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TECHNICAL NOTE

Development of microsatellite markers from four Hawaiian corals: *Acropora cytherea*, *Fungia scutaria*, *Montipora capitata* and *Porites lobata*

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Abstract Comprehensive population genetic studies of coral communities are comparatively rare, because of the scarcity of population genetic markers. The Hawaiian archipelago offers a unique perspective into understanding the population genetic structuring of this ecologically important group of organisms. Here we report the development of microsatellite marker libraries from holobiont extracts of four corals: *Acropora cytherea* ($n = 50$), *Fungia scutaria* ($n = 118$), *Montipora capitata* ($n = 140$) and *Porites lobata* ($n = 149$). Blast searches indicate that these libraries contain microsatellites from both the coral host and *Symbiodinium* endosymbionts from each coral. In addition, we also present redesigned primers for the nuclear coding region (atpsβ) for use in *M. capitata.* We report testing and optimization for seven of these microsatellites from *A. cytherea*, and eight microsatellites and the atpsβ locus from *M. capitata.* Using 25 individuals per species collected from each French Frigate Shoals (FF) and Johnston Atoll (JO), the number of alleles per locus ranged from 2 to 9. Expected heterozygosities ranged from 0.38 to 0.85 and 0.08 to 0.87 for *A. cytherea* and *M. capitata*, respectively. We expect that these libraries will be a valuable resource and provide additional useful microsatellite markers for both the coral host and zooxanthellae.

Keywords Scleractinian · Zooxanthellae · *Symbiodinium* · Connectivity · Population genetics · Phylogeography

Spanning 2,600 km, and ranging in age from a few hundred thousand to more than 60 million years, the isolated volcanic archipelago of Hawai'i offers a unique opportunity to understand population connectivity in the marine environment. For sessile benthic marine organisms, the larval phase of the life history is the only part of the life cycle that allows for gene flow between populations, but it is difficult to track miniscule larvae across oceanic scales (Levin 2006). In order to gain insight into the historical patterns of coral colonization of the archipelago, microsatellite markers are incredibly useful tool for understanding population genetic structuring and inferring patterns of connectivity (Selkoe and Toonen 2006).

Total genomic DNA of *Acropora cytherea*, *Fungia scutaria*, *Montipora capitata* and *Porites lobata* were extracted in parallel according to the chloroform extraction protocol described in Concepcion et al. (2006). DNA from *A. cytherea* and *P. lobata* were digested with restriction enzyme BstU I, while DNA from *F. scutaria* and *M. capitata* were digested with restriction enzyme Rsa I. Using a slightly modified protocol from Glenn and Schable (2005), double stranded linkers were ligated to the 5′ end of restriction fragments. Enrichment probes consisted of two mixtures of 3′ biotinylated probes—Mix I: $(AAGC)_5$, $(AACC)_5$, $(AACG)_5$, $(ATCC)_5$, $(AAGG)_5$; Mix II: $(ATC)_8$, $G(AGG)_6$, $G(CCG)_5$, $(AAT)_{10}$, $(AAG)_8$, $(ACT)_8$, $(AAC)_8$,

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Table 1 Locus name, repeat motif, primer sequences, annealing temperature (T_a) and approximate size of expected product for one nuclear coding region and eight microsatellites for *M. capitata* and seven microsatellites from *A. cytherea*

