Analyser and analysed with Sequencher 4.8. Initial results showed that the smear-based screening worked marginally well for CA- and AAAG-repeats but not at all for AAGG-repeats, and we stopped screening AAGG clones.

From the CA-enriched library, we PCR screened 912 clones and sequenced 304, yielding 49 clones with ≥ 8 uninterrupted dinucleotide repeats. From the AAAG library, we screened 1056 clones and sequenced 206, yielding 11 clones with ≥ 5 tetranucleotide repeats.

Primers were designed for 28 loci, using a three-primer system (Schuelke 2000) in which the forward primer had a T7 or M13 tail; products were labelled with T7-HEX or

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Table 1 Characteristics of 18 microsatellite loci from great frigatebirds *Fregata minor*

Locus GenBank no.	Clone	F and R primers 5′ to 3′ Parentheses surround T7 or M13 tail on F primer	Repeat	T_a (°C)	Size (bp)	N_A	H_O	H_E	P
Fmin01 EU700035	01_B11	F: (GTAAAACGACGGCCAGT)CAGCTTCCTGTCCTGACTCC R: TTCCCTCAACCTCTTGCAT	$(CA)_{16}$	55	203–217	6	0.783	0.737	0.202
Fmin02 EU700036	01_D11	F: (CGACTCACTATAGGGC)TTGCACTGACCATTGTTTCC R: TCCTATTACTCCCAATAAACTCAGAC	$(CA)_{24}$	50	190–227	12	0.957	0.888	0.145
Fmin03 EU700037	02 _C12	F: (GTAAAACGACGGCCAGT)GACTGCCCAGATCCCACATTAC R: CACAAAATGCCAGCCATACG	$(CA)_{16}$	55	125–133	4	0.522	0.691	0.069
Fmin04 EU700038	02_E06	F: (CGACTCACTATAGGGC)TGCACTTTTCCAGCTGACAC R: TTTGGGTCCTTTGTTCTTGG	$(CA)_5 \ldots (CA)_{10}$	55	121–141	6	0.565	0.673	0.070
Fmin05 EU700039	02_F09	F: (CGACTCACTATAGGGC)TGCTACATCTCTGTCTCTCT R: AAGTCACATCCCTTACTTGG	$(CA)_8 \ldots (CA)_8 \ldots (CA)_3$	60	132–143	9	0.435	0.826	0.0001*
Fmin06 EU700040	02_H11	F: (GTAAAACGACGGCCAGT)CAGCAGCAAAGAAAATTAAGAGC R: CGCAGAGTCAGCAGAAGTTG	$(CA)_{20}$	55	147–167	6	0.652	0.622	0.608
Fmin07 EU700041	09_A12	F: (GTAAAACGACGGCCAGT)ATGCCCTGAAATTCCATCAG R: TGTGCTTGCTCACTCTCCAC	$(CA)_8$	55	167–169	2	0.522	0.487	1
Fmin08 EU700042	10_A12	F: (CGACTCACTATAGGGC)TTGCAGTTTCAGAGCCAAAG R: AAGGTGTGGCATTTCTGGAC	$(CA)_8 \ldots (CA)_9$	55	162–164	2	0.391	0.477	0.415
Fmin09 EU700043	16_B07	F: (GTAAAACGACGGCCAGT)CTTGCCCCATACAATTACTCAG R: ATATGCGTGTGCATTCATCC	$(CA)_{20}$	55	176–184	4	0.304	0.579	0.003*
Fmin10 EU700044	16 _C05	F: (GTAAAACGACGGCCAGT)TGCCATGTAGGAGCAAAGTG R: TGACTCCACAGCGATTTGTC	$(CA)_{14}. (CA)_{12}$	55	186–207	8	0.826	0.827	0.184
Fmin11 EU700045	16 _C06	F: (GTAAAACGACGGCCAGT)CTGCCACAAACTATGCTTCC R: AACAGATGTGCCAGGAGGAG	$(CA)_{14}$	55	160–176	5	0.609	0.500	1
Fmin12 EU700046	18_D11	F: (GTAAAACGACGGCCAGT)TTTTTAGGTGGGGACATCTG R: CTGGAGCAAGATTGGACAGG	$(CT)_7(CA)_9$	55	127–153	8	0.783	0.833	0.488
Fmin13 EU700047	05_C11	F: (GTAAAACGACGGCCAGT)CGCATAACTAGAAGGTTGTG R: CAAAATGCCTGGAAATCTGC	$(AAAG)_5 \ldots (AAAG)_{15}$	55	235–259	5	0.609	0.515	0.328
Fmin14 EU700048	06_A09	F: (GTAAAACGACGGCCAGT)ATGCTTGTGCAAATCAATGC R: TCACGTTGAAATCCCTTTCC	$(AAAG)_{10}$	55	154–167	5	0.870	0.741	0.990
Fmin15 EU700049	11_F01	F: (GTAAAACGACGGCCAGT)TGAAGCAGGCTTTACAGAATAGG R: GATCTCCAGAGGTCCCTTCC	$(AAAG)_{18}$	55	156–202	11	0.565	0.800	0.025†
Fmin16 EU700050	13_D06	F: (CGACTCACTATAGGGC)TTTCCAAGTCCCACATCACC R: GCAGGAACCTCAAAATCTGG	$(AAAG)_{10}$	55	162–174	4	0.565	0.540	1
Fmin17 EU700051	27_E09	F: (CGACTCACTATAGGGC)TTGCTTGCTTTCTGCTTTTC R: TTCGAGTTAAAATATACAATTCAGAAC	$(AAAC)_4(AAAG)_6$ AA$(AAAG)_4$	55	402–410	4	0.545	0.568	0.914
Fmin18 EU700052	27_F11	F: (CGACTCACTATAGGGC)AACAAGTGGTCTCTCAAATCACC R: CAAACCCAAACACAAAAACC	$(AAAG)_{15}$	55	186–212	9	0.957	0.828	0.563
Mean						5.9	0.637	0.674	

