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Comparative Study of Genetic Variability of AAT and CT/GT Microsatellites in Staghorn Coral, *Acropora* (Scleractinia: Acroporidae)

Pei-Ciao Tang[1,2], Nuwei Vivian Wei[1], Chein-Wei Chen[1,4], Carden C. Wallace[3], and Chaolun Allen Chen[1,4,5,*]

[1]*Biodiversity Research Center, Academia Sinica, Nankang, Taipei 115, Taiwan*
[2]*Department of Biology, University of Louisiana at Lafayette, Lafayette, LA 70504, USA*
[3]*Museum of Tropical Queensland, Townsville Q4810, Australia*
[4]*Institute of Oceanography, National Taiwan University, Taipei 106, Taiwan*
[5]*Institute of Life Science, National Taitung University, Taitung 950, Taiwan*

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Pei-Ciao Tang, Nuwei Vivian Wei, Chein-Wei Chen, Carden C. Wallace, and Chaolun Allen Chen (2010) Comparative study of genetic variability of AAT and CT/GT microsatellites in staghorn coral, *Acropora* (Scleractinia: Acroporidae). *Zoological Studies* **49**(5): 657-668. *Acropora* is the most speciose genus of scleractinian corals. However, phylogenies among closely related species and population genetics of *Acropora* remain equivocal. In this study, 7 AAT-repeat loci developed from the Caribbean species, *A. palamta*, and 5 dinucleotide (CT/GT)-repeat microsatellites newly developed from the Indo-West-Pacific (IWP) species, *A. muricata*, were examined to determine their utility for revealing the mutation rate and genetic diversity of *Acropora* species. Five of the 8 AAT-repeat loci were successfully amplified in IWP species, with DNA sequences in the flanking region showing over 97% similarity to those of the Caribbean species, suggesting homology of these AAT loci among these cross-oceanic *Acropora*. In contrast, amplifications of all GT-repeat loci failed in the Caribbean species. Eight loci (5 AAT and 3 CT/GT ones) showed Mendelian inheritance based on crossing experiments of *A. muricata* larvae. When the mutation rate (θ) was estimated, AAT-repeat loci showed relatively higher mutation rates (θ = 39.94-112.82) compared to GT-repeat loci (θ = 2.44-53.33). Analysis of molecular variance indicated a relatively higher within-population variation at AAT-repeat loci (98.6%) than at GT-repeat loci (78.7%). The F_{ST} statistic of 2 sympatric *Acropora* species (*A. muricata* and *A. digitifera*) indicated that the value for AAT loci (F_{ST} = 0.014) was about 15 times lower than that for GT loci (F_{ST} = 0.213), although both microsatellite motifs showed statistically significant differentiation of species. Our study highlights that AAT-repeat loci might have functional constraints and result in underestimating the genetic variability of species and populations; thus, their application to revealing genetic variations within *Acropora* should be utilized with caution. http://zoolstud.sinica.edu.tw/Journals/49.5/657.pdf

Key words: Microsatellite, *Acropora*, Trinucleotide, Dinucleotide, Mutation rate.

Acropora is the most speciose genus of scleractinian corals. With more than 130 species described so far, up to 76 *Acropora* species can occur in sympatry and 35 are documented to participate in synchronous mass-spawning events (Harrison et al. 1984, Babcock et al. 1986, Wallace and Willis 1994, Wallace 1999). *Acropora* has a widespread distribution in the Indian and Pacific Oceans, suggesting a great adaptive radiation in this genus. In contrast, only 3 extant species currently exist in the tropical Caribbean of the western Atlantic (Wallace 1999).

Revealing the evolutionary history of *Acropora* is difficult because of complicated reproductive

*To whom correspondence and reprint requests should be addressed. Tel: 886-2-27899549. Fax: 886-2-27858059.
E-mail:cac@gate.sinica.edu.tw

behavior and is also hampered by a lack of efficient molecular markers (Márquez et al. 2000, Willis et al. 2006, Fukami 2008). Sympatric *Acropora* species reproduce via synchronous mass-spawning processes providing opportunities for fertilization between different species (Harrison et al. 1984, Wallace and Willis 1994, Willis et al. 2006). Laboratory crossing experiments showed that interspecific fertilization can be successful (Willis et al. 1997, van Oppen et al. 2002, Fukami et al. 2003, reviewed in Willis et al. 2006, Fukami 2008). In addition, many molecular markers were developed for *Acropora* in the last decade; however, subsequent studies revealed numerous difficulties with their efficiencies (Márquez et al. 2000, Ridgway and Gates 2006). First, mitochondrial (mt)DNA which is widely used in vertebrate phylogenetic analyses showed limited use in scleractinian corals due to its slow evolution (Shearer et al. 2002). For example, a comparison of 964 nucleotides of the cytochrome *b* gene of 2 Caribbean and 7 Pacific *Acropora* species showed only 0.3%-0.8% sequence differences, indicating that they are evolving 10-20 times more slowly than the standard vertebrate mtDNA clock (van Oppen et al. 1999). Second, nuclear DNA sequences were shown to have relatively high intra- and interspecific variations, probably resulting from either hybridization or incomplete lineage sorting, which hinders reconstruction of species phylogenies in *Acropora* (Odorico and Miller 1997, Márquez et al. 2000, van Oppen et al. 2000 2002, Vollmer and Palumbi 2004, Wei et al. 2006).

