Isolation and characterization of 29 microsatellite markers for the bumphead parrotfish, Bolbometopon muricatum, *and cross amplification in 12 related species*

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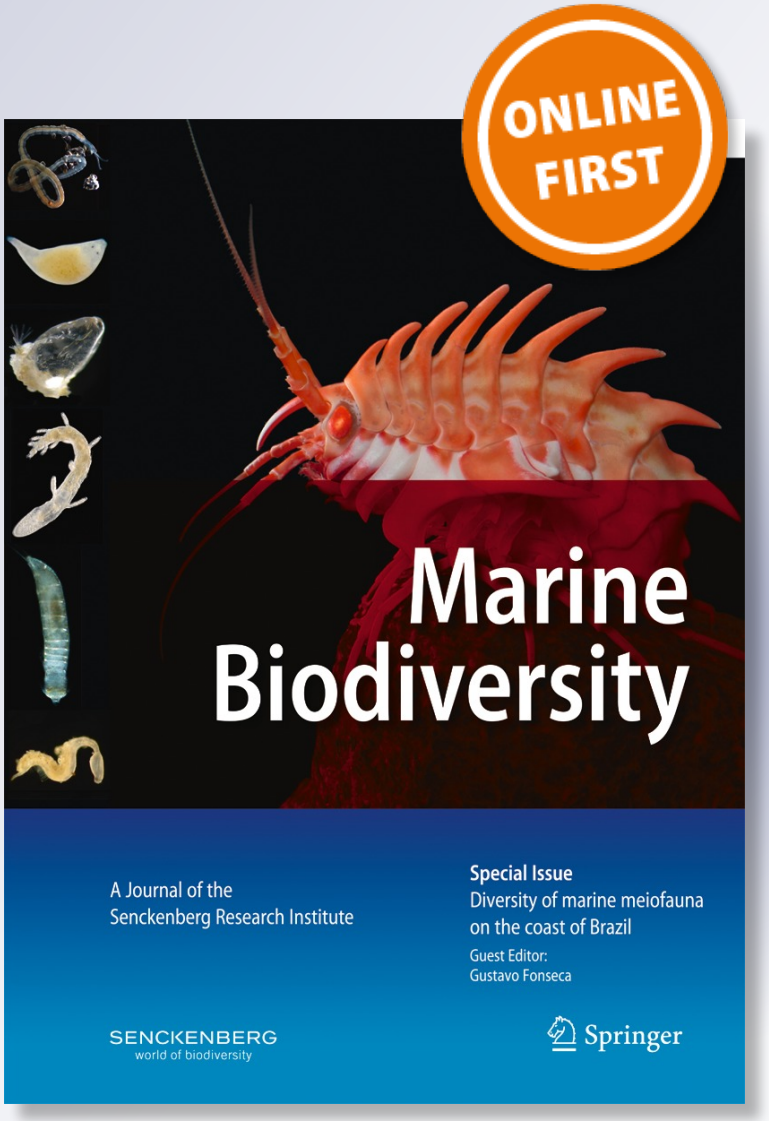

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SHORT COMMUNICATION

Isolation and characterization of 29 microsatellite markers for the bumphead parrotfish, *Bolbometopon muricatum*, and cross amplification in 12 related species

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Abstract We isolated and characterized 29 microsatellite loci for the bumphead parrotfish, *Bolbometopon muricatum*, a wide-ranging parrotfish listed as vulnerable by the International Union for Conservation of Nature (IUCN). The 29 loci were tested on 95 individuals sampled from the Solomon Islands. The number of alleles ranged from two to ten. Evidence of linkage disequilibrium was found for only one pair of loci (Bm54 and Bm112). Two loci (Bm20 and Bm119) showed significant departure from Hardy-Weinberg equilibrium. We also tested each locus for amplification and polymorphism on 11 other scarine labrid species and one labrid species. Amplification success ranged from zero to ten loci per species. These microsatellite loci are the first specific set for *B. muricatum* and will be a useful tool for assessing genetic population structure, genetic diversity, and parentage in future studies.