*significant deviation from Hardy-Weinberg equilibrium even with sequential Bonferroni adjustment of alpha.
†significant deviation from Hardy-Weinberg equilibrium only if no Bonferroni correction is used. T_a denotes the annealing temperature, size is the range of allele sizes in base pairs (including the T7 or M13 tag), N_A is the number of alleles found in 23 *F. minor* individuals, H_O and H_E denote observed and expected heterozygosities, and P is the P value for test of departure from Hardy–Weinberg equilibrium (without Bonferroni correction).

M13-FAM (5′-FAM-TGTAAAACGACGGCCAGT-3′). PCR was conducted in 10-μL reactions with 1× GeneAmp Gold buffer (ABI), 0.2 mM each dNTP, 2.5 mM $MgCl_2$, 0.05 μM unlabelled forward primer, 0.4 μM labelled M13 or T7 primer, 0.4 μM reverse primer, 0.25 U Ampli*Taq* Gold polymerase (ABI), and 10 ng DNA. Conditions were 95°C for 7 min; 15 cycles of primer-specific annealing temperature (Table 1) for 30 s, 72°C for 30 s, and 95°C for 30 s; 17 cycles of 54°C for 30 s, 72°C for 30 s, and 95°C for 30 s; and 60°C for 15 min. To reduce plus-A problems for Fmin05, we changed the 60°C final extension from 15 min to 45 min. Products were run on an ABI PRISM 3130XL, calling alleles manually in GeneMapper (ABI). We used Micro-Checker (van Oosterhout *et al*. 2004) to test for null alleles and large-allele dropout, and GenePop (Raymond & Rousset 1995) to test for gametic disequilibrium and departures from Hardy–Weinberg expectations.

Of 28 primer pairs, one would not amplify, one had unresolvable stutter, and eight were monomorphic in an initial screen of seven individuals. Thus, 18 loci (Table 1) were screened on 23 breeding individuals from a single population: 12 males and 11 females, but only a single member of each breeding pair due to the greater-than-random relatedness between mates in this population (Cohen & Dearborn 2004). There was no evidence of sex linkage. No loci exhibited large-allele dropout, but three loci had an excess of apparent homozygotes. Oosterhout null allele frequency and Weir & Cockerham (1984) F_{IS} values were $r = 0.2354$ and $F_{IS} = 0.4793$ for Fmin05; $r = 0.2015$ and $F_{IS} = 0.4797$ for Fmin09; and $r = 0.1382$ and $F_{IS} = 0.2982$ for Fmin15. However, all 23 DNA templates produced at least one size-appropriate amplicon for these three loci — that is, there was no homozygous null individual. It is unclear whether these three loci truly have null alleles or, instead, exhibit heterozygote deficiency for other reasons. Genotype frequencies of the remaining loci met Hardy–Weinberg expectations, but one pair of loci, Fmin12 and Fmin18, exhibited gametic disequilibrium. Overall, we thus found 14 moderately polymorphic loci that should be useful for studies of relatedness, and three others that might be used cautiously in some applications.

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