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Testing the Applicability of Molecular Genetic Markers to Population Analyses of Scleractinian Corals

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Abstract

The abundance of coral reefs worldwide is in decline, and despite the ecological importance of reefs, only a limited number of DNA markers have been identified for scleractinian coral genetic studies. This paper addresses the search for new coral molecular markers and investigates the applicability of the cytochrome c oxidase subunit I (COI), the internal transcribed spacer region 1 (ITS1), and the pocilloporin gene to the question of intraspecific variation in the scleractinian coral *Pocillopora verrucosa* along the southeast African coastline. The COI fragment was 710 bp long and was identical for *P. verrucosa* (n = 10) and *P. damicornis* (n = 3). Only two different ITS1 sequences were found (differing by 13 bp insertion), but more importantly, 24% of the sequences were heterogenous indicating that different multiple copies of the sequence exist. Pocilloporin is an intronless gene that was absolutely conserved throughout all *P. verrucosa* populations (n = 50). Thus, the three DNA regions studied appear unsuitable for the population genetic analyses of *P. verrucosa*.

Keywords: Coral reefs, scleractinian corals, *Pocillopora verrucosa*, COI, ITS, pocilloporin, population genetics

1. Introduction

Recent measures indicate that the abundance of healthy coral reefs worldwide is in decline, with as much as 37% of healthy coral reefs already lost and an expected 30% more to be lost over the next 10 to 30 years (Wilkinson, 2000). The degradation of coral reefs appears to be accelerating, especially as events such as mass coral bleaching have become more frequent (Hoegh-Guldberg, 1999). Mass coral bleaching is triggered by episodes of elevated sea temperatures and has occurred in six global episodes since 1979 (Hoegh-Guldberg, 1999). Since that time, mass coral bleaching has resulted in significant losses of live coral in many parts of the world. Evidence from a number of sources suggests that climate change is going to increase both the frequency and scale of coral bleaching (Hoegh-Guldberg, 1999, 2002). While considerable information is available on the disturbances associated with climate driven temperature changes, information on recovery processes and the extent to which reefs will be impacted is only in its infancy. Studies of reef connectivity appear central to any complete understanding of how these systems are likely to change as climate associated stresses increase over the next 100 years.

Effective recruitment from undisturbed areas to those in which corals have been removed is a crucial process in recovery. Measuring the rate of transport of coral larvae between reefs is almost impossible, except under unusual conditions (e.g., Helix Experiment, Sammarco and Andrews, 1988). Measures of genetic relatedness between reef areas represent a proxy to the extent of recruitment occurring between two areas. The relationship between the dispersal ability of organisms and the genetic differentiation of populations also provides a fundamental link between ecology and evolution (Ayre and Hughes, 2000). However, how far propagules actually go, where they come from, and the genetic consequences of past and present dispersal remain poorly understood for scleractinian corals. For example, Hughes and Tanner (2000) stress that the scale of planktonic dispersal of coral propagules in the Caribbean is poorly known, so it is uncertain where the larvae for recovery would come from if they are not generated locally. This uncertainty has led to widely divergent views about the importance and significance of self-seeding and inbreeding versus dispersal and outcrossing in scleractinian corals (Ayre and Hughes, 2000).

Despite the ecological importance of corals, studies of reef connectivity are not as simple or advanced as is in other organisms. Evolutionary and population genetic studies in corals have relied in the past on allozyme electrophoresis (Ayre and Resing, 1986; Ayre and Willis, 1988; Ayre and Dufty, 1994; Ayre et al., 1997; Ayre and Hughes, 2000; Benzie et al., 1995; Hellberg, 1994; Ridgway et al., 2001; Stoddart, 1984; Stobbart and Benzie, 1994). However, allozyme electrophoretic studies are hampered by the availability

of suitable material, and the limited number of testable loci result in relatively low precision. More recently developed molecular techniques, which are based on nucleotide divergence, have the potential to further our understanding of the genetic structure of coral populations (Avise, 1994). However, at present no suitable DNA markers have been identified for the study of population genetics of reef-building corals.