Keywords Bumphead parrotfish · Scarine Labridae · Microsatellites · Population genetics

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Introduction

The bumphead parrotfish, *Bolbometopon muricatum*, is the world's largest parrotfish species with a geographic range encompassing coral reefs from the Red Sea to French Polynesia (Hamilton and Choat 2012a). Where they are present, *B. muricatum* are responsible for a large amount of bioerosion on coral reefs, removing, on average, five tonnes per individual of carbonate annually, half of which is live coral (Bellwood et al. 2003). In addition to this vital ecosystem function, this species is also important in both subsistence and small scale commercial fisheries (Aswani and Hamilton 2004), yet *B. muricatum* is highly susceptible to overfishing due to life history characteristics of large size, slow growth, and the fact that this species aggregates in shallow water for feeding, spawning and sleeping activities (Hamilton et al. 2008). Consequently, continued declines in abundance throughout its range (Dulvy and Polunin 2004; Bellwood and Choat 2011), have resulted in *B. muricatum* being listed as vulnerable to extinction by the International Union for Conservation of Nature (IUCN) (Chan et al. 2012).

Previous genetic studies on parrotfishes have generally focused on phylogeography to gain insights into the evolutionary history of species (e.g., Bay et al. 2004; Winters et al. 2010; Fitzpatrick et al. 2011). However, effective management of exploited populations requires knowledge of demographically relevant connectivity over ecological time scales,

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such as the number of migrants exchanged between spatially segregated habitat patches (see review Jones et al. 2007). These direct measurements of dispersal can now be revealed using highly polymorphic co-dominant microsatellite markers in conjunction with recent statistical advances (Manel et al. 2005; Selkoe and Toonen 2006).

Here, we present 29 novel microsatellite loci for *B. muricatum* that will help determine key population parameters (i.e., genetic population structure, larval dispersal pathways) that are essential for the effective conservation and management of this species.

Materials and methods

All genomic DNA used in this study was obtained from fin clip tissues using DNeasy extraction kits (Qiagen) following the manufacture's protocol. A genomic library was created using 454 GS FLX titanium shotgun sequencing on one individual sample by the Bioscience Core Lab at the King Abdullah University of Science and Technology. Over 150,000 reads with an average length of 230 bp were generated. Microsatellites were mined from the generated 454 data using the software MSATCOMMANDER v 1.0.8 (Faircloth 2008). Specifically, we searched for dinucleotide and trinucleotide motifs with a minimum of eight perfect repeats. We found 1,748 sequences that contained dinucleotide (1,501) or trinucleotide (248) repeats. Among those, 187 had flanking regions for which primer pairs could be designed. Overall, 120 microsatellite loci were selected for polymerase chain reaction (PCR) trials (108 dinucleotide and 12 trinucleotide repeats). PCR reactions were set up following protocols associated with the Multiplex PCR kit (Qiagen). Primers were tested at annealing temperatures ranging from 55 to 63 °C. PCR reaction volume was 10 μl, consisting of 5 μL of Multiplex Mix (Qiagen), 1 μL of primers (2 μM), 3 μL of water, and 1 μL of genomic DNA (30–100 ng/μl). PCR reactions were performed using the following parameters: 15 min at 95 °C followed by 30 cycles of 30 s at 94 °C, 90 s at 55–63 °C and 90 s at 72 °C, with a final extension of 10 min at 72 °C. The 120 designed primer pairs were first tested on eight samples to identify polymorphic loci. PCR products were run on a QIAxcel genetic analyzer (Qiagen) using a high-resolution cartridge to identify polymorphic loci. Of the 120 loci tested, 29 exhibited variation and were labeled with ABI fluorescent tags: 6-FAM, PET, VIC, and NED (Fig. 1). PCR reactions were performed as described previously for 95 individuals sampled from one location in the Solomon Islands (Hamilton and Choat 2012b; Hamilton et al. 2013).

