Conservation Genet Resour (2009) 1:159–162
DOI 10.1007/s12686-009-9038-3

TECHNICAL NOTE

Isolation and characterization of microsatellite loci from blue-footed boobies (*Sula nebouxii*)

Brant C. Faircloth · Alejandra Ramos · Hugh Drummond · Patricia Adair Gowaty

Received: 29 May 2009 / Accepted: 2 June 2009 / Published online: 23 June 2009

Abstract Blue-footed boobies (*Sula nebouxii*) are socially monogamous, colonial seabirds exhibiting intra-specific nest parasitism and extra-pair copulations. Prior DNA fingerprinting assays failed to detect extra-pair offspring in the nests of congeners, and the rate of intra-specific nest parasitism has not been estimated using molecular techniques. We describe the development and characterization of 11 microsatellite DNA loci, tested using 31 individuals collected on Isla Isabel, Nayarit, México. The number of alleles per locus ranged from three to 22, averaging seven; total exclusionary power of the microsatellite panel was 0.99; no loci deviated from Hardy-Weinberg equilibrium; and we did not detect linkage disequilibrium following Bonferroni correction. This microsatellite panel will facilitate future studies of nest parasitism and extra-pair paternity in blue-footed boobies.

Keywords Microsatellites · SSRs · Blue-footed booby · *Sula nebouxii* · Sulidae

B. C. Faircloth (✉) · P. A. Gowaty
Department of Ecology and Evolutionary Biology, University of California, 621 Charles E. Young Drive South, Los Angeles, CA 90095, USA
e-mail: brant@ucla.edu

A. Ramos · H. Drummond
Departamento de Ecología Evolutiva, Instituto de Ecología, Universidad Nacional Autónoma de México, AP 70-275, México DF 04510, México

P. A. Gowaty
Institute of the Environment, University of California, Los Angeles, CA 90095, USA

P. A. Gowaty
Smithsonian Tropical Research Institute, Unit 0948, AP AA 34002-0948 Panama City, Republic of Panama

Blue-footed boobies (*Sula nebouxii*) are colonial seabirds that nest annually on small Pacific Ocean islands (Nelson 1978). They reproduce several times during their lifetime of up to 20 years, forming pair bonds lasting 1–5 years (Torres and Drummond, unpublished data). Female boobies lay clutches of 1–3 eggs and provide parental care jointly with males until chicks fledge at approximately 90 days. Though socially monogamous, both sexes perform consensual, extrapair copulations that peak in frequency during the female's presumed fertile period (Osorio-Beristain and Drummond 1998; Perez-Staples and Drummond 2005) and some females lay eggs in the nests of neighbors [intraspecific nest parasitism (INP)]. Females also lay eggs in the nests of their male extra-pair partners [quasi nest parasitism (QNP)]. Observation revealed consistently hostile responses by incubating (host) females to INP and flexible responses by males to QNP-ranging from egg destruction to acceptance (Osorio-Beristain et al. 2005).

While the occurrence of extrapair and unrelated chicks in *S. nebouxii* nests is plausible, it remains unproven. Doubts arise because fingerprinting analyses failed to detect extrapair chicks in all congeners tested: nazca (*Sula granti*, Anderson and Boag 2006), masked (*Sula dactylatra*) and brown (*Sula leucogaster*) boobies (Baumgarten et al. 2001).

Understanding the evolution of extra-pair mating, mate-guarding and nest parasitism in blue-footed boobies, as well as violent conflict between broodmates (Drummond et al. 1986; Gonzalez-Voyer et al. 2007), requires assignment of genetic parentage to and estimation of relatedness between brood mates. Mendelian inheritance, combined with high levels of polymorphism, makes microsatellite loci ideal for these purposes and for analyses of small and isolated populations, like blue-footed boobies (Hedrick 1999; Selkoe

and Toonen 2006). In this manuscript, we describe the isolation and characterization of 11 microsatellite loci, which we tested using 31 *S. nebouxii.* These are the first microsatellites characterized in members of the genus *Sula*.

We developed a double-enriched microsatellite library following Glenn and Schable (2005) using DNA purified (5-Prime ArchivePure Blood Kit) from blood collected from a female blue-footed booby on Isla Isabel, Nayarit, México. We modified the enrichment protocol of Glenn and Schable (2005) using MyOne C1 streptavidin beads (Invitrogen) with 2X (10 μM Tris HCl, pH 7.5; 1 μM EDTA; 2 M NaCl; 0.2% Tween 20) and 1X B&W Buffer (Invitrogen) during the enrichment steps. From this library, we selected 285 positive (white) colonies using the β-galactosidase gene and bi-directionally sequenced colony PCR products of 500–1,200 base pairs using 1/16th BigDye [v3.1, Applied Biosystems (ABI)] sequencing reactions and an ABI PRISM 3730*xl* sequencer. We aligned and edited sequences and assembled 179 contigs using Sequencher 4.2 (Gene Codes Corp.). Prior to primer design, we tested for sequence homology using BLAST (Altschul et al. 1990).

