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TECHNICAL NOTE

Isolation and characterization of twenty microsatellite markers for the study of hybridization in butterflyfish of the genus *Chaetodon*

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Abstract Twenty polymorphic microsatellite loci were developed via 454 sequencing for two hybridizing sister species of butterflyfish: the spot-band butterflyfish (*Chaetodon punctatofasciatus*) and peppered butterflyfish (*Chaetodon guttatissimus*), which are widely distributed in the Western Pacific and Indian Ocean, respectively. All loci were genotyped in samples collected from Christmas Island: *C. guttatissimus* (n = 25), *C. punctatofasciatus* (n = 17) and hybrids (n = 16). Mean alleles per locus (N_a) were: 9.05 for *C. guttatissimus*, 9.95 for *C. punctatofasciatus* and 9.45 for hybrids. Observed heterozygosity (H_O) ranged from 0.00 to 1.00 for *C. guttatissimus*; from 0.08 to 0.88 for *C. punctatofasciatus*; and from 0.19 to 0.94 for hybrids. Most loci conformed to Hardy–Weinberg expectations, were in linkage equilibrium, and did not contain null alleles. These markers will be useful for testing population genetic hypotheses including patterns of hybridization in this pair of butterflyfishes.

Keywords Allopatry · Butterflyfish · Christmas Island · Hybridization · Indo-Pacific · Suture zone

Hybridization is widespread in coral reef fishes, but its consequences are still poorly understood. Uni- or bidirectional parental contributions, and presence or absence of introgression, can all characterize reef fish hybridization (McMillan et al. 1999; Yaakub et al. 2006; Montanari et al. 2012). The relative importance of these processes may be explained by the magnitude of the genetic distance between parental species (Mallet 2005) but this is not known for most species.

Butterflyfishes (f. Chaetodontidae) are a young reef fish family (Cowman and Bellwood 2011) characterized by

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Table 1 Primer sequences and characteristics of 20 microsatellite loci: number of alleles (N_a), observed (H_O) and expected (H_E) heterzygosity, probability of departure from HWE (p), polymorphic information content (PIC) and estimated null-allele frequency (NULL)

Locus	Primer sequences (5′–3′)	Motif	T_a (°C)	N_a GUT HYB PUN	H_O GUT HYB PUN	H_E GUT HYB PUN	p GUT HYB PUN	PIC NULL	GenBank Accession #
Cpun1[a]	F: **FAM**GTGGAGGCAACAGAACAGGT	$(TCA)_8$	60	2	0.08	0.08	1.000	0.31	KC699732
	R: GGCCTTCATCTCACAGCTTC			3	0.19	0.17	0.982	0.28	
				7	0.39	0.67	**0.000****		
Cpun2[a]	F: **VIC**CATCAGAGGAAGCGAAGACC	$(CAA)_8$	60	4	0.40	0.44	0.220	0.45	KC699733
	R: GCCCTTGAAGCAGTCTGAAG			4	0.50	0.54	0.973	0.05	
				4	0.56	0.53	0.984		
Cpun3[b]	F: **FAM**TTCCTCTCCTATCTGACGCC	$(GGA)_8$	60	6	0.36	0.62	**0.007****	0.61	KC699734
	R: TCTGAGCGACAACAATGAGC			5	0.37	0.65	0.033*	0.20	
				6	0.53	0.63	**0.001****		
Cpun4[a]	F: **PET**GCTTGAGGTTCAACACGGAT	$(GTT)_8$	60	11	0.40	0.83	**0.000****	0.85	KC699735
	R: AAGGAGCTCGCACAAATCAC			9	0.31	0.83	**0.001****	0.35	
				11	0.53	0.88	**0.008****		
Cpun6[a]	F: **NED**ACCCTTCCCTACATGCTCCT	$(GGA)_{10}$	59	8	0.79	0.79	0.285	0.78	KC699736
	R: TGCACATATGCATTCATCTCC			9	0.69	0.82	0.593	0.03	
				10	0.76	0.77	0.973		
Cpun7[b]	F: **NED**CGAAGTCACTCTGAACGCTG	$(AGG)_{10}$	60	9	0.54	0.80	**0.000****	0.83	KC699737
	R: AGTCAACACAGGAGCGACG			8	0.73	0.80	0.222	0.19	
				8	0.50	0.81	0.050*		
Cpun9[c]	F: **FAM**CACAATGCCAGCAATGATCT	$(GAG)_{11}$	59	10	0.18	0.85	**0.000****	0.86	KC699738
	R: GCTGAAGTGCAGAATGATGG			9	0.46	0.87	**0.007****	0.46	
				8	0.35	0.80	**0.001****		
Cpun10[b]	F: **VIC**CCTTTAACGAGGCAGCTCAC	$(CAT)_{11}$	60	10	0.60	0.78	0.074	0.84	KC699739
	R: AAGTGAAGTGTTTCACCGGG			10	0.75	0.84	0.367	0.12	
				12	0.71	0.87	0.026*		
Cpun11[b]	F: **PET**AAGTGCGTCCACATTCAACA	$(GTT)_{11}$	60	16	1.00	0.91	0.400	0.93	KC699740
	R: CAGAGCCAACTCCACACTGA			16	0.94	0.92	0.427	0.00	
				14	0.88	0.91	0.793		
Cpun12[c]	F: **PET**AGGTGGAGAGCAGAAGCAGA	$(GGA)_{12}$	60	6	0.59	0.76	0.081	0.75	KC699741
	R: GTGTGACAGGTGACCCTCCT			7	0.73	0.80	0.138	0.09	
				7	0.67	0.72	0.765		
Cpun13[c]	F: **VIC**CGTCGTTAAAGCCCTGAGAG	$(GGA)_{12}$	60	11	0.29	0.88	**0.000****	0.88	KC699742
	R: TCAGAGGTCAAACTGTCGCA			9	0.67	0.88	0.073	0.48	
				7	0.08	0.83	**0.000****		
Cpun14[d]	F: **VIC**TCAGCAGCACTCCTCTCATC	$(TCT)_{13}$	59	5	0.20	0.19	1.000	0.64	KC699743
	R: GGTGGAAGACACCAGTGAGAC			11	0.75	0.69	0.377	0.15	
				13	0.72	0.88	0.878		
Cpun15[c]	F: **NED**CAGCATTTGGCTAGCTTGGT	$(TAT)_{13}$	60	6	0.39	0.37	0.934	0.71	KC699744
	R: TGGCAGCTGATCAGAAATGA			8	0.80	0.69	0.778	0.10	
				12	0.76	0.86	0.252		
Cpun17[d]	F: **FAM**TGAATGGATGAATGGATGGTT	$(ATGG)_{10}$	60	10	0.92	0.86	0.171	0.89	KC699745
	R: CCTGGGAGGAGACAAACAGA			13	0.73	0.88	**0.009****	0.05	
				14	0.78	0.91	0.567		
Cpun18[d]	F: **NED**AACAAAGCTTTCAGGCTCCA	$(CTGT)_{12}$	60	11	1.00	0.88	0.794	0.88	KC699746
	R: GTCTGTCCACACGTCACAGG			10	0.56	0.83	0.054	0.08	
				9	0.56	0.83	0.104		