In contrast to the discrete information of DNA sequences, allelic frequencies of microsatellites may reveal more information than mutational changes in detecting introgressive hybridization and in resolving species boundaries (reviewed in Goldstein and Schlötterer 2000). The characteristics of microsatellite markers, including high variability and co-dominantly inherited length variants, make them well suited for studies of hybridization and introgression (Muir et al. 2000). Their relatively rapid rates of mutation allow for differentiation between closely related species, and differences in allelic frequencies and species-specific alleles can be used to estimate levels of interspecific gene flow. For example, microsatellite data can be used to distinguish between 2 oak species, *Quercus robur* and *Q. petraea* (Muir et al. 2000 2001), and *Q. lobata* and *Q. douglasii* (Craft et al. 2002), whereas chloroplast markers, random amplified polymorphic DNAs (RAPDs), internal transcribed spacer (ITS) sequences, and allozymes cannot. Estimation of gene flow between a Hawaiian silversword alliance, *Dubautia arborea* and *D. ciliolata*, using 7 microsatellites suggested that even the presence of an active hybridization zone, the lack of genetic introgression, and the maintenance of species boundaries may be associated with natural selection in different habitats (Friar et al. 2007). *Acropora* and corals in general share similar life-history traits as well as molecular evolutionary characteristics of mitochondrial vs. nuclear genomes with plants (Chen et al. 2009), and microsatellites might be applicable to resolving species boundaries and the effects of hybridization as indicated in oaks and silversword.

Nevertheless, the development of microsatellites in *Acropora* and corals in general is thought to be difficult due to technical and biological constraints (Márquez et al. 2003). The small genomes of acroporid corals and the correlation between the genome size and microsatellite abundance suggest that biological constraints may limit the number of microsatellite loci in corals (Márquez et al. 2003). These difficulties were not overcome until recent years by several enrichment protocols in corals (in *A. palmata* by Baums et al. 2005a 2009; in *A. millepora* by van Oppen et al. 2007; in *A. nobilis* by Isomura and Hidaka 2008; and in *Pocillopora damicornis* by Starger et al. 2008) and expressed sequence tag (EST) and whole-genome shotgun (WGS) sequences (Wang et al. 2009). Baums et al. (2005a) developed 8 trinucleotide-repeat (AAT-repeat) loci from *A. palmata* from the Caribbean and tested co-dominant inheritance patterns. Furthermore, 5 of them were utilized to detect regional isolation and assess geographic variations in the clonal structure of *A. palmata* (Baums et al. 2005b 2006). Subsequently, these loci were assessed in Pacific *Acropora* species (van Oppen et al. 2007, Isomura and Hidaka 2008, Nakajima et al. 2009a). Nakajima et al. (2009b) reported a genetic connectivity study of *A. digitifera* on a reef of southwestern Japan using 6 loci, three of which were AAT loci derived from *A. palmata*. Interestingly, screening of EST and WGS microsatellite motifs indicated that trinucleotide repeats, particularly AAT, are most abundant in the genome of *A. millepora* (Wang et al. 2009). Nevertheless, the presence of trinucleotide-repeat microsatellite loci was noted in exon regions and/or associated with genes (Hammond et al. 1994, Chakraborty et al. 1997, Deka et al. 1999, Toth et

al. 2000), and selective constraints may affect the evolution of particular microsatellite loci (Goldstein and Schlötterer 2000). These shortcomings should be considered when trinucleotide-repeat motifs are utilized to assess population genetics and to phylogenetically analyze closely related species.

In this study, 3 questions were addressed: 1st, what are the utilities and molecular characteristics of the 7 Caribbean-derived AAT-repeat microsatellites (Baums et al. 2005b) applied to Indo-West Pacific (IWP) *Acropora*? Second, what are their performances compared to dinucleotide-repeat (GT/CT-repeat) microsatellites developed from an IWP species, *A. muricata*, in this study. Dobrowolski et al. (2002) indicated that co-dominant inheritance might not necessarily be assumed for microsatellite markers. In order to verify that microsatellite loci conform to co-dominant inheritance, a Mendelian inheritance test was carried out, as in the plant and yeast literature (Jakse et al. 2001, Dobrowolski et al. 2002). Third, what is the mutation rate of AAT microsatellites compared to those of GT microsatellites, and the resolution of these microsatellites when applied to species boundaries of sympatric *Acropora* species? In total, 13 microsatellite loci were tested for Mendelian inheritance in *A. muricata*. The mutation rate (θ) was estimated to test the potential functional constraints of AAT microsatellites, and F_{ST} statistics were performed to evaluate their limitations in delineating species boundaries of sympatric *Acropora*.

MATERIALS AND METHODS

Coral samples

Acropora specimens sampled in this study were taken with proper permits under conservation regulations of Taiwan, Indonesia, Panama, and the CITES treaty. Sperm of *A. muricata*, *A. valida*, *A. hyacinthus*, and *A. humilis* were collected from spawning colonies in the Penghu Archipelago, Taiwan Strait from 1998 to 2004 and were frozen in liquid nitrogen or dry ice for transfer to the laboratory. Branches of *A. muricata* and *A. digitifera* were collected from the Togian Is. (0°14'39.43"S, 122°9'32.26"E), Sulawesi, Indonesia during the 1999 *Tethyana* expedition to Indonesia by the 4th author. For those tissue samples, a small fragment of coral was clipped from each colony, placed in a labeled polyethylene bag, and preserved in 95% (v/w) ethanol. Genomic DNA of *A. palmata* isolated from colonies from Caribbean Panama was a gift from Dr. Fukami Hironobu, Seto Marine Biological Laboratory, Kyoto University, Japan.

Larval preparation for the Mendelian inheritance test of microsatellite loci

In order to examine the Mendelian inheritance of microsatellite loci isolated from this study and that of Baums et al. (2005a) using IWP *Acropora* species, crossing experiments of *A. muricata* were conducted to produce coral larvae on predicted nights in Chinwan Inner Bay (CIB), Penghu, Taiwan Strait (23°31'45.59"N, 119°33'36.78"E) in May 2005. Four coral colonies, designated A, B, C, and D, were collected from 3-5 m in depth at reefs adjacent to the Penghu Marine Biology Research Center in CIB, and shipped back to overflowing aquariums. Gamete bundles of corals were collected from each colony on the predicted nights, and eggs were separated from sperm using plankton mesh sieves. An aliquot of sperm was preserved in 80% ethanol for later genotyping (see below). The crossing procedures followed the protocol described in Willis et al. (1997). Fertilized eggs were kept in a 5-L jar to allow larvae to develop, and the seawater was changed every 2 d. Seven-day-old planula larvae were collected and preserved in 80% ethanol for genotyping. Two sets of larval batches from known parental colonies (A × B and C × D) were obtained on 2 and 15 May 2005.