PCR products were analyzed with an ABI 3730xl genetic analyzer (Applied Biosystems) and sizes were determined with GENEMAPPER 4.0 (Applied Biosystems). Number of alleles and expected heterozygosities under Hardy-Weinberg equilibrium were calculated using GENALEX v6.5 (Peakall and Smouse 2012). Tests for Hardy-Weinberg and linkage disequilibrium were conducted using genepop on the web v4.2 (Raymond and Rousset 1995; Rousset 2008), with significance levels adjusted for multiple comparisons using false discovery rates (FDR; Benjamini et al. 2006).

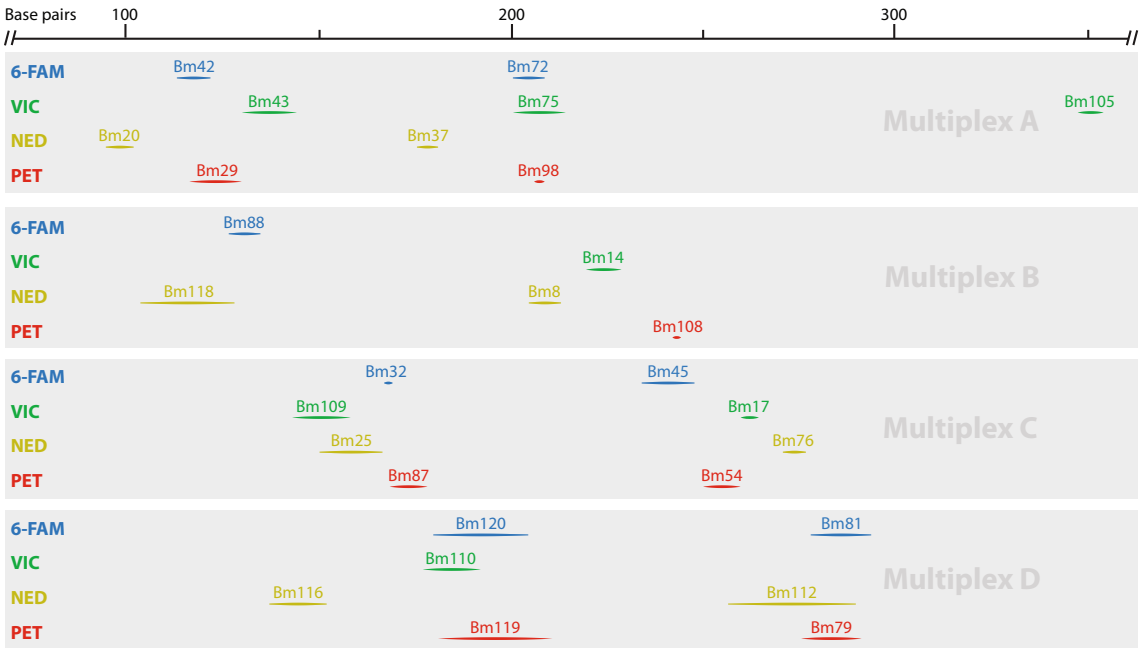

Fig. 1 Schematic of four microsatellite multiplexes designed for *Bolbometopon muricatum* representing allelic size range and fluorescent dyes used for each locus

Table 1 Primer sequences, repeat motifs and characteristics of 29 microsatellites loci developed for *Bolbometopon muricatum*: annealing temperature (Ta), number of alleles (Na), observed (Ho) and expected (Ho) heterozygosities, and probability value of Hardy-Weinberg equilibrium test (*p*)