Taxa	Locus	Motif	Primer sequence (5′-3′)	T_a (°C)	Size (bp)
M. capitata	atpsb		F: TGA TTG TGT CTG GTG TAA TCA GC	53	200–201
			R: CGG GCA CGG GCG CCG GGG GGT TCG TTC AT		
	Mc0004	$(AATC)_9$	F: **T3**—TGA AAT AAG CAG GAT CCA TGT G	55	389–414
			R: AGG TAA ATG CCA GAA TTG GAA A		
	Mc0067	$(TGAG)_{13}$	F: **T1**—AAG AAC ACC AAA CAA CCG AAC T	55	215–255
			R: GGT TAG CGC TCT TGT GCT AAA T		
	Mc0163	$(TCAC)_{12}$	F: **T4**—TTA TTT CTC GTG TAT CGC CCT T	55	239–309
			R: AGA CAG AGC GGT TGG TGT AAA T		
	Mc0701	$(TTCC)_{10}$	F: **T2**—CAG TGC GAG AAC GAC TAG AGA C	55	223–251
			R: AAA ATG ACA AGC ATG TTG GTG T		
	Mc0797	$(CCT)_6$	F: **T3**—CTT TCA AGG TGT TGA TGC CAT A	55	302–308
			R: GGC ACA TCA TGA GAA CAT CAG T		
	Mc0872	$(AAC)_7$	F: **T4**—TGG CCA GCT TAG TGT TAG TTG A	55	388–402
			R: GTT CTT GTA TTT GAC TTG CCC C		
	Mc0903	$(ATG)_9$	F: **T1**—CAT CTA GAA TTA GCG GGA TGC T	55	281–297
			R: CAG AAG TTC CGA CTT TCG ACT T		
	Mc0947	$(ATG)_7$	F: **T2**—TAG GGG TAA GGA AGG TTG AAC A	55	304–336
			R: AAG GGA AAC GGT AAG ACA TGA A		
A. cytherea	Ac0753	$(CAT)_7$	F: **T2**—GCG AAA GAG ATT CCG TTA GAG A	55	294–306
			R: AAT GGG CTC AAT TTC CCT TAA T		
	Ac0808	$(TGA)_6$	F: **T3**—TTT TAG CTG GAG ATG ACG ATG A	55	184–190
			R: TAA CAG GAA AAG GGA AAC AAG G		
	Amil2_002†	$(TG)_{10}$	F: **T1**—ACA AAA TAA CCC CTT CTA CCT	55	140–153
			R: CTT CAT CTC TAC AGC CGA TT		
	Amil2_006†	$(CA)_4TA(CA)_4$	F: **T2**—CTT GAC CTA AAA AAC TGT CGT ACA A	55	115–119
			R: GTT ATT ACT AAA AAG GAC GAG AAT AAC TTT		
	Amil2_007†	$(TG)_7AG$	F: **T4**—TAA TGA GCA AAC TCA TTC ATG G	55	116–121
			R: CTT TTC CAA GAG AAG TCA AGA A		
	Amil2_022†	$(AC)_{10}$	F: **T4**—CTG TGG CCT TGT TAG ATA GC	55	109–131
			R: AGA TTT GTG TTG TCC TGC TT		
	Amil2_023†	$(AG)_7$	F: **T3**—GCA AGT GTT ACT GCA TCA AA	55	148–150
			R: TCA TGA TGC TTT ACA GGT GA		

T1: PET-5′-GGCTAGGAAAGGTTAGTGGC-3′; **T2**: 6-Fam-5′-TCATACATGTCTCTCAGCGTAAAC-3′; **T3**: NED-5′-ACCAACCTAGG AAACACAG-3′; **T4**: VIC-5′-GACTATGGGCGTGAGTGCAT-3′

$(ACG)_6$, $(ACC)_6$, $(AGC)_6$ (Toonen 1997). Fragments with sequences complementary to these probes were captured with Streptavidin M-280 Dynabeads (Invitrogen, Carlsbad, CA, USA). DNA enriched for these tri- and tetranucleotide microsatellite repeat motifs was ligated into T-tailed pZErO-2 plasmids using a Topo-TA cloning kit (Invitrogen). *Escherichia coli* α-select chemically competent cells (Bioline Inc., Springfield, NJ, USA) were transformed with the recombinant plasmids. Across all four species, 8,064 clones were picked using a VersArray Colony Picker (Biorad) and subsequently consolidated into and re-grown in 384 well sample plates. Colonies from the 384 well plates were then imprinted and grown directly on nitrocellulose membranes resting on LB agar plates and incubated overnight at 37°C. Following Toonen (1997), DNA was extracted and fixed on the membrane and was subsequently screened for microsatellite positive colonies by hybridization with the same biotinylated oligonucleotide probe mixtures as above with a modified colorimetric detection step using chemiluminescent CDP-star (#N7001S NEB Biolabs) and subsequent exposure to Kodak® X-Ray film. 1,423 colonies were identified as containing microsatellite repeats. 1,039 clones from *A. cytherea*, *F. scutaria* and *M. capitata* were amplified and submitted for sequencing at the Hawai'i Institute of Marine Biology EPSCoR Facility, while 384 clones from *P. lobata* were amplified and

