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SHORT COMMUNICATION

Eight polymorphic microsatellite loci for the Indo-Pacific-wide zoanthid, *Zoanthus sansibaricus*

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Abstract Next generation sequencing allows rapid development of genetic markers for investigating the ecology and evolution of non-model organisms. In the present study we employed 454 sequencing and high through-put screening to ultimately generate eight highly polymorphic microsatellites for *Zoanthus sansibaricus*, a common colonial anemone in the order Zoantharia that is widely distributed throughout the Indo-Pacific. Each locus was screened against cultured *Symbiodinium* as well as on individuals harboring distantly related symbiont species to discount contamination by DNA from their obligate dinoflagellate symbionts. The range of *Z. sansibaricus* extends across the tropical and subtropical Indian and Pacific oceans. Therefore, these population genetic markers will allow examination of dispersal and gene flow in this species across large tropical and subtropical oceanic ranges.

Keywords Microsatellite · *Zoanthus* · Next generation sequencing · Marker development

Introduction

Members of the genus *Zoanthus* are common in shallow water tropical and sub-tropical environments world wide, often occurring on reef crests and shallow reef fronts, where they may dominate the benthic landscape (Irei et al. 2011). Like most tropical zoanthids, they maintain obligate symbioses with various species of photosynthetic dinoflagellate (*Symbiodinium* spp.), which may allow them to occupy different reef photic zones (Reimer et al. 2006; Kamezaki et al. 2012). Within this genus, *Zoanthus sansibaricus* Carlgren, 1900 is one of the most common species in the tropical Pacific and Indian Oceans (type locality = Zanzibar; reports from Japan: Reimer et al. 2004; Galapagos: Reimer and Hickman 2009, New Caledonia: Sinniger 2006, Singapore: Reimer and Todd 2009, etc.). Phylogenetic evidence from Cytochrome oxidase 1 indicate that various morphotypes, once considered as separate species, probably comprise a single widespread species (Reimer et al. 2004). The application of population genetic markers would allow further testing of the species boundaries in this group. Here, we use a next-generation sequencing approach to rapidly generate microsatellite markers. These single-copy, independently assorting nuclear markers provide the ability to examine clonal and population structure as well as identify the existence of reproductive boundaries in this group (i.e. species delimitation).

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Materials and methods

Development of microsatellite library

We used data generated by *454* sequencing at the Penn State University Genomics Core Facility (University Park, PA) to search for repetitive sequence motifs. Isolated DNA from three *Zoanthus sansibaricus* colonies were pooled to increase diversity in the library. A genomic library was prepared from 3,000 ng of double-stranded DNA and prepared using the Nextera DNA Sample Prep Kit (Epicentre Biotechnologies, Madison, WI) and sequenced on a 454 GS-FLX sequencer (Roche Diagnostics Corporation, Indianapolis, IN) utilizing the Titanium Sequencing Kit (Roche). Sequences containing di-, tri- or tetranucleotide repeat motifs with a minimum copy number of seven, as well as a minimum of 20 flanking

nucleotides up and downstream of the repeat, were identified using the Tandem Repeat Database (Gelfand et al. 2006). The software Primer3 (http://primer3.sourceforge.net/) was used to design 96 PCR primer sets from these putative microsatellite sequences of *Zoanthus sansibaricus.*

