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TECHNICAL NOTE

Identification of twenty one microsatellite loci for conservation genetic studies of the endemic butterflyfish *Chaetodon tricinctus*

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Abstract Coral reef habitats are increasingly under threat from global warming and this has influenced the associated reef fish communities. *Chaetodon tricinctus* is a butterflyfish endemic to the offshore reefs of Elizabeth Reef, Middleton Reef, Lord Howe Island and Norfolk Island off Australia's east coast. It is a highly specialised coral reef fish that is thought to rely on Acroporid coral species exclusively for food and shelter. We developed primers for twenty one microsatellite loci to reveal gene flow, population genetic structure and genetic diversity within and among these three reefs. Observed heterozygosities ranged from 0.185 to 0.964 and expected heterozygosities ranged from 0.230 to 0.889 in 30 individuals from Middleton Reef. When cross tested with *Chaetodon trifascialis*, a closely related species, there was poor amplification success and only a moderate level of polymorphism. Therefore, although these loci will be useful in *C. tricinctus*, it is unlikely that they can be used on other related butterflyfishes.

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

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Coral reefs support an enormous diversity of species however, there is growing concern over the global decline in coral cover (Gardner et al. 2003; Hughes et al. 2003) and how this has negatively affected reef fish communities (Doherty 2002; Spalding and Jarvis 2002). Reef fishes that specialise on one or a select few coral species for food or shelter appear to most at risk (Jones et al. 2004).

The three-striped butterflyfish *Chaetodon tricinctus* appears to be one such habitat specialist. This species isendemic to Australian waters and inhabits coral rich areas of lagoon and seaward reefs at Elizabeth Reef (ER), Middleton Reef (MR), Lord Howe Island (LHI) and Norfolk Island (NI; Randall 1976). It is the most abundant butterflyfish in the region (Choat et al. 2006a; Hobbs and Feary 2007; Hobbs et al. 2009). The feeding habits of this species are largely unknown, except that it feeds on polyps of hard corals (Kuiter 2002) and is closely associated with species of the scleractinian Acroporid genus (Hobbs et al. 2009). *Acropora* species are particularly susceptible to bleaching (Marshall and Baird 2000; Willis et al. 2004) and there is concern that local extinctions of *C. tricinctus* may occur (for evidence in other species see Graham et al. 2006) as the ability of this fish to switch diet to include other coral species or invertebrates is unclear (Hobbs et al. 2009).

Information on gene flow, population genetic structure and genetic diversity of *C. tricinctus* is lacking, however

Table 1 Details for twenty one *Chaetodon tricinctus* microsatellite loci developed from 454 shotgun sequences