Larval genotypes were tested for Mendelian inheritance. Since parental colonies provided sperm and eggs for the crossing experiments, larvae from both directions were collected for genotyping. For a cross between 2 heterozygous individuals, 4 classes of gametes are possible, and the expected Mendelian ratio is 1: 1: 1: 1. Expected and observed ratios of larval classes were compared using Chi-squared tests.

Isolation of dinucleotide-repeat microsatellites from *A. muricata*

GT and CT microsatellites were obtained from 2 partial plasmid libraries prepared from *A. muricata*. Genomic DNA was isolated from sperm by standard phenol/chloroform methods (Maniatis et al. 1982). One set of libraries was constructed by the restriction enzyme, *Sau3A* I, and ligated to the pUC19 plasmid (*BamH* I cut and phosphatased, Pharmacia, Stockholm, Sweden). The other library was constructed using a cocktail

of 4 blunt-end enzymes (*Hae* III, *Rsa* I, *Sca* I, and *EcoR* V), and ligated to the pUC19 plasmid (*Sam* I cut and phosphatased, Pharmacia). Ten micrograms of sperm DNA was digested with restriction enzymes, and run on a 1.5% low-melting-point agarose gel, and a 350-500 bp region was selected (Moore et al. 1994). Coral DNA was ligated to pre-cut plasmids as described above and transformed into fresh competent cells prepared using the CaCl/RbCl method (Maniatis et al. 1982). Transformed colonies were grown on Hybond N+ membranes (Amersham, Sydney, Australia). Overall, an estimated 50,000 clones were used to screen the GT and CT motifs in *A. muricata*. Synthetic 30 bp oligonucleotide probes, $(GT)_{15}$ and $(CT)_{15}$, were prepared by end-labeling (Maniatis et al. 1982) with α-^{32}P and overnight hybridization carried out at 65°C. Filters were washed twice in 2x standard sodium citrate (SSC) and 0.1% sodium dodecylsulfate (SDS) for 20 min each, first at room temperature and then at 65°C. A final wash was done in 0.2x SSC and 0.1x SDS at 65°C. Positive colonies were identified by exposure to x-ray film and selected from the original filters to establish the initial microsatellite library. This library was then subjected to a 2nd round of hybridization with the $(GT)_{15}$ and $(CT)_{15}$ probes to confirm positive colonies. Nucleotide sequences were determined for complementary strands of at least 2 clones from each sample using an ABI 377 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Primer pairs were designed for dinucleotide-repeat microsatellites that contained > 10 uninterrupted repeats using Oligo 4.0-s software (Rychlik 1999) to compare various primer combinations selected at either end of the flanking region.

Microsatellite analysis

Total genomic DNA of coral branches was extracted using a plant genomic DNA extraction miniprep system (Viogene, Taipei, Taiwan). The extraction procedure followed the manufacturer's protocol. The preserved sperm were resuspended in DNA isolation buffer (DNAB: 0.4 M NaCl and 50 mM EDTA; pH 8.0) and incubated at 65°C for 1 h, after which 20 μL proteinase K (10 mg/ml) was added at 55°C overnight. Lysates were extracted with phenol and chloroform. DNA was suspended in 50 μL water and stored at -20°C. Genomic DNA from planula larvae was extracted using a Chelex-boiling method (Giraffa et al. 2000).

Seven ATT-repeat microsatellite loci developed by Baums et al. (2005b) and 5 sets of dinucleotide-repeat (GT/CT-repeat) microsatellite loci isolated in this study were used (Table 1). Amplification of microsatellite DNA was carried out by a polymerase chain reaction (PCR) in a 200 μL vial, which contained 50-100 ng DNA, 1x buffer (Invitrogen, Carlsbad, CA, USA), 2 mM $MgCl_2$ (Invitrogen), 0.8 mM dNTP (Promega, Madison, WI, USA), 0.3 μM of each HEX-labeled primer, 1% DMSO (Merck, Darmstadt, Germany), and 0.03 U *Taq* polymerase (Invitrogen); then double-distilled water (ddH_2O) was added to bring the volume to 50 μL. Thermal cycles of the PCR were set to an initial denaturation at 94°C for 3 min, then 7 cycles of 94°C for 1 min, 50°C for 30 s, and 72°C for 15 s; and 38 cycles of 90°C for 30 s, 50°C for 30 s, and an extension at 72°C for 15 s; followed by a final extension for 60 s at 72°C. The PCR products were visualized by Gelscan vers. 8.0.1 (Scanalytics, BD Biosciences, Rockville, MD, USA) in a Gelscan 3000 (Corbett Research, Sydney, Australia). Accurate sizing used the internal size standard (TAMRA 500 size standard, Applied Biosystems, Foster City, CA, USA), and electropherograms were analyzed with Genetic Profiler 4.05 (Scanalytics, BD Biosciences).

In order to examine the homology of the AAT-repeat loci between IWP and Caribbean *Acropora*, the PCR products of the AAT-repeat loci were cloned into the TA-cloning system (Promega) and then transformed into *Escherichia coli* DH5α (Promega). Positive clones were sequenced on an ABI PRISM 3700 DNA Analyzer (Applied Biosystems) with T7 primers.

Data analysis

Sequences of the microsatellite loci were edited using Seqman II (DNASTAR) with manual adjustment. Microsatellite-motif formats and sequence similarities of the flanking region of AAT-repeat loci were compared with *A. palmata* using MEGA 3.1 (Kumar et al. 2004). Larval genotypes were tested for consistency with Mendelian inheritance. In this study, the expected and observed ratios of larval classes which followed Mendelian inheritance using Chi-squared tests with subsequent Bonferroni correction were compared. The allelic size and frequency of *A. muricata* and *A. digitifera* were determined using MSA vers. 4.05 (Dieringer and Schlotterer 2003). Deviation from Hardy-Weinberg equilibrium (HWE) was examined by Arlequin 3.11 (Excoffier et al. 2005). Heterozygosity deficits and excesses were tested

using the GENEPOP web version (Raymond and Rousset 1995). The mutation rate of microsatellite loci was represented using a fundamental population genetics parameter, $\theta = 4N\mu$ (where N is the population size and μ is the mutation rate), due to the direct proportion relation. θ was estimated based on the maximum likelihood using Misat 1.0 (Nielsen 1997). Differentiation between 2 motifs of microsatellites was evaluated using a *t*-test.