Locus	Primer sequence (5′-3′)	Dye	Repeat motif	Ta (°C)	Size range (bp)	Mulitplex	Na	Ho	He	*p**	Genbank accession number
Bm20	F: TTTCTGTCTGGAGGGAGCAG	NED	(AC)9	60	94–102	A	4	0.616	0.649	**0.002**	KJ489028
	R: GAGGGCTTGTTTGGATGGTG										
Bm42	F: AACCAACATACAACACGGCG	6-FAM	(AC)17	60	113–121	A	5	0.463	0.401	0.986	KJ489029
	R: TCGTGCTGCTCTGTAACCTG										
Bm29	F: ACATGGCTTCTTTGGCTCAC	PET	(AC)15	60	116–130	A	5	0.406	0.369	0.832	KJ489030
	R: GAGTGGGTTAGGCTTGTGTG										
Bm43	F: AGTTTGTTGCATACGTGGGC	VIC	(AC)10	60	130–144	A	3	0.235	0.244	0.462	KJ489031
	R: TTCCAGTGCTCCAGGGTTTC										
Bm37	F: AATCCTGGAGTGAGCAGCAG	NED	(AC)9	60	176–180	A	3	0.455	0.455	0.507	KJ489032
	R: TGAGAGTTTCTGTGGAGGGC										
Bm72	F: CAAGCAGTGGTGTTGTGGTC	6-FAM	(AC)11	60	201–209	A	4	0.136	0.130	1.000	KJ489033
	R: GTGTGCATGTCCCTGTTCAC										
Bm98	F: GTCTGAGGCGAAGTGTGTTG	PET	(AC)8	60	206–208	A	2	0.049	0.048	1.000	KJ489034
	R: GCTCAGCTTCATTCGGACAG										
Bm75	F: GTTGGGTTTCAGTGCCATCC	VIC	(AC)14	60	200–214	A	5	0.651	0.583	0.917	KJ489035
	R: TGGCGTCTTGTATCGTGGTC										
Bm105	F: TTGGGATCATGGTACCGAGC	VIC	(AC)8	60	346–354	A	2	0.395	0.396	0.576	KJ489036
	R: TGTGTGTAATTGCCCGTGAC										
Bm88	F: CCTTCATGTCGTTTGGCAGG	6-FAM	(AC)12	60	127–135	B	3	0.474	0.484	0.632	KJ489037
	R: CAACCAGCCCTATGTAGCTG										
Bm118	F: AACAATCTACAGAGGAGAGCAC	NED	(AAT)17	60	104–128	B	8	0.782	0.790	0.353	KJ489038
	R: TGGTATGTCTTTGTATCCGCC										
Bm8	F: TAAGAAGGGAGAGTGCAGGC	NED	(AC)12	60	205–213	B	5	0.699	0.699	0.500	KJ489039
	R: TGTGAGTCATGTAGGGCCTG										
Bm108	F: CATCTGTCAAGGGCTCCAAC	PET	(AT)8	60	242–244	B	2	0.397	0.431	0.312	KJ489040
	R: TATCTCTCGCTGCAGGTGTG										
Bm14	F: CAGTCCAGTGTCTGCTTTGG	VIC	(AC)13	60	219–229	B	5	0.714	0.725	0.332	KJ489041
	R: GGAGTCCAGTACCAGCAGTC										
Bm76	F: TGTCGTCCACCCTACATGAC	NED	(AC)9	60	271–277	C	4	0.154	0.155	0.466	KJ489042
	R: TTTGCCAAGAAGTCGACTGC										
Bm25	F: AACACTGCTGACAAACACCG	NED	(AC)10	60	150–166	C	3	0.211	0.193	1.000	KJ489043
	R: TCCTGTCTCTGCCTTGATGAG										
Bm32	F: GAGTCTTCGTGCTTGCATGG	6-FAM	(AC)9	60	167–169	C	2	0.110	0.104	1.000	KJ489044
	R: AGGGAGCTGACACAACATCC										
Bm87	F: AACAGTGTCATGTGGCTGAC	PET	(AC)12	60	168–178	C	5	0.658	0.675	0.471	KJ489045

