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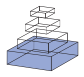

Development of novel, cross-species microsatellite markers for *Acropora* corals using next-generation sequencing technology

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The genus *Acropora* (Scleractinia, Acroporidae) is one of the most widespread coral genera, comprising the largest number of extant species among scleractinian (reef-building) corals. Molecular phylogenetic studies have suggested that *A. tenuis* belongs to the most basal clade (clade I) while *A. digitifera* belongs to a derived clade (clade IV). In order to develop microsatellite markers that would be useful for most *Acropora* species, we sequenced the genomic DNA of *A. tenuis,* using a next generation sequencer (Illumina MiSeq), and designed primer sets that amplify microsatellite loci. Afterward we selected primer pairs with perfectly matched nucleotide sequences from which at least one primer was uniquely mapped to the *A. digitifera* genome. Fourteen microsatellite markers showed non-significant departure from Hardy–Weinberg equilibrium (HWE) in both *A. tenuis* and *A. digitifera*. Thus these markers could be used for wide range of species and may provide powerful tools for population genetics studies and conservation of *Acropora* corals.

Keywords: scleractinian coral, *Acropora*, microsatellite, population genetics, cross-species, Illumina next-generation sequencing

INTRODUCTION

Acropora (Scleractinia, Acroporidae) is a common, emblematic genus of reef-building corals. It is also one of the most widespread coral genera, ranging from the Red Sea through the Indo-Pacific Ocean to the Caribbean, and has the largest number of extant species (113) (Wallace, 1999). The fossil record suggests that the genus probably originated about 50 million years ago (MYR) (Veron, 1995). There are two distinct groups of *Acropora* corals: mass spawning acroporids and "early spawners," that spawn 1.5–3 h earlier than other mass-spawning species (Hatta et al., 1999; Fukami et al., 2000). The two groups are believed to have diverged 6.6 MYA (Fukami et al., 2000). Major diversification within these groups has occurred more recently (2 MYR) (Veron, 1995). In addition, molecular phylogenetic analyses using a single copy gene PaxC intron and mitochondrial markers show that *Acropora* corals can be divided into four major clades (Van Oppen et al., 2001; Marquez et al., 2002) (**Figure 1**). Early spawning species, including *A. tenuis*, belong to the most basal clade (Clade I) (Fukami et al., 2000; Van Oppen et al., 2001), while most mass-spawning species belong to Clade III. Clade IV has relatively small number of species, including *A. digitifera* (Van Oppen et al., 2001).

Coral reefs are estimated to harbor around one-third of all described marine species (Knowlton et al., 2010); however, they face a range of anthropogenic challenges, including ocean acidification and increasing seawater temperatures (e.g., Hoegh-Guldberg et al., 2007). Although *Acropora* species are major components of coral reefs worldwide, they are the most sensitive to increased water temperatures (Loya et al., 2001) and are expected to decline in the near future (Alvarez-Filip et al., 2013). For proper maintenance and conservation of *Acropora* corals, it is important to understand genetic diversity and connectivity among populations. High-resolution genetic markers, such as microsatellites, are essential for such studies. Previous studies have succeeded in developing microsatellite markers specific to several *Acropora* species, e.g., *A. palmata* (Baums et al., 2005), *A. millepora* (Van Oppen et al., 2007), *A. cytherea* (Concepcion et al., 2010), and *Acropora* sp1 and *A. digitifera* (Nakajima et al., 2009). However, cross-species amplification was confirmed for several markers (Nakajima et al., 2009). Because microsatellite markers are currently available for only about 5 of the 113 *Acropora* species, an increased number of "universal" *Acropora* microsatellite markers would be extremely useful. In this study we developed cross-species microsatellite markers that can be applied to a variety of *Acropora* species. To achieve this we used two *Acropora* species that belong to taxonomically distant clades (I: *A. tenuis*, IV: *A. digitifera*) and we took advantage of next-generation sequencing technology to design novel microsatellite primer pairs that can be used for both species.