Species boundaries of *Acropora* are usually unclear due to the potential for hybridization by sympatric species through synchronous spawning (reviewed in Willis et al. 2006). In order to examine the utility of the 8 microsatellites in revealing genetic differentiation of *Acropora*, the genetic variability was examined in 2 sympatric species, *A. muricata* and *A. digitifera*, collected from the Togian Is., Sulawesi, Indonesia. The Togian Islands lie south of the equator, an area with great diversity of the genus *Acropora* (Wallace and Wolstenholme 1998). Differentiation of allelic frequencies between 2 species in each locus was tested using the Mann-Whitney *U*-test (2-tailed, $\alpha = 0.05$) in StatView vers. 5 (SAS Institute, Cary, NC, USA). In order to assess the capability between different motif types of microsatellite loci, within-population variation was evaluated by analysis of molecular variance (AMOVA) using Arlequin 3.11. To evaluate the species boundary, the *F*-statistic (F_{ST}) (Wright 1951) and its *p* value were calculated using Arlequin 3.11 (Excoffier et al. 2005). Recent theoretical work suggested that F_{ST} provides more-reliable estimates of genetic differentiation for microsatellite loci (Balloux et al. 2004).

RESULTS

Microsatellite amplification of *Acropora* species from 2 ocean provinces

Two partial plasmid libraries were constructed from sperm DNA of *A. muricata*, and about 50,000

Table 1. Microsatellite motifs, primer sequences, annealing temperatures, and utility of microsatellite markers in the genus *Acropora*

Locus	Primer sequence (5'-3')	Motif	Ta (°C)	Indo-West Pacific Ocean					Caribbean
				A. humilis	*A. hyacinthus*	*A. muricata*	*A. digitifera*	*A. valida*	*A. palmata*
Acr53[a]	L: GGTGAGTTTCTTCGCTGACT R: ATCTAGAATCACGCGCAAGGT	$(GT)_{10}$	50	+	+	+	+	+	-
Acr1-4[a]	L: GAAGGGAGAGAATCATGTCA R: TGTGGCAAGTTGTTCGGCTA	$(GT)_{20}$	50	+	+	+	+	+	-
Acr1-60[a]	L: GGCGACCAGACAGGCTCTTA R: TTGGCATGAAGTTGAATACGA	$(GT)_{10}$	50	+	+	+	+	+	-
Acr3-27[a]	L: TAATTGACTTCACTGCGTCA R: TCCGAACTGGTGATAACTGA	$(GT)_{13}$	50	-	+	+	+	-	-
Acr45[a]	L: AAGTGTCTCGACTCTGTAGA R: ATATTTCTCTGAGTGTACAT	$(CT)_{17}$	50	-	+	+	+	-	-
Acr166[b]	L: TCTACCCGCAATTTTCATCA R: CGCTCTCCTATGTTCGATTG	$(AAT)_{28}$	50	+	+	+	+	+	+
Acr180[b]	L: TTTCTCAGTGGGTTCCATCA R: CCTTTCGTTGCTGCAATTTT	$(AAT)_{19}$	50	+	+	+	+	+	+
Acr181[b]	L: LTTCTCCACATGCAAACAAACA R: GCCAGGATAGCGGATAATGA	$(AAT)_{10}$	50	+	+	+	+	+	+
Acr182[b]	L: TCCCACAACTCACACTCTGC R: ACGCGGAAATAGTGATGCTC	$(AAT)_{18}$	50	+	+	+	+	+	+
Acr192[b]	L: TTTGAGCATTTAAGGAGCAACA R: CAGCAGACTCAACAGCAGGA	$(AAT)_{28}$	50	-	-	-	-	-	+
Acr201[b]	L: CCAAAACTCAGAAACCCCATT R: GTTTCTTCGCAGAATCCATGTTGATAGC	$(AAT)_{12}$	50	-	-	-	-	-	+
Acr207[b]	L: ATCCACGCCCAAACAATGTA R: CTATTCGCTACCCACGCTTC	$(AAT)_{20}$	50	+	+	+	+	+	+

[a]Sequences of the clones are registered in GenBank under accession nos. EU872430-EU872434. [b]Baums et al. (2005a)

clones were produced. These clones were screened with GT/CT probes, and 128 positive clones were obtained and sequenced after a secondary screening. Sixteen of the 128 clones showing either GT or CT repeats in the DNA sequences (data not shown) were selected to design PCR primers for the primary test. Among them, primers of 1 CT and 4 GT microsatellites, which gave consistent amplifications in *A. muricata*, were used in the following analyses. The expected sizes of the GT/CT-repeat loci ranged 110-242 bp long. All 5 dinucleotide-repeat loci were successfully amplified in 5 IWP *Acropora*, except that Acr3-27 and Acr45 were not amplified in *A. humilis* or *A. valida* under the current thermal profile (Table 1); therefore, those loci were not used in the following analyses. In contrast, none of the 5 GT/CT-repeat loci was amplified in the Caribbean species, *A. palmata*, after the thermal profile and PCR conditions had been readjusted, suggesting that these microsatellite loci are specific to IWP species.

