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EXISTENCE OF INTRA-COLONIAL PARALOGUES OF THE RIBOSOMAL INTERNAL TRANSCRIBED SPACER (ITS) IMPEDES STUDIES OF INTRA-COLONIAL GENETIC VARIATION IN THE SCLERACTINIAN CORAL *DIPLOASTREA HELIOPORA* (LAMARK 1816)

Kevin K. Y. Lam, Adrian H. B. Loo, Peter A. Todd, Fook Tim Chew and **Loke Ming Chou**
Department of Biological Sciences, Faculty of Science, The National University of Singapore, Science Drive 4, Singapore 117543, Republic of Singapore

***ABSTRACT.* –** The implications of intra-colonial genetic variation in corals are relevant to any research that treats within-colony fragments or tissue samples as genetically identical. We used the fast evolving internal transcribed spacer (ITS) gene region to examine genetic structure among polyps within colonies of *Diploastrea heliopora*, a common and long-lived Indo-Pacific massive coral. Five polyps, ~ 40 cm apart, were removed from each of nine *D. heliopora* colonies found on three of Singapore's southern reefs. Based on initial coral sequences we designed ITS primers specific to *D. heliopora*, producing successful PCR reactions for DNA extracted from eight polyps. From these eight polyps, 43 good quality clonal sequences were obtained. Sequence variations, or paralogues, were observed in clones derived from individual polyps. A parsimony network using 30 non-truncated sequences showed that, even though there appeared to be some grouping of haplotypes in relation to natal reef, occasionally clones from the same polyp were more distantly related to each other than clones from colonies >10 km apart. The presence of high paralogue levels suggests that ITS is not suitable for studies of intra-colonial genetic variation for organisms such as corals where hybridization and other mechanisms may work against concerted evolution to homogenize the ITS sequences.

***KEY WORDS.* –** Singapore, paralogue, *Diploastrea heliopora*, intra-colonial genetic variation.

INTRODUCTION

Intra-colonial variation in morphology, fecundity, and pigmentation has been recorded in numerous species of hard coral (Kawaguti, 1937; Harrison & Wallace, 1990; Veron, 1995; Veron, 2000) and the possibility that the genetic composition also varies among polyps within colonies has been discussed (Fautin, 1997; Lasker & Coffroth, 1999). Two mechanisms could potentially yield colonies with more than one genotype. One is allogenic fusion, where corals with different genotypes, usually juveniles, coalesce and grow together (Fautin, 1997; Veron, 2000). Alternatively, as scleractinian corals can reach great ages, mutations in somatic tissue might occur, creating a mosaic of genotypes within the same colony (Hughes et al., 1992; Fautin, 1997; Orive, 2001). Investigations that involve transplantation of multiple coral fragments from individual colonies, e.g. studies of phenotypic plasticity (Bruno & Edmunds, 1997; Muko et al., 2000; Todd et al., 2002; Todd et al., 2004a, b), usually assume that the fragments are genetically identical, however due to the issues just raised, this supposition may be flawed. Any research that treats within-colony fragments or tissue samples as clones, or uses molecular data for population investigations or coral systematics, should be aware of the potential for genetic differentiation within a colony.

Diploastrea heliopora is easy to identify *in situ* (Veron, 2000) and common on the reefs south of mainland Singapore (Todd et al., 2004a). *D. heliopora* has a growth rate of just 2-6mm yr^{-1} (Schuhmacher et al., 2002. Watanabe et al., 2003) and can attain colony sizes in excess of 7 m diameter (Veron et al., 1977), indicating potential ages of 100's to >1000 years (Schuhmacher et al., 2002). This extreme longevity means that there is potential for somatic mutations to occur (Orive, 2001). To explore this possibility, we used the fast evolving internal transcribed spacer (ITS) gene region to identify intra-colonial genetic variation in *D. heliopora*. The ITS regions of the rRNA is a popular nuclear marker used in recent molecular studies of cnidarians (Odorico & Miller, 1997; Medina et al., 1999). ITS sequences have also provided evidence for introgression in the coral genera *Madracis* (Diekmann et al., 2001.) and *Acropora* (van Oppen et al., 2002.). More recently, ITS has been used with microsatellite data in the deep-sea coral *Lophelia pertusa* to investigate population structure and gene flow (Le Goff-Vitry et al., 2004). ITS is described as a 'double-edged tool' for eukaryote

evolutionary comparisons (Coleman, 2003) because of high variability in the sequence. However, utilisation of the secondary structure of the rRNA to improve the alignment allows the same marker to be used for both species-level comparison and mega-systematics (Schultz, et al., 2005). In this study we used the sequence identity of the *D. heliopora* ITS genes to ascertain genetic homogeneity/heterogeneity among polyps within individual colonies. Use of the conserved secondary structure as a aid to ITS2 alignment was not considered here because we have amplified the ITS1, 5.8s nrDNA and ITS2 as a single fragment. The conserved region of the 5.8s nrDNA aided proper alignment considerably.