MATERIALS AND METHODS

Genomic DNA was isolated from an *A. tenuis* colony collected at Sesoko Island, Okinawa, Japan, under Okinawa prefectural permit (Number: 24–48), using the guanidinium reagent, CHAOS

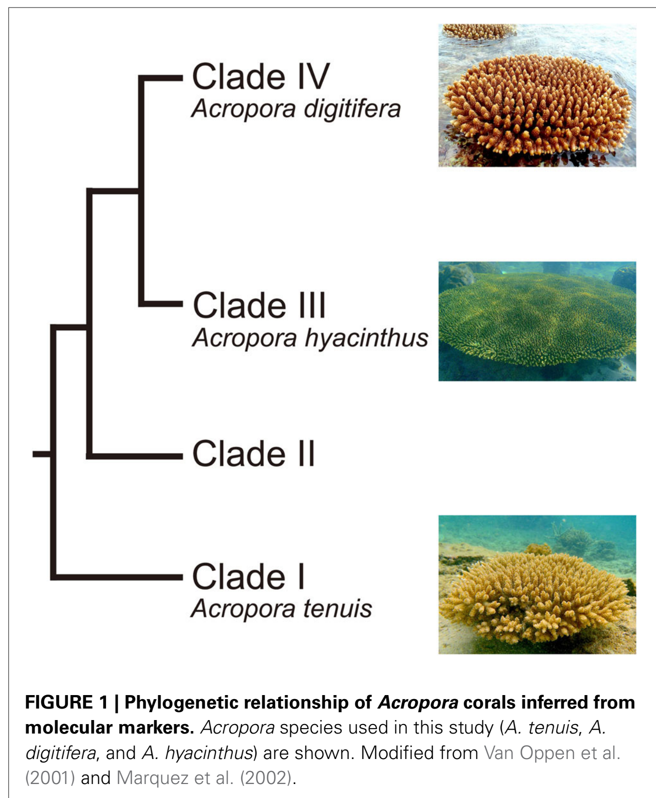

FIGURE 1 | Phylogenetic relationship of *Acropora* corals inferred from molecular markers. *Acropora* species used in this study (*A. tenuis*, *A. digitifera*, and *A. hyacinthus*) are shown. Modified from Van Oppen et al. (2001) and Marquez et al. (2002).

(Fukami et al., 2000). We sequenced 250 bp paired end reads using a MiSeq sequencer (Illumina) according to manufacturer's instructions. Low quality bases (Phred quality value, QV ≥ 20) were trimmed from raw data and read pairs of at least 80 bp were retained using SolexaQA (Cox et al., 2010). We used PAL_FINDER (Castoe et al., 2012) for detection of simple sequence repeats (SSRs) and PCR primer design from paired end sequencing data. In order to select microsatellite loci that may be highly variable, we selected primer pairs amplifying longer repeat stretches (thresholds: 2 mer; 15 repeats more, 3 mer; 10, 4 mer; 7, 5 mer; 5 and 6 mer; 4, respectively). To remove primers originating from DNA of the symbiotic *Symbiodinium*, nucleotide sequences of both primers in each pair were mapped to the recently decoded *A. digitifera* genome (Shinzato et al., 2011), using *Symbiodinium*-free sperm DNA, and employing BLASTN software (Altschul et al., 1990). In addition, primer pairs from which at least one primer was mapped uniquely to the *A. digitifera* genome were selected in order to avoid selecting primer pairs that could produce nonspecific PCR amplification.