Table 1 continued

Locus	Primer sequences (5′–3′)	Motif	T_a (°C)	N_a GUT HYB PUN	H_O GUT HYB PUN	H_E GUT HYB PUN	p GUT HYB PUN	PIC NULL	GenBank Accession #
Cpun19[e]	F: **VIC**TCCTCTCCATCGTCTCCAAC	$(GTCT)_{12}$	60	11	0.67	0.84	0.027*	0.86	KC699747
	R: GTTGTAGAGGTGCCATGCAG			14	0.62	0.86	**0.007****	0.19	
				13	0.41	0.84	**0.000****		
Cpun20[d]	F: **PET**GGCAACTGGGTTCAGATGAT	$(TGGA)_{13}$	60	15	0.70	0.92	0.230	0.93	KC699748
	R: CTGTTCGTCCTTGGATTGCT			16	0.79	0.91	0.307	0.13	
				16	0.69	0.92	0.102		
Cpun21[e]	F: **PET**CTCTTCTGACGGACGGTGAT	$(GACGT)_{10}$	60	16	0.76	0.90	0.751	0.90	KC699749
	R: TGACTTTCTATTGAGCCGCA			12	0.87	0.88	0.384	0.06	
				11	0.76	0.85	0.093		
Cpun22[e]	F: **FAM**GAAGGCTGTGCTGACACTGA	$(AGGAC)_{11}$	60	6	0.6	0.75	0.505	0.79	KC699750
	R: GAGTTTGAAGCCGTGTGGAG			7	0.81	0.81	0.906	0.04	
				8	0.83	0.82	0.665		
Cpun23[e]	F: **NED**GACAGAGCGATGTGCTATGG	$(GGAGA)_{13}$	60	8	0.00	0.86	**0.000****	0.88	KC699751
	R: AGGTCCCTCAGCAAGGAGAT			9	0.62	0.86	0.087	0.41	
				9	0.47	0.81	0.038*		

Chaetodon guttatissimus (n = 25, GUT), hybrids (n = 16, HYB) and *C. punctatofasciatus* (n = 17, PUN) were all collected from Christmas Island

[a,b,c,d,e] Multiplex plate allocation; * $p < 0.05$; ** $p < 0.0167$ after sequential Bonferroni correction (highlighted in bold)

having high levels of gene flow among geographically isolated populations (Lawton et al. 2011) and a high proportion of hybridizing species (Hobbs et al. 2013). The peppered butterflyfish (*Chaetodon guttatissimus*) and the spot-band butterflyfish (*Chaetodon punctatofasciatus*) are allopatric sister species with large geographical ranges spanning the Indian and Western Pacific Oceans (Allen et al. 1998). Both taxa occur at Christmas Island (Indian Ocean, Australia) at the edge of their distributions (Hobbs and Salmond 2008) and hybridize at this reef fish suture zone (Hobbs et al. 2009).