MATERIAL AND METHODS

Diploastrea heliopora was sampled from three haphazardly selected colonies from each of three reefs off the southern coast of Singapore, i.e. Cyrene Reef (1º15'N 103º45'E), Pulau [=Island] Hantu (1º13'N 103º45'E) and Raffles Lighthouse (1º10'N 103º45'E). Full descriptions of these sites can be found in Todd et al. (2004a). Using a hammer and small chisel, five living single-polyp samples (~ 40 cm apart on a ~2 m high colony) were removed along a vertical transect laid out on the coral with a fiberglass measuring tape. A total of 45 polyps (five per colony) were collected from three colonies from each of the three reefs. A voucher specimen was collected for each colony (KYLamC2, KYLamP1, KYLamR1 and KYLamR2; coll. K. Y. Lam and J. R. Guest Aug. 15. 2002.) and deposited in the Zoological Reference Collection (ZRC) of the Raffles Museum of Biodiversity Research, National University of Singapore.

Approximately 200 mg (fresh weight) of coral was flash-frozen in liquid nitrogen and homogenized using a pestle and mortar. The powder was incubated in 1 ml cetyltrimethylammonium bromide (CTAB) buffer (1.875% CTAB, 37.5 mM Tris HCl, 7.5 mM EDTA, 1.3 m NaCl, 2.0 mg/ml PVP). 100 µl of 20% SDS and 2 µl of ß-mercaptoethanol were added fresh and the mixture was incubated at 60°C for 30 min (inverting every 10 min). 500 µl of chloroform:isoamyl alcohol (24:1) was added and the mixture centrifuged at 13 000 rpm for 10 min. The supernatant was pipetted out and cleaned using Qiaquick PCR purification kit (Qiagen) according to the manufacturer's recommendations.

All PCR conditions were modified from Medina et al. (1999) with a final extension step of 5 min at 72°C. The universal primers ITS-4 (5'-TCC TCC GCT TAT TGA TAT GC-3') and ITS-1 (5'-TCC GTA GGT GAA CCT GCG G-3') from White et al. (1990) were first used to amplify ITS sequences. The amplicons were cloned and screened by sequencing 10 random clones. Besides coral sequences, the amplicons occasionally consisted of fungal and bacterial sequences, which were rejected. Based on the coral sequences obtained, we designed ITS primers specific to *D. heliopora*, ITSF6 (5'-AAC CTG CGG AAG GAT CAT TAC-3') and ITSR2 (5'-TAG CCT TGC CTG ATC TGA GGT CAA GAC-3'). PCR products were cloned into pGEM®-T Easy vector and transformed into XL1-Blu competent *E. coli* cells (Stratagene). The clones were sequenced from the SP6 and T7 vector promoter sites using the BigDye™ Terminator v3.0 Ready Reaction Cycle sequencing kit on an ABI Prism" 3100 Genetic Analyzer. Approximately ten bacterial colonies were sequenced for each PCR amplification.

The reverse and forward sequences were edited and assembled into a contig using GAP v4.7b4 (Bonfield et al., 1995.). The sequences were then compared against known entries in Genbank using BLAST (Altschul et al., 1997.) to verify that the sequences were coral ITS sequences. Each sequence was named using a code with the first letter signifying the locality, i.e. C for Cyrene, P for Pulau Hantu and R for Raffles Lighthouse. The first numeral indicated the colony, the next letter identified the individual polyp, and the last number denoted the clone (Table 1). The sequences were aligned using ClustalW (Thompson et al., 1994.), manually optimized, and saved in nexus format. A statistical parsimony network algorithm (Templeton et al. 1992) was used to provide a 95% plausible set of all haplotype linkages in an unrooted tree. This algorithm was implemented using the TCS v1.13 program (Clement et al. 2000). Thirty ITS rDNA haplotypes of the 43 sequenced clones for *D. heliopora* were included in the analysis. As gaps were treated as a fifth state in the analysis, 13 sequences that were truncated were excluded from the analysis as the resultant gaps are parsimony informative.

