Conservation Genet Resour (2012) 4:247–250
DOI 10.1007/s12686-011-9517-1

TECHNICAL NOTE

Identification of seventeen microsatellite markers for conservation genetic studies of the endemic anemonefish, *Amphiprion mccullochi*

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Received: 25 August 2011 / Accepted: 28 August 2011 / Published online: 14 September 2011

Abstract Endemic species at remote islands have a high risk of extinction because they often exhibit ecological, biological and genetic traits that make them particularly vulnerable to disturbances. McCulloch's anemonefish (*Amphiprion mccullochi*) is endemic to a few oceanic reefs off Australia's east coast and is an habitat specialist. Using 454 shotgun sequencing, we developed and evaluated primers for seventeen independent microsatellite loci to reveal gene flow, population genetic structure and genetic diversity across three isolated reefs. Observed and expected heterozygosities ranged from 0.556 to 1 and 0.543 to 0.907, respectively, in 30 Lord Howe Island individuals. When cross tested with the close relative, *Amphiprion akindynos*, amplification was successful with high levels of polymorphism. These loci will therefore be useful in studies of *A. mccullochi, A. akindynos* and possibly other closely related anemonefish.

Keywords Coral reef fish · Island endemics · Genetic diversity · Cross-species amplification

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Remote offshore islands usually contain high numbers of endemic species (Gillespie et al. 2008), which makes them a high conservation priority (Gillespie et al. 2008). Conserving endemics is challenging because of their high extinction risk due to small population size, ecological specialisation, limited dispersal and low genetic diversity (McKinney 1997; Frankham 1998). The McCulloch's anemonefish (*Amphiprion mccullochi*) has arguably the smallest geographic range size of any anemonefish (endemic to offshore Australian waters, Coleman 1980) and is a habitat specialist living on a single host anemone, *Entacmaea quadricolor* (Fautin and Allen 1992). To date, gene flow, population genetic structure and genetic diversity of *A. mccullochi* have not been examined. This paper describes the development of 17 polymorphic microsatellite markers for *A. mccullochi* using 454 shotgun pyrosequencing on a Roche GS-FLX (Australain Genome Research Facility, AGRF, Brisbane, Australia).

Genomic DNA was extracted using a Qiagen Gentra Puregene extraction protocol and was RNAse treated. The DNA (1 μg) was shotgun sequenced on 12.5% of a Roche GS-FLX (Australian Genome Research Facility, AGRF, Brisbane, Australia) following Gardner et al. (2011). The resulting sequences (totalling 114,272 reads, average sequence length of 350, total GC content of 40.08%) were screened for pure di to hexanucleotide microsatellite loci with six or more repeats. QDD v1.3 (Meglecz et al. 2009) identified 7,224 (6.32% of sequences) microsatellite loci,

of which 923 (12.78% of loci found) were successfully designed. Of these, primers for the best of the best 24 loci were synthesised with a 5′ 17 base pair lambda tag (5-GGTGGCGACTCCTGGAG-3) to enable indirect fluorolabelling (Shimizu et al. 2002). Loci were initially tested for amplification success and specificity in eight individuals using Type-it microsatellite PCR kit (Qiagen). Individual amplifications were performed in 10 µl reactions, containing 1× Type-it Multiplex PCR Master Mix (Qiagen, Doncaster/Australia), 20–50 ng template, and 0.2 µM each primer (forward and reverse). Indirectly labelled reactions contained a tailed forward primer and a reporter primer (5′ labelled with fluorescent dye modification HEX, TET or FAM) at a 1:4 ratio (total = 0.2 µM). All primers were tested and optimised using a Bio-Rad C1000 Thermal Cycler (Table 1) with an initial denaturation of 94°C for 3 min followed by 28 cycles of 94°C for 40 s, 58°C for 40 s and 72°C for 40 s followed by 5 min at