Another pair of butterflyfishes hybridize at Christmas Island (Montanari et al. 2012). Comparisons between these hybridizations will allow to identify patterns of directionality and introgression in reef fish hybridization. To examine the specific consequences of hybridization in the *C. punctatofasciatus–C. guttatissimus* complex, we developed markers for the parental species, rather than attempting cross-amplification of available markers (e.g. Lawton et al. 2010).

All loci were developed from *C. punctatofasciatus* DNA, extracted using Gentra Puregene (Qiagen). DNA (1 μg) was shotgun sequenced on 12.5 % of a Roche GS-FLX (AGRF, Brisbane, Australia) (Gardner et al. 2011). All 454 sequencing results were deposited on Dryad (Meglécz et al. 2012; doi:10.5061/dryad.jd183).

A total of 113,361 reads (average sequence length = 348 bp; total GC content = 43.07 %) were screened for di-hexanucleotide repeats using the default settings of QDD (Meglécz et al. 2010). Microsatellite coverage was 0.03 %, within the average obtained for the Actinopterigii (Meglécz et al. 2012). PCR primers with the lowest pair penalty (Meglécz et al. 2010) were synthesized for 24 tri-hexanucleotide loci.

Initial testing for amplification and marker diversity was performed on five individuals of each taxon. PCR contained 1 X Type-it Multiplex PCR Master Mix (Qiagen), 20–50 ng template, and 0.2 M each primer. Each tailed forward primer and a reporter primer (5′ TET-labeled) were mixed at a 1:4 ratio (total = 0.2 M) for indirect labeling (Shimizu et al. 2002). PCRs included an initial denaturation of 95 °C for 5 min followed by 28 cycles of 95 °C for 30 s, 60 °C for 90 s and 72 °C for 30 s followed by 30 min at 60 °C on a Bio-Rad C1000 Thermal Cycler (Bio-Rad). Genotypes were run on an ABI 3730XL Genetic Analyzer (Applied Biosystems) with a LIZ-500 size standard and scored using Microsatellite Plugin v1.1.0 (Biomatters Ltd.).

Twenty loci reliably amplified and were polymorphic (Table 1). All these were directly fluoro-labeled and genotyped in 58 individuals collected from Christmas Island, using Chelex-extracted DNA: *C. guttatissimus* (n = 25), *C. punctatofasciatus* (n = 17) and hybrids (n = 16). Multiplexing (Table 1) was carried out using PCR conditions described above.

Number of alleles (N_a), observed (H_O) and expected (H_E) heterozygosities and probabilities of departure from

Hardy–Weinberg Equilibrium (HWE) were calculated using the R package *adegenet* (Jombart 2008). GENEPOP v4.2 (Rousset 2008) and MICROCHECKER v2.2.3 (van Oosterhout et al. 2004) were used to check for linkage disequilibrium and null alleles. CERVUS v3.0 (Kalinowski et al. 2007) and COLONY v2.0.4 (Wang 2004) were used to calculate polymorphic information content (PIC) and sibship probabilities.

For *C. guttatissimus* H_O ranged from 0.00 to 1.00 and for *C. punctatofasciatus* from 0.08 to 0.88 (Table 1). Mean N_a was 9.05 ± 0.84 SE and 9.95 ± 0.69 SE, respectively (Table 1). Eight loci departed significantly from HWE in the parental species (Table 1), possibly due to siblings in the sample. All loci departing from HWE showed presence of null alleles (frequency = 0.16–0.48), with the exception of Cpun3 in *C. punctatofasciatus*. Two between-locus comparisons deviated from linkage equilibrium (Bonferroni-adjusted $\alpha = 0.0026$; *C. punctatofasciatus*: Cpun6-Cpun7, $p = 0.001$ and Cpun19-Cpun21, $p < 0.001$).

For hybrids, mean N_a was 9.45 ± 0.77 SE and H_O ranged from 0.19 to 0.94 (Table 1). Four loci departed significantly from HWE (Table 1): all but Cpun17 displayed evidence of null alleles (frequency = 0.13–0.30), whereas no loci deviated from linkage equilibrium.

Markers reported here are polymorphic, amplify in two sister species of genus *Chaetodon* and will be used for resolving population structure, patterns of hybridization and speciation in this species pair and closely related taxa.

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