For fragment analyses, 30 colonies of *A. tenuis* were collected at Sesoko Island, Okinawa, Japan, and 45 colonies of *A. digitifera* were collected in the Kerama Islands, Okinawa, Japan, respectively (Okinawa prefecture permit number: 24–48). To avoid multiple collections of colonies that could have been produced through asexual fragmentation or propagation, only colonies that were physically distinct and at least 2 m from other colonies were sampled. Genomic DNA was extracted using a DNeasy kit (QIAGEN). The reaction mixture (10 μL) contained template DNA (<1 ng/L), AmpliTaq Gold 360 Master Mix (Qiagen), and three primers for each locus: a non-tailed reverse primer (0.1 μM), a forward primer with an M13 Reverse (5′- CAGGAAACAGCTATGAC-3′) sequence tail (0.5 μM), and an M13 Reverse primer (0.5 μM) fluorescently labeled with FAM, based on the method of Schuelke (2000). PCR cycling conditions were 15 min at 95°C, followed by 32 cycles of 30 s at 94°C, 90 s at 58°C (all loci), and 60 s at 72°C, with an extension of 30 min at 60°C in the final cycle. In addition to *A. tenuis* and *A. digitifera*, we also used *A. hyacinthus* (Clade III) (**Figure 1**) (Marquez et al., 2002) genomic DNA to confirm PCR amplification (data not shown). PCR products from *A. tenuis* and *A. digitifera* were identified and analyzed with the ABI 3130 capillary sequencer (Applied Biosystems) and GeneMapper v4.1 (Applied Biosystems). The number of alleles and observed and expected heterozygosities were calculated and the probability of deviation from Hardy–Weinberg equilibrium (HWE) was tested for each locus and species, using GenAlEx ver. 6.5 (Peakall and Smouse, 2012). Linkage disequilibrium between the loci was tested after Bonferroni correction ($P < 0.05$) using Genepop v4.2 at http://genepop.curtin.edu.au/index.html (Raymond and Rousset, 1995; Rousset, 2008).

RESULTS AND DISCUSSION

We obtained 6,327,391,737 bp (12,802,836 read pairs) of raw sequence data from *A. tenuis* genomic DNA. From those we selected high quality 2,534,049,158 bp (6,783,510 read pairs), which were used for microsatellite detection and primer design. Primer pairs (7,200) were produced by PAL_FINDER. In order to eliminate primer pairs that could have produced nonspecific PCR amplification and that originated from symbiotic *Symbiodinium*, we selected pairs from which at least one primer sequence was unique to the *A. digitifera* genome sequence. Subsequently 141 primer pairs were selected. Among those, we confirmed that 74 pairs could produce PCR amplicons in three *Acropora* species (*A. tenuis*, *A. digitifera*, and *A. hyacinthus*) and performed fragment analyses. We identified 14 polymorphic nuclear microsatellite DNA makers that did not show significant deviation from HWE after applying Bonferroni correction ($P < 0.05$) in both *A. tenuis* and *A. digitifera* (**Table 1**). Four markers (9079m3, 11192m4, 8010m6, and 12198m3) showed significant deviation from HWE in *A. digitifera*, but not in *A. tenuis*, and one marker (7805m4) showed significant deviation from HWE in *A. tenuis*, but not in *A. digitifera* (**Table 1**). We confirmed that no previously reported *Acropora* microsatellite primer sequences (Baums et al., 2005; Van Oppen et al., 2007; Nakajima et al., 2009; Concepcion et al., 2010) were detected in the PCR amplicon sequences, indicating that all microsatellite loci identified in this study are novel. The number of alleles per locus ranged from 3 to 14 in *A. tenuis* and 2 to 13 in *A. digitifera*, respectively (**Table 1**). Observed and expected heterozygosities ranged from 0.192 to 0.933 and 0.341 to 0.892 in *A. tenuis* and 0.200 to 0.911 and 0.241 to 0.862 in *A. digitifera*, respectively (**Table 1**). Although only the linkage disequilibrium between 11401m4 and 11745m3 was significant in *A. digitifera*, linkage disequilibrium between 11 loci combinations (11401m4-11745m3, 11401m4-11543m5, 11401m4-7203m5, 11401m4-12406m3, 11745m3-12406m3, 12406m3-10366m5, 441m6-12406m3, 530m4-11543m5, 530m4-8346m3, 7203m5-11745m3, and 8346m3-11543m5) was significant in *A. tenuis*.

Table 1 | Characteristics of the 19 developed polymorphic microsatellite loci from *A. tenuis* and *A. digitifera*: locus name, repeat motif, primers sequence, number of alleles, size range, observed (*Ho*) and expected (*He*) heterozygotes and GenBank accession number.