Despite the rapid expansion of molecular biology, DNA studies on corals are still in their infancy, largely due to two historical technical difficulties associated with the application of DNA-based techniques to studies on scleractinian corals. Firstly, there was the difficulty in preparing high molecular weight DNA necessary for DNA hybridisation analysis (see van Oppen et al., 2002 for more detail). This has been overcome by the use of the Polymerase Chain Reaction (PCR), as high molecular weight DNA is no longer necessary for molecular analyses. The second problem is the presence of symbiotic dinoflagellates from the genus *Symbiodinium* that inhabit the endodermis of hermatypic corals, as the DNA from these symbionts invariably contaminate the DNA preparations; i.e. extracted DNA contains both coral host and symbiont DNA. The use of DNA from symbiont-free gametes and the development of 'coral specific' PCR primers can overcome this problem, however, obtaining gametes from coral species is not always possible, and the development of 'coral specific' primers requires prior knowledge of the coral genome.

To date the majority of coral molecular genetic studies have been based on the use of nuclear DNA markers, with the primary focus being rDNA ITS (internal transcribed spacer) sequences (Lopez and Knowlton, 1997; Odorico and Miller, 1997; Medina et al., 1999; Takabayashi, 2000; Diekmann et al., 2001; Rodriguez-Lanetty and Hoegh-Guldberg, 2002; van Oppen et al., 2002). While ITS appears useful to study the evolution of different organisms, the application to population genetic and gene flow studies has not been fully tested. In most animal groups, the mitochondrial genome provides genetic markers appropriate for population level studies (Avise, 1994). However, the mitochondrial genome of corals seems to evolve at an incredibly slow rate (Romano and Palumbi, 1996; Medina et al., 1999; van Oppen et al., 1999). There has been a recent proliferation of the use of microsatellite markers in the population genetic literature. Despite significant effort, only a single microsatellite marker has been published for the genus *Montastrea* (Lopez et al., 1999). More recently, five microsatellite loci have been published for the genus *Seriatopora* (Maier et al., 2001), but the applicability of these markers to population genetic studies has not been established. Furthermore, it appears that microsatellite loci are not necessarily transferable between coral species (Dr. Karen Miller, University of Wollongong, pers. comm.). Single-locus intron regions (mini-collagen second intron, PaxC 46/47 intron) have also been used in

phylogenetic studies of the scleractinian coral genus *Acropora* (Hatta et al., 1999; van Oppen et al., 2001), but once again their applicability to population level analyses has not yet been assessed.

Given the lack of a suitably tested population genetic marker for scleractinian corals, and the fact that the precision of allozymes is perhaps not as comprehensive as newer molecular based approaches, it is imperative that new DNA markers be developed in order to address the much needed question of coral reef connectivity. This paper investigates the applicability of the cytochrome c oxidase subunit I (COI) mitochondrial gene, the internal transcribed spacer region 1 (ITS1) in the nuclear ribosomal DNA, and the pocilloporin gene to the question of intraspecific variation in the scleractinian coral species *Pocillopora verrucosa*.

2. Materials and Methods

Study sites and sample collection

The development of new markers requires a reef system in which the performance of the new markers can be compared against the performance of established methods (i.e., allozymes, Ridgway et al., 2001). Ideally, a clear geographical relationship between reef populations would simplify the interpretation of population genetic data as it is generated. In this regard, the reef complex of East Africa represents an ideal location. Reefs in this area extend in a linear sequence down to 27°51'S. Reefs at the southern end include approximately 1,200 km of reefs that are separated by distances ranging from one to 250 kilometres, and hence represent an opportunity to test assumptions about geographic distance and genetic connectivity. Given this opportunity, the collection of material for this study occurred at multiple sites along the southeast African coastline.

The branching pocilloporid coral *P. verrucosa* Ellis and Solander, 1780, was chosen for this study because it is locally abundant within reefs fringing the South African and Mozambican coastline (Veron, 2000). Furthermore, it is a broadcast spawning coral (Kruger and Schleyer, 1998) with a branching morphology and this life history seems likely to favour widespread larval dispersal (see Ayre and Hughes, 2000).