Table 2 From each sampling location for each of eight microsatellites and one nuclear coding region isolated for *Montipora capitata* and two microsatellites isolated from *Acropora cytherea* plus five additional microsatellites isolated from congener *Acropora millepora*, we report sample size (N), number of alleles (N_a), observed heterozygosity (H_o); expected heterozygosity (N_e); inbreeding coefficient (F_{is}), P-values for tests of departure from HWE (P_{hwe}), and Brookfield-1 estimator of null alleles ($Null_{est}$). Significant values for P_{hwe} after correction with a false discovery rate $\alpha = 0.05$ shown in bold. Corrected α for *M. capitata* ($P < 0.006$) and *A. cytherea* ($P < 0.014$)

		Montipora capitata								*Acropora cytherea*							
		atpsb	Mc0004	Mc0067	Mc0163	Mc0701	Mc0797	Mc0872	Mc0903	Mc0947	Ac0753	Ac0808	Amil2_002[†]	Amil2_006[†]	Amil2_007[†]	Amil2_022[†]	Amil2_023[†]
FF	N	19	25	25	23	25	25	24	25	25	25	25	23	25	25	25	25
	N_a	6	4	8	7	5	2	6	4	3	5	4	9	3	4	7	2
	H_o	0.737	0.480	0.800	0.304	0.400	0.080	0.417	0.480	0.520	0.160	0.577	0.478	0.400	0.400	0.640	0.560
	H_e	0.804	0.631	0.801	0.801	0.580	0.078	0.479	0.500	0.530	0.521	0.669	0.851	0.431	0.520	0.734	0.470
	Fis	0.083	0.239	0.001	0.620	0.310	-0.021	0.130	0.041	0.018	0.693	0.138	0.438	0.072	0.231	0.128	-0.191
	P_{hwe}	0.498	0.110	0.391	**0.000**	0.117	1.000	0.544	0.445	0.510	**0.000**	0.201	**0.000**	0.032	**0.000**	0.409	0.408
	$Null_{est}$	0.026	0.086	-0.009	**0.269**	0.107	-0.003	0.036	0.007	-0.001	**0.232**	0.048	**0.193**	0.016	**0.073**	0.046	-0.068
JO	N	20	22	25	12	23	25	25	25	25	24	24	22	24	24	24	24
	N_a	7	8	8	6	3	2	5	4	3	3	3	7	2	6	5	2
	H_o	0.750	0.682	0.560	0.667	0.478	0.520	0.440	0.400	0.320	0.458	0.458	0.818	0.417	0.292	0.667	0.458
	H_e	0.723	0.715	0.867	0.768	0.541	0.507	0.388	0.484	0.358	0.377	0.582	0.794	0.496	0.785	0.735	0.510
	Fis	-0.036	0.048	0.548	0.152	0.131	-0.025	-0.119	0.210	0.120	-0.178	0.271	-0.030	0.191	1.690	0.102	0.112
	P_{hwe}	0.586	0.859	**0.000**	0.400	0.232	1.000	1.000	0.601	0.228	0.710	0.321	0.020	0.672	**0.000**	0.231	0.694
	$Null_{est}$	-0.026	0.010	**0.157**	0.040	0.033	-0.016	-0.044	0.051	0.023	-0.065	0.071	-0.024	0.047	**0.270**	0.031	0.027

[†] Van Oppen et al. (2007)

sequenced at the Huck Institute for the Life Sciences, Pennsylvania State University. The Staden-Troll pipeline (Martins et al. 2006) was used to identify tandem repeats. Primers were designed using Primer3 for each of 50, 118, 140, and 149 sequences from *A. cytherea*, *F. scutaria*, *M. capitata* and *P. lobata*, respectively. Primer sequences as well as the genus, species, bitscore and e-value for the top BLASTn hit (Altschul et al. 1990) can be found in Supplemental Table 1.