Screening for variable loci

Ninety-six primer sets (i.e. loci) were then screened using DNA from eight *Zoanthus sansibaricus* specimens, representing a cross section of the species range: from Otsuki (Kochi), Kushimoto (Wakayama), Yoron (Kagoshima) and Manza (Okinawa), Japan. PCR reactions were preformed using 1 μl reaction solution containing 25–50 ng template DNA in a standard Taq buffer (New England Biolabs; 10 mM Tris-HCl [pH 8.3], 50 mM KCl, 2.5 mM MgCl) and 0.075 U/μl of Taq (New England Biolabs) and bovine serum albumin (BSA, 0.1 mg/ml). Primer concentrations of 0.2 μM tailed forward primer (Table 1), 1 μM reverse primer, and 1 μM dye-labeled T-oligonuclotide were used for amplifying PCR products. All loci were amplified using the following touchdown thermal cycle profile: 94 °C for 2 min (1 cycle); 94 °C for 30 s, 5 °C above the primer-specific annealing temperature (Table 1) for 30 s decreased by 0.5 °C per cycle, 72 °C for 30 s (10 cycles) 94 °C for 30 s, primer-specific annealing temperature for 30 s, 72 °C for 30 s (20 cycles); 72 °C for 5 min, on a Mastercycler gradient thermal cycler (Eppendorf, Westbury, NY). The PCR product was then screened by capillary gel electrophoresis (Penn State University Genomics Core Facility, University Park, PA). Primer sets that returned repeatable, polymorphic peaks that were >1,000 relative florescent units in intensity and within the size range expected were retained for further analysis. Screening yielded a total of ten polymorphic microsatellite loci from eight individuals initially examined.

Testing for "contaminant" loci that amplify *Symbiodinium*

The target organism, *Zoanthus sansibaricus,* maintains an obligate symbiotic relationship with photosynthetic dinoflagellates in the genus *Symbiodinium*. To insure against cross amplification of *Symbiodinium*, we tested loci on cultured *Symbiodinium* cells as well as *Zoanthus* samples with divergent Symbiodinium types. Tests were performed on *Symbiodinium goreaui* cultures (rt113 and rt152 ITS2 type C1 *sensu* LaJeunesse, 2001, from the Robert K. Trench collection) and *Zoanthus sansibaricus* samples collected from Hachijojima, Tokyo, Japan that harbored a Clade D symbiont. Two loci produced strong amplification products from the DNA of cultured *Symbiodinium* and also failed to amplify the *Z. sansibaricus* samples from Hachijojima. Therefore, two of ten loci were markers that amplified *Symbiodinium* Clade C, which were symbionts in the animals used to generate these loci. The remaining eight loci, which did not amplify cultured symbiont DNA, amplified in the sample obtained from Hachijojima confirming their host origin.

Zoanthus sansibaricus collections for population genetic analysis

Zoanthus sansibaricus samples were collected from Okinawa-Jima Island, Japan in June 2011. Two sites were

Table 1 Locus name, repeat motif, primer sequences, annealing temperature (T_a) and approximate size range of expected product for nine microsatellite loci for *Zoanthus sansibaricus*

Locus name	Fragment length	Motif	Primer sequences (5′–3′)	T_a (°C)	Accession number
Zsan_2	186–259	TAA	F: **M13-**ATCGTGCAGCGACCTAAATA R: AGTTTGAAGGGCCTGAACTT	56	JX129533
Zsan_14	72–114	ATTT	F: **M13-**CTTTTTACTCTGCACGAGGG R: TGAGGTTGCACAAACGTAGA	54	JX129529
Zsan_30	119–167	TTA	F: **M13-**ATTATTAGGCCTTTGGCTGG R: CGGAGGTTTACCGAGAAAAT	55	JX129527
Zsan_67	131–155	TA	F: **M13-**ATGGGGTAATTAAACGCACA R: CACACACACACACACGTGAC	56	JX129526
Zsan_71	121–165	TAT	F: **M13-**ATTTCTTGTGGTGAAGCTGG R: AGCTTTTCCCACTTTCGTTT	53	JX129528
Zsan_102	150–190	TAA	F: **M13-**AAATTTGAAGCATTTTCGCA R: TAGCAGGCCCTCACTGTAAT	55	JX129522
Zsan_104	150–215	TAT	F: **M13-**AAAACATTCTCTGCAAACCG R: GCAGACAAGTGCTGCCTTAG	55	JX129523
Zsan_130	130–172	TGA	F: **M13-**CAAGCGAAAGTTGACTGGTT R: GCCGCATAACTCCATTTGTA	55	JX129531

Primers were developed from 454 sequence data and amplified with M13 tail sequences (TGTAAAACGACGGCCAGT) on the 5′ ends of each forward primer amplifications included these primers along with separate dye-labeled M13 oligonucleotides