In contrast to the dinucleotide-repeat loci, the cross-oceanic amplification of AAT-repeat loci in IWP species was relatively successful. Five of 7 AAT-repeat loci (71.43%) were successfully amplified in IWP *Acropora* (Table 1). Comparing flanking regions of IWP species with *A. palmata*, the *p*-distances showed high similarities which ranged 89.2%-100% (Table 2). This finding indicates that these AAT-repeat loci in IWP species are homologous to those in *A. palmata*. These 5 loci in IWP species showed the core motif of AAT repeats (Table 2) as indicated in the Caribbean species, *A. palmata* (Baums et al. 2005a). In contrast to the perfect AAT motif in *A. palmata*, most of the core motifs in IWP species were imperfect AAT repeats containing point mutations (Table 2).

Table 2. Core motifs and similarities of the AAT microsatellite flanking regions between Indian-West Pacific Ocean (IWP) and Caribbean *Acropora*

Locus	Species	Core repeat	Flanking region length (bp)	Identity (%)
Acr166	*A. humilis*	n.a.	n.a.	n.a.
	A. hyacinthus	$(AAT)_{10}AGTAAT$	99	89.2
	A. muricata	$(AAT)_4AAC(AAT)_4$, $(AAT)_6AAC(AAT)_7AGTAAT$	99	91.8
	A. valida	n.a.	n.a.	n.a.
	A. palmata[a]	$(AAT)_{28}$	84	100
Acr180	*A. humilis*	n.a.	n.a.	n.a.
	A. hyacinthus	$(AAT)_{10}$, $(AAT)_9AACAAT$, $(AAT)_{15}(AAG)_{11}AAT$	77	98.8
	A. muricata	$(AAT)_4TATACT(AAT)_{12}$	77	98.5
	A. valida	$(AAT)_{18}$, $(AAT)_{21}$	77	98.4
	A. palmata[a]	$(AAT)_{19}$	77	100
Acr181	*A. humilis*	n.a.	n.a.	n.a.
	A. hyacinthus	n.a.	n.a.	n.a.
	A. muricata	$(AAT)_{18}$	126	96.9
	A. valida	$(AAT)_6GAT(AAT)_5ATT(AAT)_3$, $GAT(AAT)_{15}$	118	96.9
	A. palmata[a]	$(AAT)_{10}$	122	100
Acr182	*A. humilis*	$(AAT)_9ATTAATTATAAT$	108	97.1
	A. hyacinthus	$(AAT)_3AAA(AAT)_{14}$, $(AAT)_{27}$	108	98
	A. muricata	$(AAT)_{15}$, $(AAT)_{16}$	108	100
	A. valida	$(AAT)_{16}$, $(AAT)_3AAA(AAT)_{14}$	108	97.8
	A. palmata[a]	$(AAT)_{18}$	111	100
Acr207	*A. humilis*	$(AAT)_5$,$(AAT)_8$	101	97.7
	A. hyacinthus	$(AAT)_5AGT(AAT)_6$, $(AAT)_{11}ACTAAT$	109	98.1
	A. muricata	$(AAT)_5AGT(AAT)_6$, $(AAT)_5(AGT)_2(AAT)_5$	109	98.6
	A. valida	$(AAT)_5$, $(AAT)_2GAT(AAT)_3(GAT)_2(AAT)_3ATT(AAT)_{14}$	109	97.7
	A. palmata[a]	$(AAT)_{12}$	98	100
	Average			97

n.a., PCR was not successful in that species, thus comparisons were not available. [a]Baums et al. (2005a)

Mendelian inheritance test of 8 microsatellite loci

Eight microsatellite loci, Acr1-4, Acr1-60, Acr53, Acr166, Acr180, Acr181, Acr182, and Acr207, were examined for Mendelian inheritance. Numbers of scorable larval genotypes of sets A × B and C × D were 69-92 and 46-64, respectively. Results of the crossing experiments showed 4 inheritance types (Table 3). First, both parental colonies were heterozygous and included 4 different alleles. The ratio of the 4 different larval genotypes was 1: 1: 1: 1; for example, A × B at loci Acr1-4 and Acr180, and C × D at loci Acr166, Acr180, and Acr182. Second, both parental colonies were heterozygous but identical in genotypes. Therefore, the ratio of larval genotypes was 1: 2: 1 which was exhibited by C × D at locus Acr181. The 3rd pattern was one of parental colonies being homozygous and another being heterozygous which led to a ratio of larval genotypes of 1: 1. For example, A × B at loci Acr53, Acr166, and Acr181, and C × D at loci Acr1-4 and Acr1-60. In the 4th pattern, the ratio of larval genotypes could not be calculated due to homozygosity in both parental colonies. This pattern was observed for A × B at loci Acr1-60 and Acr207, and C × D at loci Acr53 and Acr207. Most loci fit the Mendelian law, except for 1 set (A × B) at locus Acr182 ($p < 0.001$).

Mutation rates of the 2 microsatellite motifs

In order to examine the molecular characteristics of the 2 microsatellite motifs, the mutation rate and population differentiation were estimated. The mutation rate is represented by θ, because it is in direct proportion to the mutation rate. θ values ranged from 2.44 for Acr53 to 53.33 for Acr1-60 in *A. muricata* and from 14.52 for Acr53 to 49.04 for Acr1-60 for GT-repeat loci. For AAT-repeat loci, θ values ranged from 46.18 for Acr166 to 112.82 for Acr207 in *A. muricata* and from 39.94 for Acr166 to 85.10 for Acr180 (Fig. 1). Significant differentiation between the 2 motif types was shown in *A. muricata* ($p = 0.029$) but not in *A. digitifera* ($p = 0.184$).

Genetic variation of GT and AAT microsatellites in *A. muricata* and *A. digitifera*

The range of allele sizes of the GT-repeat loci was smaller than that of AAT-repeat loci (Table 4). Average numbers of samples of *A. muricata* and *A. digitifera* were 61.75 and 72.25, respectively. Numbers of alleles ranged 87-213 in *A. muricata* and 86-243 in *A. digitifera* among the 8 microsatellite loci, and average numbers of alleles in *A. muricata* and *A. digitifera* were 16.6 and 19.4. The mean expected and observed heterozygosities of *A. muricata* were 0.820 and 0.546; those of *A. digitifera* were 0.872 and 0.559, respectively (Table 4). Departure from HWE was present in most loci except Acr53 and Acr1-4 in *A. muricata* ($p < 0.001$). The majority of loci were recognized as having a heterozygous deficit except at loci Acr53, Acr1-4, and Acr1-53 in *A. muricata* ($p < 0.001$).