Locus	Repeat motif		Primer sequences (5′–3′)	No. of alleles		Size range of alleles (bp)		*Ho*/*He*		Accession No.	
	A. tenuis	*A. digitifera*		*A. tenuis*	*A. digitifera*	*A. tenuis*	*A. digitifera*	*A. tenuis*	*A. digitifera*	*A. tenuis*	*A. digitifera*
8346m3	$(ATT)_{12}$	$(ATT)_{7}$	M13R-CGACAAAGATTGGAGACCC TTTCAATGCAGTGTGATTCC	8	8	162–213	186–210	0.933/0.794	0.733/0.720	AB915217	DF093698.1
7961m4	$(AAAG)_{7}$	$(AAAG)_{3}$	M13R-AAGCATCACCAAAACGGC TTACATTTGCGTCTCGGC	3	4	192–208	174–190	0.192/0.341	0.556/0.481	AB915218	DF093718.1
11745m3	$(AAT)_{16}$ GATAATGAT$(AAT)_{11}$	$(AAT)_{4}$	M13R-TTCTGTTCGCGTGTTCCC TGTTCTGCCACTGGAGGG	14	5	218–269	171–183	0.900/0.859	0.644/0.598	AB915219	DF093961.1
12406m3	$(AAC)_{12}$	$(AAC)_{7}$AGC$(AAC)_{3}$	M13R-GCTGAAGTTGTCTCCGTGC TTTTCAGGCATATCAGGAGC	5	9	163–175	164–188	0.733/0.776	0.600/0.570	AB915220	DF093708.1
11543m5	$(AAAAG)_{5}$	$(AAAAG)_{4}$	M13R-TTCTGACACAGCCATGAACC CCCCTTTCCAAAATTCACC	3	4	132–142	125–140	0.483/0.469	0.200/0.224	AB915221	DF094105.1
530m4	$(AATG)_{3}$ GATG(AATG)7	$(AATG)_{3}$	M13R-GTTCACAGGAGTGTTATGCC TCTCATTTGCACGTTCTCC	6	4	269–405	382–398	0.571/0.546	0.533/0.662	AB915222	DF093793.1
11401m4	$(ATTT)_{8}$	$(ATTT)_{5}$	M13R-TGCAGACAGAACCGAGAAGG TGGGCCACGATTCTTACG	7	6	382–418	364–400	0.833/0.729	0.267/0.345	AB915223	DF093776.1
441m6	$(CTCGGT)_{4}$	$(CTCGGT)_{3}$	M13R-GCCTTCCGGAACTATCGC TGCGAAGATGGTGTCACG	5	6	288–312	268–298	0.733/0.697	0.666/0.674	AB915224	DF094515.1
11292m4	$(AATG)_{4}$	$(AATG)_{7}$	M13R-TGCGAATGGAGCTCTGG TCATTTCGTCCATTCATGC	7	9	443–495	466–502	0.633/0.647	0.733/0.756	AB915225	DF093646.1
8499m4	$(CGGT)_{5}$	$(CGGT)_{7}$	M13R-AAACCGTGGGTTAAGGGC CGATGGAATTATTCGCGG	5	6	340–356	341–361	0.733/0.527	0.778/0.669	AB915226	DF096297.1
7203m5	$(AAAAT)_{4}$	$(AAAAT)_{5}$	M13R-ATTTCCTCACCATTCCCC TGAGGGAAAACAACACTCC	5	2	279–319	293–298	0.448/0.552	0.311/0.346	AB915227	DF093797.1
10366m5	$(AAAAC)_{5}$	$(AAAAC)_{2}$	M13R-CAACGACTGAAAGGCAGC GGCTTTCGACTTTTATGTCC	3	3	222–232	216–226	0.833/0.569	0.578/0.447	AB915228	DF095728.1
12130m5	$(AAAAC)_{6}$	AAAACAAAAAA$(AAAAC)_{2}$	M13R-TGAGGGTAAAGGCGGACC TTTTGCTTTATCCGCATCG	4	5	248–283	265–295	0.893/0.538	0.302/0.268	AB915229	DF095024.1
4546m2	$(AT)_{26}$	$(AT)_{2}$AATT$(AT)_{2}$AC$(AT)_{3}$	M13R-TGTGCAATGAAAATTTCCCC CAGTTCCCTTGTTCCTGGG	5	4	250–282	231–255	0.367/0.471	0.068/0.067	AB915230	DF093922.1
DEVIATION FROM HWE IN *A. digitifera*											
9079m3	$(TAA)_{11}$	$(TAA)_{12}$	M13R-TTTCGTGTTATAGCTCCCG CCTGGCTTTTAATCTGAGG	7	13	269–287	219–303	0.767/0.779	0.911*/0.862	AB915231	DF093956.1
11192m4	$(AAAC)_{7}$	$(AAAC)_{6}$	M13R-TGAGGACCCTCCCTTGC AGGCTGCATCTGGTTTCC	4	5	127–139	104–120	0.633/0.660	0.205*/0.490	AB915232	DF094226.1
8010m6	$(AAAGGG)_{4}$	$(AAAGGG)_{2}$	M13R-ACGCTGTGGTAAAGCACG CACTTGACACCACGCTGC	4	4	204–228	189–213	0.500/0.442	0.200*/0.241	AB915233	DF093908.1