Seven populations were sampled from South African reefs (Leadsman Shoal (27°51'S); Red Sands reef (27°45'S); Two-mile reef (27°31'S); Five-mile reef (27°29'S); Seven-mile reef (27°27'S); Nine-mile reef (27°25'S); Kosi Bay (26°50'S)), and three populations sampled from the reefs of southern Mozambique (Baixo Danae, Inhaca Island (26°01'S); Anchor Bay, Inhambane (23°50'S); and Lighthouse Reef, Bazaruto Island (21°31'S) (Fig. 1). All reefs
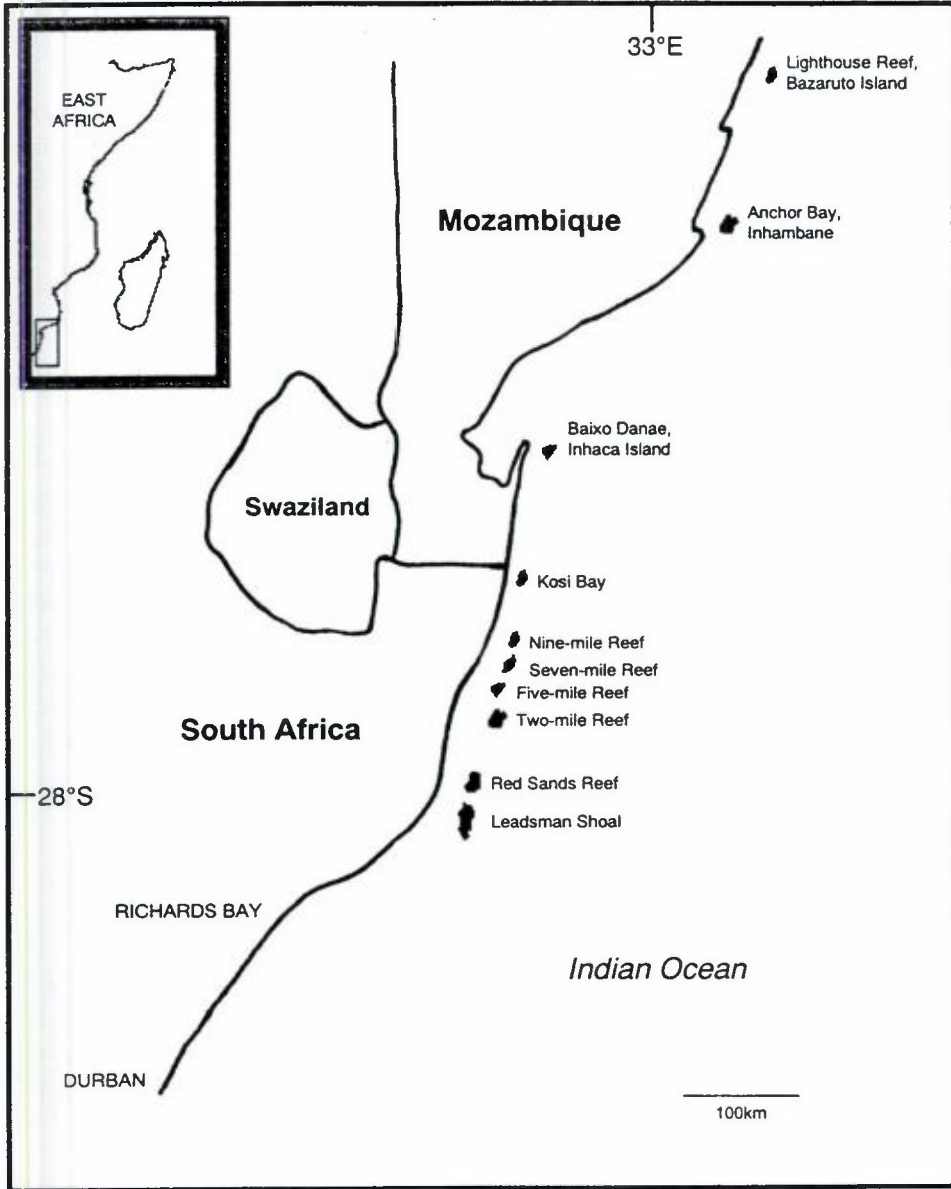
Figure 1. Location of the 10 sampling sites of *Pocillopora verrucosa* along the coastlines of South Africa and southern Mozambique.