Results of the Mann-Whitney *U*-test revealed significant differences in allelic frequencies between *A. digitifera* and *A. muricata* at loci Acr53, Acr1-60, Acr180, and Acr181 (Table 4). Results of the AMOVA showed higher within-population variation at AAT-repeat loci (98.6%) than at GT-repeat loci (78.7%). Between *A. muricata* and *A. digitifera*, F_{ST} values of the 3 GT-repeat loci ($F_{ST} = 0.213$, $p < 0.001$) were 15 times higher than those of AAT-repeat loci ($F_{ST} = 0.014$, $p < 0.05$), which was attributed to 5 loci. Overall, the level of differentiation between *A. muricata* and *A. digitifera* was significant ($F_{ST} = 0.089$, $p < 0.001$), suggesting that a clear species boundary existed between the sympatric species of Togian I., Sulawesi, Indonesia.

DISCUSSION

Microsatellite markers, Mendelian inheritance test, and cross-specific amplification from different ocean provinces

Sixteen dinucleotide-repeat (GT/CT-repeat) clones isolated from approximately 50,000 clones of 2 partial plasmid libraries represented a frequency of success of merely 3.2×10^{-4}, suggesting a very low abundance of dinucleotide microsatellites in the genome of *Acropora*. A previous study showed that microsatellite loci are difficult to obtain in the Acroporidae due to technical difficulties and a low genomic density (Márquez et al. 2000). An abundance of different types of motif repeats was observed, which might explain why microsatellite probes are difficult to develop (Baums et al. 2005a). Recently, 191 microsatellites from 10,258 expressed sequence tags and 618 microsatellites from 14,625 whole genome sequences were successfully isolated from *A. millepora* (Wang et al. 2009). Among

Table 3. Chi-squared analysis of larval genotypes observed from *Acropora muricata* crosses at 8 microsatellite loci. Alleles were shown by their amplification product size (bp)

			Parental genotype				No. of larval genotypes observed			Expected ratio	Chi-square
Motif type	Locus	Cross	Father	Mother	No. of progeny						*p* value
GT	Acr53	$A_M \times B_F$	159/159	163/159	36	20 (163/159)	16 (159/159)			01:01	0.5
		$B_M \times A_F$	163/159	159/159	33	15 (163/159)	18 (159/159)			01:01	0.6
		Total			69	35	34			01:01	0.9
		$C_M \times D_F$	159/159	159/159	32	32 (159/159)					
		$D_M \times C_F$	159/159	159/159	30	30 (159/159)					
		Total			62	62					
GT	Acr1-4	$A_M \times B_F$	161/157	171/163	36	11 (171/161)	9 (171/157)	7 (163/161)	9 (161/157)	1:1:1:1	0.83
		$B_M \times A_F$	171/163	161/157	37	4 (171/161)	13 (171/157)	7 (163/161)	13 (161/157)	1:1:1:1	0.09
		Total			73	15	22	14	22	1:1:1:1	0.38
		$C_M \times D_F$	147/147	163/147	32	14 (163/147)	18 (147/147)			01:01	0.48
		$D_M \times C_F$	163/147	147/147	30	17 (163/147)	13 (147/147)			01:01	0.47
		Total			62	31	31			01:01	1
GT	Acr1-60	$A_M \times B_F$	194/194	194/194	39	39 (194/194)					
		$B_M \times A_F$	194/194	194/194	48	48 (194/194)					
		Total			87	87					
		$C_M \times D_F$	194/194	200/194	32	16 (200/194)	16 (194/194)			01:01	1
		$D_M \times C_F$	200/194	194/194	31	18 (200/194)	13 (194/194)			01:01	0.37
		Total			63	34	29			01:01	0.53
AAT	Acr166	$A_M \times B_F$	149/128	152/152	35	16 (152/149)	19 (152/128)			01:01	0.61
		$B_M \times A_F$	152/152	149/128	46	19 (152/149)	27 (152/128)			01:01	0.24
		Total			81	35	46			01:01	0.22
		$C_M \times D_F$	152/143	152/149	32	8 (152/152)	6 (152/149)	6 (152/143)	12 (149/143)	1:1:1:1	0.39
		$D_M \times C_F$	152/149	152/143	32	8 (152/152)	11 (152/149)	6 (152/143)	7 (149/143)	1:1:1:1	0.63
		Total			64	16	17	12	19	1:1:1:1	0.65
AAT	Acr180	$A_M \times B_F$	142/109	142/130	39	6 (142/142)	13(142/130)	10(142/109)	10 (130/109)	1:1:1:1	0.47
		$B_M \times A_F$	142/130	142/109	40	8 (142/142)	12(142/130)	7 (142/109)	13 (130/109)	1:1:1:1	0.46
		Total			79	14	25	17	23	1:1:1:1	0.26
		$C_M \times D_F$	130/109	130/121	31	6 (130/130)	11 (130/121)	5 (130/109)	9 (121/109)	1:1:1:1	0.4
		$D_M \times C_F$	130/121	130/109	32	8 (130/130)	10(130/121)	5 (130/109)	9 (121/109)	1:1:1:1	0.63
		Total			63	14	21	10	18	1:1:1:1	0.22
AAT	Acr181	$A_M \times B_F$	182/161	161/161	45	21 (182/161)	24 (161/161)			01:01	0.64
		$B_M \times A_F$	161/161	182/161	47	20 (182/161)	27 (161/161)			01:01	0.3
		Total			92	41	51			01:01	0.3
		$C_M \times D_F$	164/161	164/161	24	10 (164/164)	9 (164/161)	5 (161/161)		01:02:01	0.17
		$D_M \times C_F$	164/161	164/161	22	5 (164/164)	10(164/161)	7 (161/161)		01:02:01	0.76
		Total			46	15	19	12		01:02:01	0.41
AAT	Acr182	$A_M \times B_F$	157/139	157/139	45	26 (157/157)	11 (157/139)	8 (139/139)		01:02:01	< 0.001*
		$B_M \times A_F$	157/139	157/139	47	20 (157/157)	17(157/139)	10(139/139)		01:02:01	0.02
		Total			92	46	28	18		01:02:01	< 0.001*
		$C_M \times D_F$	163/148	157/148	30	7 (163/157)	7 (163/148)	7 (157/148)	9 (148/148)	1:1:1:1	0.94
		$D_M \times C_F$	157/148	163/148	31	5 (163/157)	4 (163/148)	6 (157/148)	16 (148/148)	1:1:1:1	0.01
		Total			61	12	11	13	25	1:1:1:1	0.04
AAT	Acr207	$A_M \times B_F$	145/145	145/145	38	38 (145/145)					
		$B_M \times A_F$	145/145	145/145	34	34 (145/145)					
		Total			72						
		$C_M \times D_F$	145/145	145/145	29	29 (145/145)					
		$D_M \times C_F$	145/145	145/145	20	20 (145/145)					
		Total			49						