Locus	Repeat motif	Primer sequence	Size range (bp)	N	N_a	H_o	H_e	pHWE	PIC	Genbank accession no.
Chaetodon tricinctus										
Ct2[1C]	$(ACAG)_9$	[HEX]TGTCTTTGGATGTGAGCAGG ACCACAGGCCAGTTTTTCAC	237–245	30	3	0.300	0.383	0.544	0.321	JN604067
Ct3[3C]	$(ATCT)_{11}$	[HEX]CCAAAGCTTGGCTTTTGTGT CTAAGTCTTGGCGAACAGCC	227–255	24	5	0.417	0.444	0.821	0.419	JN604068
Ct4[1B]	$(CTAT)_{12}$	[FAM]AGCTGAGTTCAGGTCAGGGA GCATAGGACTCGGGAACAAG	130–158	30	7	0.633	0.618	0.047	0.569	JN604069
Ct5[3B]	$(ATAG)_{11}$	[FAM]CTGACACAGCCAAACGATGT TACGGTCAGGTTCGGCTTAC	191–207	24	5	0.583	0.694	0.446	0.64	JN604070
Ct7[7C]	$(TCTA)_{10}$	[HEX]CATTCATTCCTGCTGCCATA GGCCAAGTAACGTTTGCTGT	56–156	28	7	0.964	0.654	0.280	0.61	JN604071
Ct8[1A]	$(TCCA)_{10}$	[TET]GCACTTATGTGCTCCATCCA GACCTCCAATCTGGCAACTC	102–140	30	7	0.733	0.654	0.950	0.591	JN604072
Ct9[5C]	$(GATA)_{13}$	[HEX]TGGACCAGCACACTCAGAAG ACAGATGTGAATTCCCGTGG	203–223	27	6	0.741	0.628	0.993	0.587	JN604073
Ct10[4C]	$(GATA)_{10}$	[HEX]TCGCAAGCTTGGATTATGAA GCCTCAGCAGTGCAAGTACA	191–203	29	4	0.586	0.499	0.532	0.453	JN604074
Ct11[2A]	$(ATAG)_{10}$	[TET]TGACCAAAATGACAACCAGG TACTTTTATCGGCGGCAAAG	95–139	30	9	0.833	0.767	0.983	0.734	JN604075
Ct12[4A]	$(TCAA)_{13}$	[TET]GTTTGTCAGCCGACTCACTG AAACAGTGTCTGGAGGCTGC	107–119	30	4	0.533	0.563	0.307	0.505	JN604076
Ct13[3A]	$(GATA)_{13}$	[TET]ACAAGACAGCGAATGAGTGC AGGATCTCAGCCACGAAAGA	117–145	24	8	0.667	0.778	0.945	0.747	JN604077
Ct14[6B]	$(TAGA)_9$	[FAM]GACATGACCACCAAGCAGC AGGGTGGGAGGCATAAAAGT	113–137	28	7	0.750	0.736	0.254	0.699	JN604078
Ct15[5B]	$(ATAG)_{12}$	[FAM]TTTGTGCAACATGACAATGAA AATGATGGCTTTGCTCTGCT	120–164	27	9	0.926	0.848	0.509	0.83	JN604079
Ct16[4B]	$(ATAG)_{13}$	[FAM]GTTAATGATGGCGTTGGAGC AACACTAAATTTCCCGGATCA	107–147	30	7	0.567	0.553	0.002	0.501	JN604080
Ct17[7A]	$(TAGA)_{12}$	[TET]GCAAGATCTGGCAAGAAACC GGCTCACTACATTATGCACTGGT	108–136	28	7	0.643	0.719	0.728	0.682	JN604081
Ct18[6A]	$(GTTT)_9$	[TET]TGTAAGGCTTGTAGTCAAGTCAGG GGAAAGCCTTTAGGGGACAG	89–101[a]	28	4	0.214	0.339	0.004	0.318	JN604082
Ct20[2C]	$(AGGAG)_9$	[HEX]AGAGGCAGTCCTGGAAAATG TGTTTGGATGCTCAGGTCAG	150–300	29	9	0.793	0.822	0.860	0.799	JN604083
Ct21[5B]	$(TTGTG)_{16}$	[FAM]GGCCCACAACTTCTCATTGT TACTCCACAGGGTTGAATGC	105–175	28	13	0.857	0.889	0.006	0.879	JN604084
Ct22[2B]	$(TATCT)_{10}$	[FAM]CCTTCTGTGCCACTGGTTTT AGAACATGAGGACATCAGAGCA	140–180	30	8	0.833	0.846	0.438	0.828	JN604085
Ct23[6C]	$(TTCAA)_{10}$	[HEX]CAAACCAACTTTTCTGTGAAGTG CCCCTCGTGAGACGAATAAA	135–150	28	4	0.929	0.675	0.002	0.618	JN604086
Ct24[5A]	$(TAGATT)_6$	[TET]ATTTTAGGCTGTGGCCTGTG ACCAGCTGTCCTTGCAGAAG	90–102	27	3	0.185	0.230	0.621	0.211	JN604087

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

[a] Locus may have null alleles

these data are vital for inferring if populations at the 4 different locations (ER, MR, LHI and NI) could replenish or re-stock each other following a major bleaching event. Additionally, genetic diversity data will help determine if these populations may or may not be resilient under future environmental conditions when oceans are expected to be warmer and more acidic. This study describes the development of 21 polymorphic microsatellite markers and for *C. tricinctus* using 454 shotgun pyrosequencing on a Roche GS-FLX (Australain Genome Research Facility, AGRF, Brisbane, Australia). These markers will be useful to quantify population connectivity of *C. tricinctus.* Preliminary tests of cross-species amplification of these loci on a closely related species, *C. trifascilis*, are also described.