were <2 km^2 and samples were taken from depths ranging from 5 m to 15 m on reef slopes. At each site, tissue samples were collected randomly by removing

small fragments from the branch tips of up to 20 attached adult colonies, from reef areas averaging 650 m^2. Collection of multiple fragments from adjacent colonies was avoided by sampling colonies that were separated by at least 3 m. The fragments were taken ashore and approximately 3 cm^2 of tissue was removed from the skeletons of live fragments of corals by tissue blowing using direct jets of high-pressure air (using a modified airgun connected to a SCUBA cylinder). The tissue slurry was collected in a small plastic bag, mixed and preserved with an equal volume of 20% DMSO in 0.25 M EDTA and saturated NaCl (pH 8.0). The samples were stored and transported at 4°C for subsequent analysis.

DNA extraction

Three hundred and fifty µl of the tissue slurry was used per DNA extraction. DNA extraction was performed following the protocol of the Nucleon HT Extraction kit (Amersham Pharmacia Biotech Inc.), and the samples were resuspended in 50 µl sterile Milli-Q water and stored at –20°C. Obtaining symbiont-free gametes for *P. verrucosa* was not possible, thus it was important to isolate and extract pure *Symbiodinium* sp. (zooxanthellae) cells. This 'coral-free' zooxanthellae DNA was then used as symbiont PCR controls. Viable zooxanthellae were isolated from the coral hosts using a density gradient centrifugation separation method (Tytler and Spencer Davies, 1983). DNA extraction of the 300 µl isolated zooxanthellae slurry was performed following the protocol of the Nucleon HT Extraction kit (Amersham Pharmacia Biotech Inc.)

PCR amplification

PCR amplifications were carried out in 30 µl volumes and consisted of 0.6 µl of each primer (20 µM), 3 µl 10x PCR buffer (Invitrogen), 0.9 µl 50 mM $MgCl_2$ (Invitrogen), 6 µl 1.25 mM dNTP, 0.2 µl of PLATINUM® *Taq* DNA polymerase (5 units/µl, Invitrogen), 16.7 µl H_2O and 1 µl template DNA (approximately 100 ng). Amplifications were performed using a Hybaid PCR Express thermal cycler with the following thermal cycle: 94°C for 1 min (1 cycle); 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min (35 cycles); 94°C for 1 min, 55°C for 1 min, and 72°C for 10 min (1 cycle).

COI: The COI region from 10 individuals of *P. verrucosa* from South Africa (5 individuals each from Leadsman Shoal and Kosi Bay), and three individuals of *Pocillopora damicornis* from One Tree Island (23°30'S, 152°06'E) on the Great Barrier Reef (GBR) was amplified using the 'universal' primers LCO1490 (5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3') and HCO2198 (5'-TAA ACT

TCA GGG TGA CCA AAA AAT CA-3') (Folmer et al., 1994). Amplified DNA was purified, cloned and sequenced. Sequences were aligned using the software package CLUSTALW (Thompson et al., 1994). Additional COI sequences from the *Montastrea annularis* species complex (Medina et al., 1999) were obtained from GenBank (AF013736–AF013738) and included in the analysis.

ITS: The ITS1-5.8S-ITS2 rDNA was amplified, cloned and sequenced from five individuals of *P. verrucosa* using the 'universal' primers ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') and ITS5 (5'-GGA AGT AAA AGT CGT AAC AAG G-3') (White et al., 1990). 'Coral specific' primers (PVITS1-F and PVITS1-R) were then designed using the Primer Select option in the software program DNASTAR (DNAStar, Madison, Wisconsin, USA), to amplify an approximately 220 bp region of ITS1. Five individuals of *P. verrucosa* from each of the seven South African populations and the three southern Mozambican populations were amplified (n = 50) using these coral specific primers: PVITS1-F (5'-GGA AGG ATC ATT ACC GAA AGA GA-3') and PVITS1-R (5'-TGC ACG CAC TAG ATA GAC ACG GAG AG-3'). Three samples of 'coral-free' zooxanthellae DNA were included as symbiont PCR controls to test the 'coral' specificity of the primers.