A_M, sperm (male) of colony A; B_F, egg (female) of colony B; C_M, sperm (male) of colony C; D_F, egg (female) of colony D. *$p < 0.001$ following the Bonferroni correction.

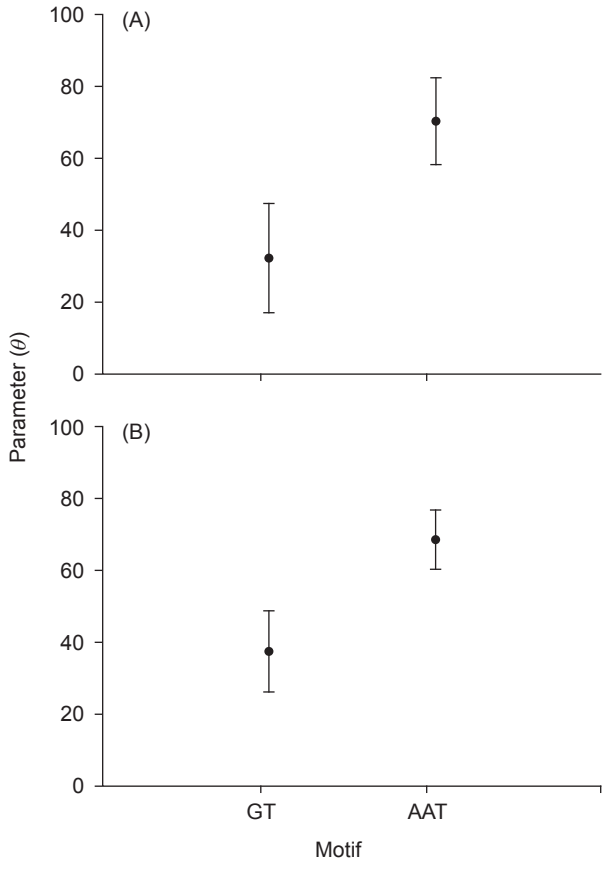

Fig. 1. Two different microsatellite motifs in different *Acropora* species. The mean and standard errors of the mutation rate (θ) for microsatellite loci grouped by motif type: (A) data from *Acropora muricata* and (B) data from *A. digitifera*.

these microsatellites, trinucleotide repeats (66% in EST and 57.9% in WGS) were the most frequently observed motif. Within the trinucleotide repeats, AAT was the most abundant motif (38.89% in EST and 63.38% in WGS of all trinucleotide repeats). In contrast, dinucleotide repeats were only < 1/2 (21.5% in EST and 23.1% in WGS) of their relative abundances of the trinucleotide counterpart. This suggests that the *Acropora* genome is unique and contrasts with the general observation that dinucleotides are more abundant in animal genomes.

In this study, 7 trinucleotide-repeat (AAT-repeat) loci and 5 dinucleotide-repeat (GT/CT-repeat) loci were assessed in IWP and Caribbean *Acropora* species. Three of the 5 dinucleotide-repeat microsatellite loci with the GT/CT-repeat motif were isolated from the IWP species, *A. muricata*, which were stably amplified among IWP species, while none was amplified in Caribbean species. This suggests the null-allele effect of these GT/CT loci in Caribbean species, i.e., the GT/CT core repeats still exist in the loci, but the flanking regions of these loci in the Caribbean species accumulated enough mutations after divergence from the common ancestor of the IWP *Acropora* to cause the failure of primer binding in PCRs. In contrast, 5 of 7 AAT-repeat loci previously developed from Caribbean species were successfully amplified in IWP samples, which

Table 4. Characteristics of eight microsatellite loci and comparisons of allele frequencies between *Acropora muricata* and *A. digitifera* in the Togian Is. using the Mann-Whitney *U*-test

Locus	Species	No. of samples	Allele size range (bp)	No. of alleles	Ho	He	*U* value
Acr53	*A. muricata*	62	159-171	7	0.233	0.27	
	A. digitifera	74	161-193	9	0.292	0.698	1382.5*
Acr1-4	*A. muricata*	62	137-201	14	0.8	0.844	
	A. digitifera	72	129-243	21	0.708	0.899	8862.5
Acr1-60	*A. muricata*	62	182-213	14	0.783	0.873	
	A. digitifera	73	171-233	21	0.646	0.801	6134.5*
Acr166	*A. muricata*	62	87-162	17	0.5	0.876	
	A. digitifera	72	96-162	17	0.554	0.903	8862.5
Acr180	*A. muricata*	61	92-158	18	0.55	0.923	
	A. digitifera	73	86-167	20	0.538	0.908	6614.0*
Acr181	*A. muricata*	62	125-197	23	0.467	0.939	
	A. digitifera	69	122-239	23	0.6	0.907	3415.5*
Acr182	*A. muricata*	62	129-183	17	0.667	0.918	
	A. digitifera	73	123-195	20	0.677	0.919	8869.5
Acr207	*A. muricata*	61	101-188	23	0.367	0.919	
	A. digitifera	72	110-188	24	0.462	0.937	7861.5