Table 1 (continued)

Locus	Primer sequence (5′-3′)	Dye	Repeat motif	Ta (°C)	Size range (bp)	Mulitplex	Na	Ho	He	*p**	Genbank accession number
	R: TGTTTGCTCTTCTAGGCTTGG										
Bm109	F: GCTTCTGCCATGATAACAACTC	VIC	(AAG)11	60	143–158	C	4	0.159	0.167	0.429	KJ489046
	R: TGGAAATAGGGACCTCTCGC										
Bm45	F: GAGGAGAACGAAGAGAGCCC	6-FAM	(AC)10	60	234–248	C	5	0.620	0.575	0.862	KJ489047
	R: GTGTGTGTACTCTGCAGCTG										
Bm54	F: CTTGAGCCGGCTGTGTTAAG	PET	(AG)12	60	250–260	C	4	0.187	0.233	0.040	KJ489048
	R: TATCTCCAGTCCAGCACAGC										
Bm17	F: AACTGAGGGTACTGGTGCTG	VIC	(AC)11	60	260–264	C	3	0.527	0.487	0.811	KJ489049
	R: TCGGATAGAAAGTCAGCCTGG										
Bm79	F: AACACACACATTTCTCTGCATG	PET	(AC)9	60	275–291	D	5	0.717	0.674	0.574	KJ489050
	R: GAGACTGCCATCTAGAGGCG										
Bm81	F: CGGCTGTTCGAGTAGATTCC	6-FAM	(AC)11	60	278–294	D	6	0.244	0.245	0.552	KJ489051
	R: CTCGTCTTCATCCCTCACCC										
Bm112	F: AGATGCCAGTATTATGCAGGTG	NED	(AAT)12	60	256–289	D	8	0.670	0.653	0.592	KJ489052
	R: TGGGATTCTGTGTACAACTACG										
Bm110	F: GGTCCTGTCTGTTTATCAAAGC	VIC	(AAT)11	60	177–192	D	5	0.298	0.303	0.439	KJ489053
	R: CTGGGCAGACACACTTCAAC										
Bm116	F: GGGTCTGGGATTAGGGTAGG	NED	(AAC)14	60	137–152	D	6	0.613	0.671	0.323	KJ489054
	R: ATGAGGTCAGAGGTCAGAGC										
Bm119	F: GAAGATGACGTGACGCTGAG	PET	(AAT)17	60	181–211	D	10	0.274	0.795	**0.000**	KJ489055
	R: TGAACAGAGGGATTACAGCG										
Bm120	F: GCAATTTCTTAAGCCTCTCAGC	6-FAM	(AAT)19	60	180–204	D	8	0.761	0.728	0.955	KJ489056
	R: CTTACTGTACTCAAGTCCTGCC										

*Numbers in bold indicate significant departures from expected Hardy-Weinberg equilibrium after correction for multiple tests

Finally, all 29 loci were tested for amplification and polymorphism on 11 other scarine labrid species (three individuals per species), and *Cheilinus undulatus,* using the same PCR and genotyping conditions described above.

Results and discussion

Allelic diversity was generally low, ranging from two to ten (mean allelic diversity 4.621). Observed and expected heterozygosities ranged from 0.049 to 0.782, and 0.048 to 0.795, respectively (mean observed and expected heterozygosities were 0.437 and 0.450, respectively) (Table 1). Two loci (Bm20, and Bm119) showed significant departure from Hardy-Weinberg equilibrium after corrections for multiple testing [FDR, $q < 0.05$] due to heterozygote deficiency. Only one pair of loci showed evidence of linkage based on the genotypic disequilibrium test using the log likelihood ratio statistic implemented in Genepop (Bm54 and Bm112, $p < 0.001$ after FDR correction, $q < 0.05$). Allelic diversity was generally low compared to microsatellites identified for some other coral reef fishes (e.g., *Chaetodon vagabundus*, Almany et al. 2009; *Dascyllus marginatus*, Aluana et al. 2012; but see also *Coris bulbifrons*, van der Meer et al. 2013). This could be a result of testing our markers on individuals from only one geographic location, and further testing may reveal greater