Table 1 Details for seventeen *Amphiprion mccullochi* microsatellite loci

Locus	Repeat motif	Primer sequence 5′–3′	Size range	N	N_a	H_o	H_e	pHWE	PIC	Genbank
Amphiprion mccullochi										
Am1[1A]	$(TG)_{12}$	[TET] ACAAAGCCTTCATGTGGGTC CGCAAGTGTTGCCTCATAGA	108–112	27	2	0.556	0.543	0.007	0.444	JN604050
Am4[2A]	$(GT)_{13}$	[TET] AGCTGGTTGGGTGTTACCTG ATGGCTTCAGTCTGCTGGTT	120–156	30	11	0.900	0.819	0.901	0.802	JN604051
Am5[2C]	$(AC)_{20}$	[FAM] CCACTAGAGGCTCCCTGTTG CGAGCATGAGCGAATGTATG	77–95	30	9	0.800	0.844	0.910	0.827	JN604052
Am6[1C]	$(TGT)_{10}$	[HEX] AGCAGAGAGGAAAGAAGGGC CAAGTGCCTGGCAGAAGATT	262–274	27	4	0.667	0.620	0.724	0.542	JN604053
Am7[3A]	$(ATG)_{19}$	[TET] TGTCGCTACGACAGACTGCT GCATGAGTGATTGGACCCTA	86–128	28	10	0.786	0.851	0.972	0.834	JN604054
Am9[1B]	$(TTA)_{17}$	[FAM] TGCTGCACTCTGTCTATTTTGT GTGACTGAAGGCAAGGCAAT	151–202	27	13	1.000	0.907	0.203	0.899	JN604055
Am10[4C]	$(ACAG)_{16}$	[HEX] GGAAGCAGCAATAAAGACGC AGAGACGCCTGATGGTGAGT	286–302	30	5	0.600	0.635	0.981	0.586	JN604056
Am11[4B]	$(CTAT)_{12}$	[FAM] ATTCCCCGACGGAGAGTAGT TGTCGCTTTGTGACACCTTC	124–172^	29	7	0.621	0.782	0.127	0.751	JN604057
Am12[3C]	$(TTCA)_{20}$	[HEX] ATGAGCAGCTTTGACGGAAT ACCTACATGGTTGGAGCCTG	182–210	28	8	0.821	0.833	0.830	0.811	JN604058
Am14[2C]	$(GATG)_{13}$	[HEX] CAGCAGCCTCAAGTGACTGT GCAGCATTCTCACACACCAC	170–198	28	8	0.750	0.763	0.898	0.741	JN604059
Am15[5A]	$(GTCT)_{12}$	[TET] ACTAGGCTCAGAGCAGGGTC CAAGTCAATCAAAGCAGCCA	100–160	27	11	0.889	0.875	0.647	0.863	JN604060
Am17[6A]	$(AATA)_{14}$	[TET] GGCTGTCTGGGATGAGATGT TGTTCTGCAGATGGACTGTTTT	105–138	27	9	0.593	0.823	0.219	0.8	JN604061
Am18[5B]	$(TGAA)_{19}$	[FAM] TGGTCCTAGCAGCTGTCTGT GGCTACATCTGCAACGACAA	87-–119	28	8	0.929	0.790	0.809	0.76	JN604062
Am19[5C]	$(TCCA)_{12}$	[HEX] CTGTAATGAATCCAAGGAGCTG TGGATAATGAAGAAATGGATGG	102–146^	27	13	0.704	0.853	0.346	0.837	JN604063
Am21[5B]	$(TTCTA)_{11}$	[FAM] TCTCGTCTGGTGTTGACTGC CAATGGCTTTACTTTTCTCTGC	95–140	27	9	0.778	0.800	0.446	0.774	JN604064
Am22[6C]	$(TGGGTC)_6$	[HEX] GCCGAATATGCCGTACAACT TATCTTCAGACCCACCTGGC	110–155	27	10	0.889	0.843	0.502	0.824	JN604065
Am24[3B]	$(TCAGGA)_6$	[FAM] CTGCTGGATCAGGGTTAGGA ACCATGCCCAGGTACTGTCT	141–165	28	4	0.571	0.554	0.192	0.475	JN604066