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 113,794 reads with an average sequence length of 350 and total GC content of 42.53%) were screened for di, tri, tetra, penta, and hexanucleotide microsatellite loci with six or more repeats using the default settings of QDD1 (Meglecz et al. 2009) which identified 13,381 (11.76% of sequences) microsatellite loci. However, PCR primers were successfully designed for the best 1,664 (12.44%) of loci found. Of these, primers for the best of the best 24 loci were synthesised with a 5′ 17 base pair lambda tag (5-GGTG GCGACTCCTGGAG-3) to enable indirect fluorolabelling (Shimizu et al. 2002) and minimize costs. 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 (see 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 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 Bioscinces Megabase Capillary Sequencer with a 400 bp size standard and scored using Fragment Profiler 1,2 (Amersham, Buckinghamshire, England).

Primer pairs for 21 loci reliably amplified products of the expected size, with no additional products and were polymorphic, representing one hexamer, four pentamers and sixteen tetramers. Directly-labelled forward primers (HEX, TET or FAM) were synthesised for the 21 polymorphic *C. tricinctus* loci to allow PCR multiplexing of at least three loci (Table 1). Loci were genotyped in directly labelled multiplex reactions in 30 *C. tricinctus* individuals from MR. DNA extraction protocol and PCR conditions as described above. Multiplex PCR combinations (Table 1) were designed and tested using PCR conditions described above.

Characteristics of the 21 loci are summarised in Table 1. GENALEX 6 (Peakall and Smouse 2006) was used to examine 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) and MICRO-CHECKER 2.2.3 (Van Oosterhout et al. 2004) were used to test linkage disequilibrium and the presence of null-alleles, respectively.

Twenty of the 21 loci were in HWE (not Ct16) and few locus pairs (x out of y) with significant linkage disequilibrium were detected after FDR correction (Benjamini and Hochberg 1995): Ct2 and Ct21, Ct3 and Ct7, Ct5 and Ct18, Ct5 and Ct23, Ct7 and Ct9, Ct10 and Ct13, Ct14 and Ct23 and, Ct18 and Ct21 (Table 1). In addition, null alleles were suggested for only one loci (Ct18) due to homozygote excess. The *C. tricinctus* markers displayed high allelic richness (mean $N_A = 6.5 \pm 0.54$, range 3–13) and high levels of expected heterozygosity ($H_E = 0.635 \pm 0.039$, range 0.230–0.889). The polymorphic information content

Table 2 Cross species amplification potential for the novel *Chaetodon tricinctus* microsatellite loci on the test species *C. trifascialis*, number of successful amplifications, amplicon size range and number of alleles (N_a)

Locus	Amplification	Size range (bp)	N_a
Ct2	14/16	221–265	10
Ct4	12/16	132–160	8
Ct5	14/16	179–279	11
Ct7	5/16	52	1
Ct8	15/16	132–160	8
Ct12	16/16	139–171	6
Ct13	1/16	162	1
Ct14	1/16	121	1
Ct15	2/16	180–189	2
Ct16	9/16	139–181	6
Ct18	15/16	67–71	2
Ct20	15/16	260–305	9
Ct21	1/16	95	1
Ct22	5/16	135–155	2
Ct24	15/16	78	1

(PIC) for the combined microsatellite loci was 0.616 indicating a reasonable discrimination between individuals, making the loci useful for studies of connectivity and population genetic structure in this taxon.

All 21 loci were cross-tested on sixteen individuals of *Chaetodon trifascialis* (Heron Island, Australia). Fifteen loci amplified successfully and ten were polymorphic with 2–11 alleles per locus (Table 2). The newly developed primers reported here will provide a useful tool to examine the gene flow, population genetic structure and genetic diversity in *C. tricinctus* but may require optimization in other related butterflyfish species.

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