Using MSATCOMMANDER (Faircloth 2008), we located microsatellite repeat arrays within 68 contigs, designed 45 primers, and applied 5′-tags (CAG or M13R) to primer pairs for subsequent polymorphism testing (Boutin-Ganache et al. 2001; Glenn and Schable 2005). We reviewed contigs containing repeats for which the automated software indicated primer design errors, and we manually designed primers for these regions ($N = 15$) using Oligo 6.0 (Molecular Biology Insights) and the 5′-tagging approach. We added GTTT "pigtails" to the 5′ end of each primer lacking either CAG or M13R tag to facilitate the addition of adenosine by *Taq* polymerase (Brownstein et al. 1996).

We tested 60 primer pairs for amplification using phenol-chloroform purified DNA collected from three blue-footed boobies on Isla Isabel during 1994. We performed PCR amplifications in 10 μL volumes using ABI 9700 thermal cyclers. Concentrations for reactions were 10 mM Tris pH 8.4, 50 mM KCl, 0.5 μM "pigtailed" primer, 0.05 μM CAG or M13-reverse (M13R) tagged primer (CAG or M13R + primer), 0.45 μM dye labeled tag (Dye + CAG or M13R), 1.5 mM $MgCl_2$, 0.5 mM dNTPs, 0.5 U AmpliTaq Gold DNA Polymerase (ABI), and 20 ng DNA. We labeled M13R and CAG universal primers with VIC, NED, FAM, or PET fluorescent dyes (ABI). We performed reactions using a touchdown thermal cycling program (Don et al. 1991), and we evaluated each primer at a starting annealing temperature of 60°C. We used the following cycling parameters: 1 cycle of 95°C for 5 m; followed by 20 cycles of 95°C for 20 s, 60°C for 30 s minus 0.5°C per annealing cycle, and 72°C for 90 s; followed by 20 cycles of 95°C for 20 s, 50°C for 30 s, 72°C for 90 s; and a final extension period of 10 m at 72°C. We scored each amplicon using an ABI Prism 3730*xl* DNA Sequencer in combination with LIZ600 fluorescent size standard (ABI), GeneMapper 4.0 Software (ABI) and the Local Southern size calling method.

Based on the performance of primers during the initial test, we selected 18 primer pairs for subsequent optimization and polymorphism testing. The primers we did not select for additional testing were monomorphic ($N = 25$, 78%) or failed to amplify ($N = 7$, 22%) during the initial test. We optimized and screened primers using phenol-chloroform purified DNA collected from 31 individuals sampled on Isla Isabel and amplification conditions identical to those presented above. When visual assessment of resulting peak morphologies indicated that increased annealing temperatures might improve amplification quality, we also genotyped and scored loci using a starting annealing temperature of 65°C (−0.5°C step). We selected the starting annealing temperature resulting in the most precise peaks without inducing allelic dropout, and we removed any loci from the candidate set yielding ambiguous peaks or inconsistent results.

We calculated observed (H_O) and expected (H_E) heterozygosity and polymorphic information content (PIC) using Cervus 3.0 (Marshall et al. 1998; Kalinowski et al. 2007), and we tested for deviations from Hardy-Weinberg equilibrium (HWE) and evaluated genotypic linkage disequilibrium (LD) using Genepop (Raymond and Rousset 1995). We conducted *a posteriori* Bonferroni correction (Rice 1989) for each analysis consisting of multiple, concurrent statistical tests (HWE and LD).

Table 1 presents the characteristics of 11 primer pairs amplifying microsatellite loci in blue-footed boobies. The number of alleles ranged from three to 22, averaging seven. No loci deviated from HWE, and we did not detect LD following Bonferroni correction. Total exclusionary power with both parents unknown was 0.99.

Incorporation of the MyOne C1 Streptavidin beads to the process did not appear to increase the efficiency of enrichment for fragments containing microsatellite repeats (38%) when compared to the older M-280 beads ($\approx$50–60%, Crawford et al. 2009, BC Faircloth *unpublished data*). This observation may be species-specific or a general indication that the M-280 beads are more efficient for the purpose of microsatellite enrichment. Others (S Lance and T Glenn, University of Georgia, *personal communication*) have observed reduced enrichment efficiencies using MyOne C1 beads and B&W hybridization buffer.