Pocilloporin: The cDNA sequence of the pocilloporin pigment protein from *P. damicornis* (sequence obtained from Dr. Sophie Dove, University of Queensland, unpubl. data) was found to contain no introns. Primers POC-F (5'-ATG AGT GTG ATC GCT ACA CAA-3') and POC-R (5'-TGG CAT CTT CAC AGG CTT CTT TG-3') were therefore designed from the sequence, and five individuals of *P. verrucosa* from each of the seven South African populations and the three southern Mozambican populations were amplified (n = 50) using these primers. Three samples of 'coral-free' zooxanthellae DNA were included as symbiont PCR controls to test the 'coral' specificity of the primers.

DNA cloning and sequencing

DNA bands were excised from agarose gels and purified using the GFX™ PCR Product and Gel Band Purification kit (Amersham Pharmacia Biotech Inc.). 1.5 μl of the gel purified DNA of each sample was ligated into the vector using the pGEM-T Easy Vector System I (Promega), and plasmids were purified using the High Pure Plasmid Isolation Kit (Roche). Samples of gel- or plasmid-purified DNA were sequenced using an ABI377 automatic DNA sequencer using Big Dye terminator chemistry.

SSCP

Single-stranded conformation polymorphism (SSCP) of the ITS1 and

pocilloporin PCR products was done using a method adapted from Sunnucks et al. (2000). Five µl of PCR product was denatured by adding it to 5 µl standard formamide loading dye, heating to 95°C for 5 min, and then snap-cooled on ice to allow each single DNA strand to self anneal. Eight µl was loaded onto 8 cm long 6% non-denaturing polyacrylamide gels. A double-stranded DNA sample was run on each gel to aid in the interpretation. Recurrent banding patterns were run on consecutive gels as suitable references. Gels were run in a Hoefer Mighty Small II vertical gel apparatus at 50V for 3.5 hours. Gels were stained with SYBR® Gold (Molecular Probes) for 10 min and visualised with a UVIDOC camera system.

SSCP was used to survey the sequence variability of the amplified ITS1 and pocilloporin fragments from the 50 amplified samples. For ITS1, seven representative samples of gel-purified DNA of each of the different banding patterns on the SSCP gels were sequenced as a reference to verify the scored differences on the gel. For pocilloporin, six representative samples of the banding patterns on the SSCP gels were cloned, and five clones per individual sequenced. All sequences were aligned using the software package CLUSTALW (Thompson et al., 1994).

3. Results

Cytochrome c oxidase subunit I (COI)

The sequence of the mitochondrial COI fragment (710 bp) was identical for the 10 samples of *Pocillopora verrucosa* sequenced. In addition, the sequences of *P. damicornis* from the GBR were identical to those of *P. verrucosa* from the Western Indian Ocean (WIO, Fig. 2). The COI nucleotide composition was 22% A, 16% C, 21% G, and 41% T, which is the same as reported for the *Montastrea annularis* species complex (Medina et al., 1999). The COI sequences of *P. verrucosa* showed 87% sequence identity to the COI sequences of *M. annularis*, *M. cavernosa*, and *M. faveolata* (Medina et al., 1999). The lack of sequence variability of the COI region renders it unsuitable for the study of the population genetics of *P. verrucosa*.

Internal transcribed spacer region (ITS)

The ITS4/ITS5 primer pair produced two differently sized bands when *P. verrucosa* DNA was used. BLAST search analysis (http://www.ncbi.nlm.nih.gov) of the sequences obtained from these two bands found that the lower (ca. 600 bp) band sequence had homology with ITS1-5.8S-ITS2 for *Symbiodinium* sp., and the upper (ca. 1,000 bp) band had homology

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                          10        20        30        40        50        60
                           .         .         .         .         .         .
P. verrucosa     GGTCAACAAATCATAAAGATATTGGTACTTTGTATTTAATCTTCGGAGCAGGAGCTGGTT
P. damicornis    GGTCAACAAATCATAAAGATATTGGTACTTTGTATTTAATCTTCGGAGCAGGAGCTGGTT
                 ************************************************************

P. verrucosa     TAATTGGAACTGCTTTTAGTATGCTTATACGATTGGAGCTTTCTGCGCCGGGGGCGATGT
P. damicornis    TAATTGGAACTGCTTTTAGTATGCTTATACGATTGGAGCTTTCTGCGCCGGGGGCGATGT
                 ************************************************************