(Continued)

Table 1 | Continued

Locus	Repeat motif		Primer sequences (5′–3′)	No. of alleles		Size range of alleles (bp)		*Ho*/*He*		Accession No.	
	A. tenuis	*A. digitifera*		*A. tenuis*	*A. digitifera*	*A. tenuis*	*A. digitifera*	*A. tenuis*	*A. digitifera*	*A. tenuis*	*A. digitifera*
12198m3	$(TAA)_{14}$	$(TAA)_7$	M13R-CATCTCCAAGGAACTTTGC TTCACGTTGTGTTTTGGC	13	6	304–346	294–339	0.767/0.892	0.222*/0.619	AB915234	DF093689.1
DEVIATION FROM HWE IN *A. tenuis*											
7850m4	$(AATC)_9$	$(AATC)_4$	M13R-ATGCCTGCAAGTGTTTGG GTTTCTTTAACGTCACGCGTTGTCC	9	6	225–265	229–253	0.414*/0.829	0.733/0.637	AB915235	DF093738.1

Numbers of alleles and Ho and He were calculated using GenAlEx (ver. 6.5; Peakall and Smouse, 2012). Asterisks indicate significant deviation from Hardy–Weinberg equilibrium after Bonferroni correction ($P < 0.05$) using Genepop v4.2 (Raymond and Rousset, 1995; Rousset, 2008). Accession numbers in A. digitifera are the scaffold nucleotide sequence in which each locus is located.

Alignment of the *A. tenuis* nucleotide sequences to the *A. digitifera* genome revealed high nucleotide conservation between *A. tenuis* and *A. digitifera* (about 93%, BLASTN, 1e-5, alignment length longer than 100 bp), indicating high genomic similarity between the two species. In addition, all microsatellite loci are located in different scaffold sequences in the *A. digitifer*a genome (**Table 1**), suggesting that the loci are evenly distributed across these *Acropora* genomes. Since genome structures of *A. digitifera* and *A. tenuis* should be similar, this may reflect the significant *A. tenuis* population decrease after a massive 1998 bleaching event around Sesoko island (Loya et al., 2001). Thus, the population used in this study was new and recently recruited, possibly resulting in more loci combinations with significant linkage disequilibrium. Although not tested on a large number of *Acropora* species, 14 primer pairs showing no significant deviation from HWE in two phylogenetically distant species. These can be used for a variety of *Acropora* species and may provide powerful tools for *Acropora* population genetic studies. We hope that they will contribute to establishment of reef conservation guidelines and coral reef transplantation and restoration.

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