*$p < 0.05$.

indicates that the flanking regions of AAT-repeat loci have evolved relatively more slowly than those of GT/CT-repeat loci in the genus *Acropora*. This is supported by the high similarities (with an average of 97%) of flanking regions between IWP and Caribbean species. However, variable success in amplifying these AAT loci is also expected depending on the PCR conditions or species. AAT-repeat loci were tested in 2 other IWP *Acropora* species, *A. millepora* and *A. nobilis*, but fewer loci were successfully amplified (van Oppen et al. 2007, Isomura and Hidaka 2008). In contrast, Nakajima et al. (2009a b) successfully applied 3 of the AAT loci (Acr166, Acr181, and Acr182) to the genetic connectivity of *A. digitifera* and *Acropora* sp 1.

Microsatellite markers might not necessarily show co-dominant inheritance (Dobrowolski et al. 2002). Eight AAT microsatellite markers were developed for *A. palmata* (Baums et al. 2005a). Of those, only 5 had simple Mendelian inheritance as shown by controlled crosses (Baums et al. 2005a). Genotyping larvae produced from controlled crossing in this study indicated that all microsatellite markers exhibited Mendelian inheritance in *A. muricata*, with the exception of the coral-specific but partial set (A × B) at locus Acr182 which deviated from Mendelian expectations. However, another set (C × D) at locus Acr182 showed correspondence with Mendelian inheritance. The Mendelian inheritability at locus Acr207 needs to be further confirmed by other crossing experiments due to homozygosity in the 2 sets of colonies used for the crossing experiment.

High mutation rates and low pairwise differentiation of AAT loci

Data mining by EST and WGS can be an alternative way to obtain a large quantity of microsatellite loci, thus resolving the difficulty of isolating microsatellites by Southern hybridization and enrichment for corals (Wang et al. 2009). However, trinucleotide repeats, particularly AAT, are the most abundant motifs in the EST and WGS of *A. millepora*, raising concerns of selective constraints of trinucleotide motifs (Goldstein and Schlötterer 2000), and evaluation of their molecular patterns is needed before their application to genetic diversity studies of corals.

Two distinct molecular characteristics were observed in *Acropora* AAT-repeat loci when compared to GT/CT-repeat loci. First, higher mutation rates were observed at AAT-repeat loci than at GT/CT-repeat loci in *Acropora*, although a statistical difference was observed in *A. muricata* but not in *A. digitifera*. In general, the mutation rates of dinucleotide-repeat loci were higher than those of trinucleotide-repeat loci, but an exception can occur when trinucleotide-repeat loci are related to disease causes, i.e., a functional association (Chakraborty et al. 1997). Comparisons between AAT- and GT-repeat microsatellites in *Acropora* fit this exception, although the possible functions of these AAT loci remain to be discovered. In addition, the imperfect core motifs at AAT-loci in IWP *Acropora* spp. can be explained by the high mutation rates, which led to an interruption of perfect repeats (Goldstein and Schlötterer 2000). Second, low values of the pairwise *F*-statistics (F_{ST}) were present in AAT-repeat loci. Five AAT-repeat loci examined in *A. palmata* (Baums et al. 2005b) showed low F_{ST} values (0.032-0.04). Low F_{ST} values (-0.006-0.015) were observed in *A. digitifera* on Sekisei Reef, southwestern Japan, using 6 loci, three of which were Caribbean-derived AAT loci (Nakajima et al. 2009b). When these AAT-repeat loci were applied to 2 sympatric IWP *Acropora* spp. for a species boundary examination, the F_{ST} was even 3-4 times smaller (0.014) than that of the *A. palmata* population subdivision. In contrast, the F_{ST} value of GT-repeat loci (0.213) was 15 times higher than that of AAT-repeat loci.

Application of microsatellites to species delineation of *Acropora*

Our study demonstrated that with cautionary evaluation, microsatellites can be potential markers to delineate species boundaries of sympatric *Acropora*. First, allelic frequencies between *A. digitifera* and *A. muricata* significantly differed at loci Acr53, Acr1-60, Acr180, and Acr181 (Table 4). Second, between *A. muricata* and *A. digitifera* F_{ST} values of the 3 GT-repeat loci, 5 AAT-repeat loci, and the combined data all significantly differed, suggesting that species boundaries exist among sympatric species of *Acropora*. Similar approaches successfully uncovered limited hybridization between mixed-stand oak species (Craft et al. 2002) and a Hawaiian silversword alliance for which even an active hybrid zone is present. Natural selection in different habitats might be associated with maintenance of species boundaries and a lack of genetic introgression (Friar et al. 2007). Further application of these loci to species that show cross-specific fertilization under artificial conditions (e.g., *A. hyacinthus* and

A. cyatherea) is needed to confirm the utility of microsatellites in delineating species of *Acropora*.

In conclusion, 12 microsatellite loci including 5 dinucleotide-repeat and 7 trinucleotide-repeat loci were assessed in the genus *Acropora*. Five of the 7 Caribbean-derived AAT microsatellites were successfully amplified in IWP *Acropora* with very high homologies in the flanking regions of these loci. In contrast, 3 IWP-derived GT/CT microsatellites failed to be amplified in Caribbean *Acropora*. Results indicated that GT-repeat loci are restricted to IWP species, but AAT-repeat loci are common to both Caribbean and IWP species. Trinucleotide-repeat loci may have functional constraints due to the high mutation rates and within-population variations compared to dinucleotide-repeat loci. The F_{ST} values of 3 GT-repeat loci, 5 AAT-repeat loci, and the combined data all significantly differed, suggesting that species boundaries exist among sympatric species of *Acropora*. With careful evaluation of molecular evolution of different motifs and awareness of their limitation, microsatellites can serve as potential markers for species boundary examinations among sympatric *Acropora* species.

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