Due to time constraints and cost, primers were ordered and tested for only the first 50 putative loci from *M. capitata* and *A. cytherea.* Primers were screened with a low annealing temperature (48°C) PCR against DNA extracts from pure zooxanthellae cultures provided by RA Kinzie believed to represent clades A, B and C isolated from *Cassiopea* sp. (KB8), *Aiptasia pulchella* (HIAp) and *Montipora verrucosa* (Mv), respectively. They were subsequently screened against host genomic DNA with an annealing temperature of 55°C. We used the tailed three primer method described by Gaither et al. (2009). Tails were added to the 5′ end of all forward primers that successfully amplified host genomic DNA, but not symbiont DNA (Table 1). PCRs were as follows: each 10 µl reaction contained: 1 µl 10×NH_4 reaction buffer, 0.6 µl 50 mM $MgCl_2$, 0.4 µl 10 mM total dNTPs (2.5 mM each), 0.35 pmol tailed forward primer, 1.5 pmol reverse primer, 1.5 pmol oligonucleotide dye label, 2–25 ng of template DNA, 0.1 µl of Biolase polymerase (Bioline Inc.), and deionized water to volume. PCR amplification was performed on a BioRad MyCycler™ as follows: 95°C for 10 min (1 cycle), 94°C for 30 s, 55°C for 30 s, 72°C for 30 s (35 cycles), followed by a final extension of 72°C for 30 min (1 cycle).

Eight primer pairs for *Montipora capitata*, and two primer pairs for *Acropora cytherea* in addition to five primer pairs from van Oppen et al. (2007) successfully amplified polymorphic loci and were selected for further genotyping of samples (Table 1). Fragments were analyzed on an ABI 3130XL Genetic Analyzer at the Hawai'i Institute of Marine Biology and sized using Genemapper v4.0 and GS500LZ size standards (Applied Biosystems, Inc.). Arlequin 3.11 (Excoffier et al. 2005) was used to calculate genotypic disequilibrium, heterozygosity, and probability of departure from HWE. Each primer pair was initially screened for variability against 25 individuals sampled from each of French Frigate Shoals and Johnston Atoll (Table 2). The number of alleles for each locus ranged from 2 to 9 for both species (Table 2). No linkage was detected in either population for *A. cytherea*, and *M. capitata*. After controlling for false discovery rate (Benjamini and Yekutieli 2001), significant departures from HWE were detected in six out of 32 comparisons (Table 2). MicroChecker (van Oosterhout et al. 2004) detected no evidence of scoring errors, or large-allele dropout in either species, although possible null alleles were likely at each locus that showed significant departure from HWE in a population (Table 2).

Additionally, forward and reverse primers for ATP synthethase β (atpsβ) (Jarman et al. 2002) were redesigned for specificity in the genus *Montipora* with PCR conditions following Concepcion et al. (2008). This locus was also sequenced directly for each individual from each population ($n = 50$). Computational methods for determining phase of diploid sequence data is more cost effective and can be as accurate as cloning (Harrigan et al. 2008). Therefore, we used Phase (Stephens et al. 2001; Stephens and Donnelly 2003) as implemented in DnaSP (Librado and Rozas 2009) for determining gametic phases (Table 2).

The use of microsatellites to study coral population genetics is still in its infancy. We hope this library of both characterized, and untested microsatellite loci will provide a wealth of population genetic tools to aid studies both inside and outside of Hawai'i, in species of corals other than the four for which they were isolated, as well as providing possible microsatellite markers for the symbionts (*Symbiodinium* spp.)

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