The microsatellite loci identified here will serve as a useful resource for future studies of blue-footed boobies requiring a panel of polymorphic DNA markers.

Table 1 Characteristics of 11 primer pairs amplifying microsatellite DNA loci in blue-footed boobies (*Sula nebouxii*) sampled from Isla Isabel, Nayarit, México

Locus	Sequence	Accession	Anneal	N	A	Range	Repeat	HO	HE	PIC
BOOB-RM2-F07 U	GTTTGCTAATCCCCTGGTGCAAGTG	FJ587350	60	30	3	389–397	(CT)^8	0.60	0.59	0.50
BOOB-RM2-F07 L	***GGAAACAGCTATGACCAT***ACATCCGGTGCTCAGTTGTG									
BOOB-RM3-D07 U	GTTTGGCCTACTGCCATCCAGGG	FJ587394	60	31	15	325–399	(AAAG)^35… (AAAG)$^{\wedge 5}$	0.87	0.90	0.87
BOOB-RM3-D07 L	***CAGTCGGGCGTCATC***AGTTGCTCATAAACAGGCTGGTATTC									
BOOB-RM3-F11 U	GTTTAGAGGGTGTAGGCTGAAATC	FJ587411	60	31	22	281–420	(CTTTT)^41	0.97	0.95	0.94
BOOB-RM3-F11 L	***CAGTCGGGCGTCATC***ATTCTCTAGGCAAGGATCAATACC									
BOOB-RM4-A08 U	GTTTCCTGAAAGTCCTTTATTGTGGAGGG	FJ587430	60	31	3	398–406	(AG)^6…(AGAT)^7…(ACAG)^6	0.58	0.54	0.42
BOOB-RM4-A08 L	***CAGTCGGGCGTCA***TCAGCCTAGCACAGGGGTG									
BOOB-RM4-B03 U	***CAGTCGGGCGTCATCA***TGGACCAGCAGCTAAGGC	FJ587372	65	31	3	385–389	(AT)^6…(ATGT)^5	0.29	0.37	0.32
BOOB-RM4-B03 L	GTTTAGGTGAGTGGCTGGGTGG									
BOOB-RM4-C03 U	***CAGTCGGGCGTCATCA***TGGTCAAACAGTAACATGACAAGGAC	FJ587442	60	31	11	336–432	(AAAG)^29	0.84	0.80	0.77
BOOB-RM4-C03 L	GTTTCCAGTAACAACCACTCAGTTCAGG									
BOOB-RM4-D07 U	***CAGTCGGGCGTCATCA***GCCACCCTCAAGCCATTCC	FJ587311	65	31	3	213–239	(GGAT)^13	0.52	0.45	0.36
BOOB-RM4-D07 L	GTTTCCAACAGTTCTGCTGCTCAC									
BOOB-RM4-E03 U	GTTTGAGCTGTGTTCCAAAGTTGCTC	FJ587457	65	31	3	313–317	(ATCT)^7…(CT)^10…(ATCC)^4	0.39	0.36	0.33
BOOB-RM4-E03 L	***CAGTCGGGCGTCA***TCATTTCTGTCCTGGTTAAAATTCCC									
BOOB-RM4-E10 U	***CAGTCGGGCGTCA***TCAAGTGCGCAGGGAAAACAG	FJ587461	65	31	5	251–267	(ATCT)^15…(ATCT)^4	0.65	0.62	0.53
BOOB-RM4-E10 L	GTTTGGCTGGCAGGGCTTTGTG									
BOOB-RM4-F11 U	GTTTGTTGCTCAGGACATGCATCC	FJ587469	60	31	5	287–307	(AATAG)^12	0.61	0.61	0.54
BOOB-RM4-F11 L	***CAGTCGGGCGTCATC***ACAAAGTCCAATTGACAGCCAG									
BOOB-RM4-G03 U	***CAGTCGGGCGTCATCA***GGCAGCACTCAAGCTGAAGG	FJ587472	65	30	5	167–197	(ATCCT)^11	0.67	0.64	0.56
BOOB-RM4-G03 L	GTTTCTCAAGGTAGGGCAGGGTC									

Sequences used to introduce sites for the universal, fluorescent tags are in bold italics. Underlined bases indicate sharing of nucleotides between the CAG tag (5′ - CAGTCGGGCGTCATCA - 3′), the M13R tag (5′ - GGAAACAGCTATGACCAT - 3′), or the locus-specific primer binding site and the GTTT "pigtail". *U* upper primer; *L* lower primer; *N* number of individuals successfully genotyped at each locus; *A* number of alleles; *HO* observed heterozygosity; *HE* expected heterozygosity; *PIC* polymorphic information content