P. verrucosa     TAGGAGATGATCATCTTTATAATGTAATTGTTACAGCACATGCTTTTATTATGATTTTTT
P. damicornis    TAGGAGATGATCATCTTTATAATGTAATTGTTACAGCACATGCTTTTATTATGATTTTTT
                 ************************************************************

P. verrucosa     TTTTGGTTATGCCGGTCATGATTGGGGGATTTGGTAATTGATTAGTCCCATTATATATTG
P. damicornis    TTTTGGTTATGCCGGTCATGATTGGGGGATTTGGTAATTGATTAGTCCCATTATATATTG
                 ************************************************************

P. verrucosa     GGGCGCCGGATATGGCGTTTCCTCGATTAAACAATATTAGTTTTTGACTTTTGCCTCCTG
P. damicornis    GGGCGCCGGATATGGCGTTTCCTCGATTAAACAATATTAGTTTTTGACTTTTGCCTCCTG
                 ************************************************************

P. verrucosa     CGCTTTTTTTATTATTAGGCTCTGCTTTTATTGAACAAGGGGCGGGGACGGGGTGAACAG
P. damicornis    CGCTTTTTTTATTATTAGGCTCTGCTTTTATTGAACAAGGGGCGGGGACGGGGTGAACAG
                 ************************************************************

P. verrucosa     TTTATCCTCCTCTTTCTAGTATTCAAGCACACTCCGGAGGTTCTGTTGATATGGTTATTT
P. damicornis    TTTATCCTCCTCTTTCTAGTATTCAAGCACACTCCGGAGGTTCTGTTGATATGGTTATTT
                 ************************************************************

P. verrucosa     TTAGTCTTCATTTAGCTGGGGTTTCTTCTATTTTAGGTGCTATTAACTTTATTACTACAA
P. damicornis    TTAGTCTTCATTTAGCTGGGGTTTCTTCTATTTTAGGTGCTATTAACTTTATTACTACAA
                 ************************************************************

P. verrucosa     TTTTTAATATGCGAGCCCCGGGTGTGTCTTTTAATAAACTACCTTTATTTGTTTGATCTA
P. damicornis    TTTTTAATATGCGAGCCCCGGGTGTGTCTTTTAATAAACTACCTTTATTTGTTTGATCTA
                 ************************************************************

P. verrucosa     TTTTAATAACAGCTTTTTTATTACTTTTATCTTTACCTGTTTTAGCTGGTGCTATTACTA
P. damicornis    TTTTAATAACAGCTTTTTTATTACTTTTATCTTTACCTGTTTTAGCTGGTGCTATTACTA
                 ************************************************************

P. verrucosa     TGTTGTTAACAGATAGAAACTTTAATACGACTTTTTTCGATCCAGCGGGGTGGCGGGGAC
P. damicornis    TGTTGTTAACAGATAGAAACTTTAATACGACTTTTTTCGATCCAGCGGGGTGGCGGGGAC
                 ************************************************************