DNA was extracted from 13 Raffles Lighthouse samples, 15 Cyrene Reef samples, and 4 Pulau Hantu samples. The typical yield using the CTAB extraction method was 4.68 µg. However, only eight individual polyps (from four corals - one colony each from Cyrene Reef and Pulau Hantu, and two from Raffles Lighthouse) produced successful PCR reactions. From these eight polyps 43 good quality clonal sequences (submitted to Genbank, Table 1) suitable for further investigation, were obtained.

RESULTS AND DISCUSSION

BLAST analysis confirmed the coral genomic origin of the sequences, i.e. the closest match was most frequently AF483813 (the Faviid coral *Plesiastrea versipora*). We found an average GC content of 56.5%, similar to *P. versipora*'s 55.1% for the same region of ITS sequence. Several sequence variations were observed in clones derived from individual polyps, for example, some of these paralogues had indels of one to 4 bases within the ITS regions. ITS paralogues have been found in a variety of taxa (Ko & Jung, 2002; Wei & Wang, 2004) including corals (van Oppen et al., 2002, Rodriguez-Lanetty & Hoegh-Guldberg, 2002; Vollmer & Palumbu, 2004). Paralogues represent the inability of concerted evolution to homogenize the ITS sequences. Several explanations can be offered for this phenomenon, including recent speciation, maintenance of ancestral polymorphism as a result of the paralogues occupying loci

Table 1. The sampling sites from which the polyps were collected, clones of the ITS sequences, and their Genbank accession numbers. Asterisks denote non-truncated sequences used in the parsimony analysis (Fig. 1).

Sampling site	Specimen polyp	Individual ITS clone	Genbank accession
Cyrene Reef	C2a	C2a10*	AY509698
Cyrene Reef	C2a	C2a11	AY509699
Cyrene Reef	C2a	C2a8*	AY509700
Cyrene Reef	C2a	C2a9*	AY509701
Cyrene Reef	C2b	C2b10*	AY509702
Cyrene Reef	C2b	C2b11*	AY509703
Cyrene Reef	C2b	C2b12*	AY509704
Cyrene Reef	C2b	C2b1*	AY509705
Cyrene Reef	C2b	C2b2*	AY509706
Cyrene Reef	C2b	C2b6*	AY509707
Cyrene Reef	C2b	C2b7*	AY509708
Cyrene Reef	C2b	C2b8*	AY509709
Cyrene Reef	C2b	C2b9*	AY509710
Cyrene Reef	C2d	C2d10*	AY509711
Cyrene Reef	C2d	C2d11	AY509712
Raffles Lighthouse	R1a	R1a2	AY509718
Raffles Lighthouse	R1c	R1c3*	AY509719
Raffles Lighthouse	R1c	R1c4*	AY509720
Raffles Lighthouse	R1c	R1c5	AY509721
Raffles Lighthouse	R1c	R1c6	AY509722
Raffles Lighthouse	R1c	R1c7	AY509723
Raffles Lighthouse	R1c	R1c8*	AY509724
Raffles Lighthouse	R2b	R2b1	AY509725
Raffles Lighthouse	R2b	R2b3*	AY509726
Raffles Lighthouse	R2b	R2b4*	AY509727
Raffles Lighthouse	R2b	R2b5	AY509728
Raffles Lighthouse	R2b	R2b6*	AY509729
Raffles Lighthouse	R2b	R2b7*	AY509730
Raffles Lighthouse	R2c	R2c1*	AY509731
Raffles Lighthouse	R2c	R2c10*	AY509732
Raffles Lighthouse	R2c	R2c2*	AY509733
Raffles Lighthouse	R2c	R2c3*	AY509734
Raffles Lighthouse	R2c	R2c4*	AY509735
Raffles Lighthouse	R2c	R2c5*	AY509736
Raffles Lighthouse	R2c	R2c6*	AY509737
Raffles Lighthouse	R2c	R2c7*	AY509738
Raffles Lighthouse	R2c	R2c8	AY509739
Raffles Lighthouse	R2c	R2c9	AY509740
Pulau Hantu	P1c	P1c10*	AY509713
Pulau Hantu	P1c	P1c11*	AY509714
Pulau Hantu	P1c	P1c12	AY509715
Pulau Hantu	P1c	P1c6	AY509716
Pulau Hantu	P1c	P1c7	AY509717