Acknowledgements Financial support was provided by: Universidad Nacional Autónoma de México; a UC-Mexus Small Grant to PAG and HD; and setup funds for PAG from the University of California, Los Angeles. We conducted blue-footed booby blood collection on Isla Isabel, Nayarit, Mexico under permits granted to HD by SEDESOL and SEMARNAT as part of his long-term research program on the island. The authors thank Tania del Rio Albrechtsen, Alejandra Nuñez and Sergio Ancona for collecting blood, and the Mexican Navy, San Blas fishermen and Isla Isabel staff for providing transportation and logistical support. BCF thanks Jonathan Drury, Kevin Tan and Alexandra Title for their assistance during the microsatellite enrichment process. PAG and HD acquired funding for this research and provided the physical and intellectual resources for its pursuit, BCF and AR conducted the data collection, BCF conducted the data analysis, and BCF and AR wrote the manuscript.

References

Altschul SF, Gish W, Miller W et al (1990) Basic local alignment search tool. J Mol Biol 215:403–410

Anderson DJ, Boag PT (2006) No extra-pair fertilization observed in nazca booby (*Sula granti*) broods. Wilson J Ornithol 118: 244–247

Baumgarten MM, Kohlrausch AB, Miyaki CY et al (2001) DNA fingerprinting and parentage in masked (*Sula dactylatra*) and brown (*S.* leucogaster) boobies. Ornitol Neotrop 12:319–326

Boutin-Ganache I, Raposo M, Raymond M et al (2001) M13-tailed primers improve the readability and usability of microsatellite analyses performed with two different allele-sizing methods. BioTechniques 31:24–28

Brownstein MJ, Carpenter JD, Smith JR (1996) Modulation of non-templated nucleotide addition by *Taq* polymerase: primer modifications that facilitate genotyping. BioTechniques 20: 1004–1010

Crawford NG, Peters MB, Hagen C et al (2009) Polymorphic microsatellite loci from Sprague's pipit (*Anthus spragueii*), a grassland endemic passerine bird. Mol Ecol Res 9:315–317

Don RH, Cox PT, Wainwright BJ et al (1991) Touchdown PCR to circumvent spurious priming during gene amplification. Nucleic Acids Res 19:4008

Drummond H, González E, Osorno JL (1986) Parent-offspring cooperation in the blue-footed booby (*Sula nebouxii*): social roles in infanticidal brood reduction. Behav Ecol Sociobiol 19:365–372

Faircloth BC (2008) MSATCOMMANDER: detection of microsatellite repeat arrays and automated, locus-specific primer design. Mol Ecol Res 8:92–94

Glenn TC, Schable NA (2005) Isolating microsatellite DNA loci. In: Zimmer EA, Roalson EH (eds) Methods in enzymology 395, molecular evolution: producing the biochemical data, Part B. Academic Press, San Diego, CA

Gonzalez-Voyer A, Székely T, Drummond H (2007) Why do some siblings attack each other? Comparative analysis of aggression in avian broods. Evol Int J org Evol 61:1946–1955

Hedrick PW (1999) Perspective: highly variable loci and their interpretation in evolution and conservation. Evol Int J org Evol 53:313–318

Kalinowski ST, Taper ML, Marshall TC (2007) Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. Mol Ecol 16:1006–1099

Marshall TC, Slate J, Kruuk LEB et al (1998) Statistical confidence for likelihood-based paternity inference in natural populations. Mol Ecol 7:639–655

Nelson B (1978) The Sulidae: gannets and boobies. Oxford University Press, Oxford

Osorio-Beristain M, Drummond H (1998) Non-aggressive mate guarding by the blue-footed booby: a balance of female and male control. Behav Ecol Sociobiol 43:307–315

Osorio-Beristain M, Perez-Staples D, Drummond H (2005) Does booby egg dumping amount to quasi-parasitism? Ethology 112:625–630

Perez-Staples D, Drummond H (2005) Tactics, effectiveness and avoidance of mate guarding in the blue-footed booby (*Sula nebouxii*). Behav Ecol Sociobiol 59:115–123

Raymond M, Rousset F (1995) GENEPOP (version 1.2) population genetics software for exact tests and ecumenicism. J Hered 86:248–249

Rice WR (1989) Analyzing tables of statistical tests. Evol Int J org Evol 43:223–225

Selkoe KA, Toonen RJ (2006) Microsatellites for ecologists: a practical guide using and evaluating microsatellite markers. Ecol Lett 9:615–629