Table 2 Number of alleles, Observed and expected heterozygosity for samples collected at Manza

Locus name	Number of alleles	Heterozygosity observed	Heterozygosity expected
Zsan_2	24	0.621	0.952[a]
Zsan_14	8	0.300	0.700[a]
Zsan_30	25	0.677	0.952[a]
Zsan_67	9	0.538	0.704[a]
Zsan_71	16	0.839	0.924[a]
Zsan_102	21	0.679	0.927[a]
Zsan_104	21	0.556	0.944[a]
Zsan_130	17	0.615	0.879[a]

[a]Significant departure from HWE with a Bonferroni-corrected alpha of 0.0063912

chosen, one on the west side of the island facing the East China Sea (Manza; n=31), and the other on the east side facing the Pacific Ocean (Teniya; n=31). Samples were collected by SCUBA diving and snorkeling from the intertidal zone to a maximum depth of 3 m. Small clusters of 1–30 polyps/colony were individually bagged and labeled at the collection site. Colonies were separated by 5–100 m. Upon returning to the University of the Ryukyus, samples were transferred into 99 % ethanol for preservation. DNA was then extracted using a Qiagen DNA isolation kit from a single polyp from each sample .

The allelic variation of each locus was evaluated from a total of 62 samples. Alleles were scored using GeneMarker v. 1.91 (SoftGenetics). The number of alleles per collection site ranged from 6 to 25. Tests for departure from Hardy-Weinberg expectations (HWE) as well as linkage disequilibrium (LD) were preformed by GenPop on the Web http://genepop.curtin.edu.au/ (Raymond and Rousset 1995). Departure from HWE was observed in all loci at Manza (Table 2) and at most loci at Tenyia (Table 3) caused by a heterozygote deficiency. These highly variable loci spanned large size ranges. PCR is biased toward smaller fragments, the drop out of large alleles therefore might explain the observed deficiency in heterozygotes. Tests of LD showed no significant (Bonferroni-corrected alpha to 0.0018302) pattern of linkage among loci.

Conclusion

The markers generated in this study will be used in the investigation of connectivity, diversity, species identity and clonality of *Zoanthus sansibaricus*. Although not reef building, *Z. sansibaricus* is extremely widespread, with longitudinal distributions from the Red Sea and eastern coast of Africa to the Hawaiian Islands and the Galapagos, and extends into sub-tropical environments, spanning latitudes from mainland Japan to the southern Great Barrier Reef (Reimer et al. 2006; Reimer and Hickman 2009). As such, investigations into patterns of clonal structure and connectivity between populations of *Z. sansibaricus* and its symbionts may be useful in examining patterns of relative connectivity and host-symbiont co-evolution across this entire region. The genotypic diversity of *Z. sansibaricus* observed in this small dataset was greater than the genotypic diversity reported for *Acropora palmata* and *Pocillopora* spp., two scleractinians with wide geographic ranges and limited population genetic structure (Baums et al. 2005a, 2005b; Pinzón and LaJeunesse 2011). This remarkable degree of diversity would suggest a large effective population size perhaps from a high degree of connectivity across large geographic areas due to potentially long planktonic larval stages (Polak et al. 2011; Hirose et al. 2011). Investigation of gene flow across seemingly disjunct geographic locations will be an important area of future research.

Table 3 Number of alleles, Observed and expected heterozygosity for samples collected at Teniya

Locus name	Number of alleles	Heterozygosity observed	Heterozygosity expected
Zsan_2	21	0.786	0.937
Zsan_14	6	0.536	0.638[a]
Zsan_30	24	0.552	0.929
Zsan_67	6	0.500	0.752[a]
Zsan_71	13	0.704	0.857
Zsan_102	13	0.480	0.846[a]
Zsan_104	15	0.621	0.927[a]
Zsan_130	15	0.607	0.884[a]

[a]Significant departure from HWE with a Bonferroni-corrected alpha of 0.0063912

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