on different chromosomes, incomplete lineage sorting, prolonged asexual reproduction, and polyploidy (Modrich & Lahue, 1996; Vollmer & Palumbu, 2004). Odorico & Miller (1997) and van Oppen et al. (2002) proposed inter-specific genetic exchange (introgression) as a model for the high sequence diversity observed in two different Acroporid coral groups. The *D. heliopora* colonies used in this study were very old (>1 to >5 m in diameter), and it is possible that prolonged asexual reproduction through polyp fission has maintained the paralogues.

To elucidate the relationship of the ITS clones, a parsimony network was determined using 30 non-truncated sequences (Table 1). The resultant network representation reveals three ITS haplotype clades (I, II and III; Fig. 1); note that it is important to treat this plot with caution - it is used here simply to illustrate the observed paralogy. All Cyrene Reef and Pulau Hantu haplotypes, and four from Raffles Lighthouse (R2b3, R1c8, R1c4 and R1c3) form networks in clades I and II. Clade III consists of the remaining Raffles Lighthouse R2b and R2c polyps. The single R2 haplotype found in clade I is more closely related to the Cyrene Reef colony than other R2 haplotypes originating from the same polyp. Similarly, relative to the network as a whole, the two haplotypes from the Pulau Hantu polyp (P1c10 and P1c11) are only related distantly.

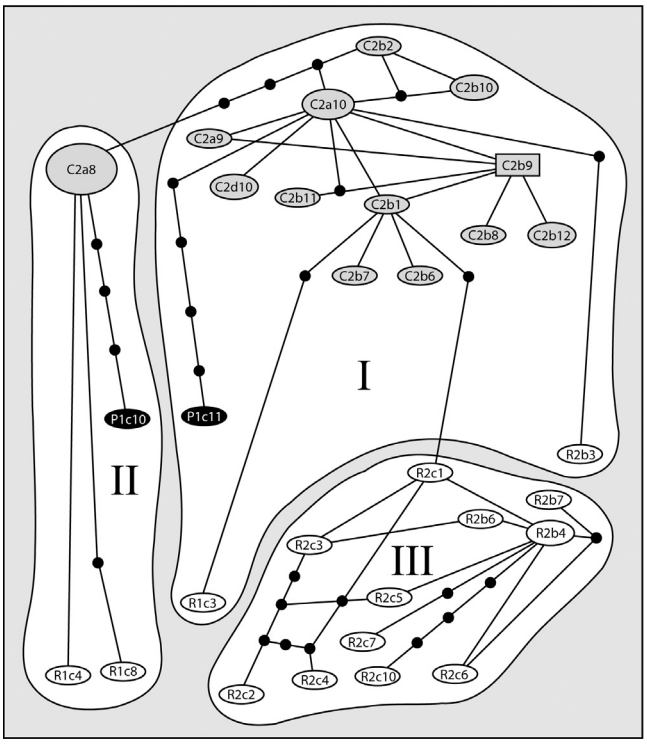

Fig. 1. Unrooted parsimony network of 30 ITS rDNA haplotypes of *Diploastrea heliopora*, representing a 95% plausible set of cladograms. The square represents the haplotype with the highest outgroup probability and the size of the square and ovals correspond with the haplotype frequency. On the connecting lines, each filled circle represents one step linking two haplotypes. To some extent, the ITS rRNA haplotypes are segregated according to their natal sites and have thus been arranged to reflect this geography, i.e. with the haplotypes to the top of the figure originating from Cyrene Reef (C prefix, light shading) and the haplotypes to the bottom of the figure coming from Raffles Lighthouse (R prefix, no shading). The two haplotypes from Pulau Hantu (P prefix, dark shading) have been placed in the middle. Three haplotype clades (I, II and III) are outlined.

As the parsimony network shows that clones from colonies >10 km apart can be more closely related to each other than clones from the same polyp, it is clearly unsatisfactory to use these data to identify among-polyp within-colony genetic variation in *D. heliopora*. The presence of high paralogue levels suggest that ITS is not suitable for studies of intra-colonial genetic variation for organisms such as corals where hybridization and other mechanisms may work against the homogenization of ITS sequences.

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