Table 2 Cross-amplification success of 29 microsatellite loci developed for *Bolbometopon muricatum* when tested on 11 other scarine labrid species (three individuals per species)

Locus	Species										
	Calatomus viridescens	*Cetoscarus bicolor*	*Chlorurus sordidus*	*Scarus arabicus*	*Scarus collana*	*Scarus fuscopurpureus*	*Scarus ghobban*	*Scarus niger*	*Scarus rubroviolaceus*	*Chlorurus gibbus*	*Hipposcarus harid*
Bm20	–	++	–	–	–	–	–	–	+	–	–
Bm42	++	+	–	–	–	–	–	–	+	+	–
Bm29	–	–	–	–	–	–	–	–	–	+	–
Bm43	+	–	–	–	–	–	–	+	–	+	–
Bm37	+	+	–	–	–	–	–	–	–	–	–
Bm72	++	++	+	+	+	+	++	++	++	++	++
Bm98	+	++	–	+	++	–	++	+	++	++	++
Bm75	+	–	–	–	–	–	–	–	–	+	+
Bm105	–	–	–	–	–	–	–	–	–	–	–
Bm88	–	–	–	–	–	–	–	–	–	–	–
Bm118	–	–	–	–	–	–	–	–	–	–	–
Bm8	–	+	–	–	–	–	–	–	–	–	–
Bm108	–	–	–	–	–	–	–	–	–	–	–
Bm14	–	–	–	–	–	–	–	–	–	–	+
Bm76	++	++	–	–	–	–	–	–	–	–	–
Bm25	–	–	–	–	–	–	–	–	–	–	–
Bm32	++	+	++	–	–	++	++	+	++	–	++
Bm87	–	–	–	–	–	–	–	–	–	–	–
Bm109	–	–	–	–	–	–	–	–	–	–	–
Bm45	–	++	+	–	–	–	–	–	++	–	–
Bm54	–	–	–	–	–	–	–	–	+	–	–
Bm17	–	++	+	–	–	+	–	–	+	–	+
Bm79	–	–	–	–	–	–	–	–	–	–	–
Bm81	–	–	–	–	–	–	–	–	–	–	–
Bm112	–	–	–	–	–	–	–	–	–	–	–
Bm110	–	–	–	–	–	–	–	–	–	–	–
Bm116	–	–	–	–	–	–	–	–	–	–	–
Bm119	–	–	–	–	–	–	–	–	–	–	–
Bm120	–	–	–	–	–	–	–	–	–	–	–

– denotes no amplification; + denotes one observed allele; ++ denotes multiple alleles

range-wide allelic diversity. Nonetheless, given the large number of markers presented here, parentage and kinship, and thus dispersal, can still be confidently estimated (Harrison et al. 2013).

Cross-amplification tests revealed contrasting results among several other species. Amplification success per species in the scarine labrids ranged from two loci (in *Scarus arabicus* and *Scarus collana*) to ten loci (in *Cetoscarus bicolor*), with successful amplification of locus Bm72 in all species (Table 2). Thus, these markers may also be useful for further studies on other scarine labrid species. We also tested all loci on the humphead wrasse, *Cheilinus undulatus*, as this species is more closely related to large parrotfishes than many of the smaller wrasse families (Kazancioğlu et al. 2009), and *C. undulatus* is also listed as endangered by the IUCN (Russell 2004). However, no amplification success was observed at any locus.

The microsatellite loci presented here are the first specific set for *B. muricatum* and will be a useful tool for evaluating many key population parameters, such as stock structure, and larval dispersal distances. This information is essential for improved management and effective conservation of this functionally and commercially important coral reef fish.

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