T_A, annealing temperature, *N,* sample size, N_A, number of alleles, H_O, observed heterozygosity, H_E, expected heterozygosity, *P* HWE, Hardy–Weinberg equilibrium significance value at $P < 0.05$ after FDR correction, *PIC*, polymorphic information content. ^, locus may have null alleles

[1A,B,C] *A. mccullochi* Multiplex corresponding to group 1 with three loci (A, B and C)

72°C in a Bio-Rad C1000 Thermal Cycler (Bio-Rad, Australia). PCR products were column purified using an Ammonium Acetate protocol. Genotypes were run on an Amersham Biosciences Megabase Capillary Sequencer with a 400 bp size standard and scored using Fragment Profiler 1,2 (Amersham, Buckinghamshire, England).

Primer pairs for 17 loci reliably amplified products and were polymorphic. Directly-labelled forward primers (HEX, TET or FAM) were synthesised for the 17 polymorphic *A. mccullochi* loci with PCR multiplexing of three loci (Table 1). Loci were genotyped in directly labelled multiplex reactions in 30 *A. mccullochi* individuals from LHI. DNA extraction protocol and PCR conditions as described above. GENALEX 6 (Peakall and Smouse 2006) was used to determine the number of alleles, observed and expected heterozygosities and, conformation to Hardy- Weinberg Equilibrium (HWE). CERVUS 3.0 (Kalinowski et al. 2007) was used to calculate polymorphic information content (PIC) for each locus, GENEPOP 4.0.10 (Rousset 2008) was used to test linkage disequilibrium and, MICRO-CHECKER 2.2.3 (Van Oosterhout et al. 2004) was used to detect the presence of null-alleles.

All seventeen loci were in HWE after FDR correction and less than 2% (4 out of 272 of locus pairs had significant linkage disequilibrium) were detected after FDR correction (Benjamini and Hochberg 1995): Am1/Am5, Am1/Am24, Am11/Am14 and Am12/Am 14 (Table 1). In addition, null alleles were suggested for only two loci (Am11, Am19) due to homozygote excess. The *A. mccullochi* markers displayed a high allelic richness (mean $N_A = 8.35 \pm 0.73$, range 2–13) and high levels of expected heterozygosity ($H_E = 0.773 \pm 0.027$, range 0.543–0.907). The polymorphic information content (PIC) for the combined microsatellite loci was 0.724 indicating a high level of discrimination between individuals making them useful for studies of connectivity and population genetic structure in this species.

Loci were cross-tested on fourteen different individuals of *A. akindynos* from Lizard Island (Australia). All loci amplified successfully and were polymorphic with 4–11 alleles per locus (Table 2). The newly developed primers reported here will provide a useful tool to examine gene flow, population genetic structure and genetic diversity in the endemic *A. mccullochi* and potentially other related anemonefish species.

Table 2 Cross species amplification of *Amphiprion mccullochi* microsatellite loci on the test species *A. akindynos*, number of successful amplifications, amplicon size range and number of alleles (N_a)

Locus	Amplification	Size range (bp)	N_a
Am4	14/14	118–166	10
Am5	14/14	71–113	11
Am6	12/14	262–274	4
Am7	12/15	86–134	10
Am9	13/14	151–223	15
Am10	13/14	286–298	4
Am11	14/14	116–148	6
Am12	13/14	166–222	11
Am14	13/14	162–198	7
Am15	14/14	100–144	11
Am17	13/14	100–144	10
Am18	13/14	108–141	7
Am19	13/14	99–139	11
Am21	14/14	94–142	11
Am22	14/14	90–160	11
Am24	13/14	115–180	4

Acknowledgments We are grateful for valuable support and assistance provided by Sallyann Gudge and Ian Kerr, the Lord Howe Island Board, Envirofund Australia (Natural Heritage Trust) and the Lord Howe Island Marine Park. We also thank L. Bay for the *Amphiprion akindynos* samples.

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