P. verrucosa     CCAATATTATTTCAGCATTTATTTTGATTTTTTGGTCACCCTGAAGTTTA
P. damicornis    CCAATATTATTTCAGCATTTATTTTGATTTTTTGGTCACCCTGAAGTTTA
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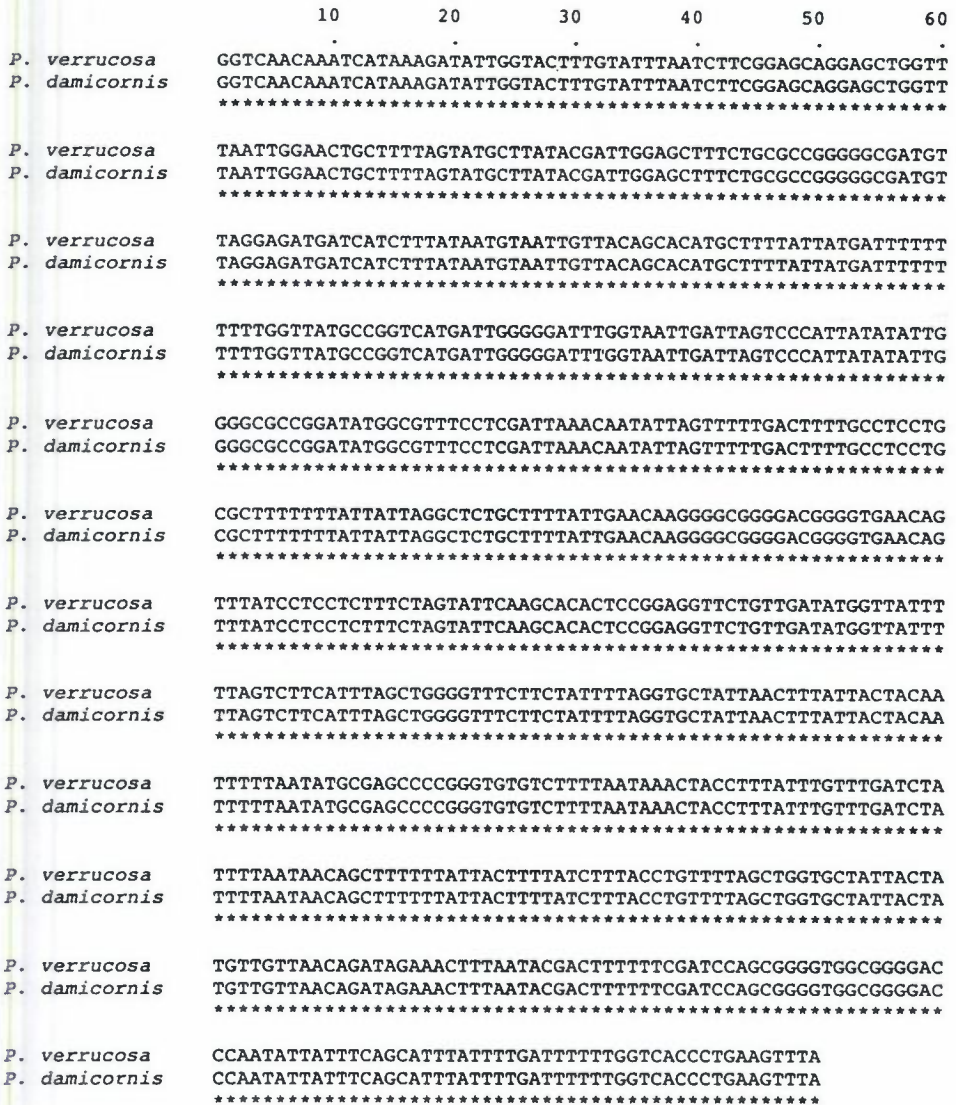
Figure 2. Comparison of the COI sequences of *Pocillopora verrucosa* and *P. damicornis*.
The sequences show 100% homology. Asterisks indicate sequence position is
conserved throughout all sequences.

with ITS1-5.8S-ITS2 from scleractinian coral species. The full sequence of the
complete coral ITS1-5.8S-ITS2 was 956 bp, which is the largest reported to date
for any scleractinian coral species (see van Oppen et al., 2002). Both the ITS1

and ITS2 regions were 399 bp, with GC contents of 53% and 61%, respectively. The length of the 5.8S region was 158 bp, which is consistent with that of the scleractinian coral *Plesiastrea versipora* (Rodriguez-Lanetty, 2001).

No amplification was ever recorded using 'coral-free' zooxanthellae DNA despite consistent positive amplifications from *P. verrucosa* DNA. DNA from fifty individuals of *P. verrucosa* was therefore amplified using the PVITS1-F/PVITS1-R primer pair, and the sequence variability of the partial ITS1 sequence screened using SSCP. Fig. 3a shows an example of an SSCP gel obtained. Of the 50 samples screened, only two different banding patterns were recorded. Alignment of the sequences showed that identical banding patterns correlated to identical sequences. Moreover, alignment of the sequences of the two banding patterns (I and II) showed that both had different sequences, with banding patterns I and II differing by a 13 bp insertion between nucleotide positions 120 and 121 (Fig. 4). The most striking result obtained was that of the 50 individuals screened, 12 (24%) displayed multiple banding patterns (multi-copy, Fig. 3a) on the SSCP gels, indicating that these individuals contained multiple copies of the ITS1 sequence. Direct sequencing of these multi-copy samples was consistent with sequence heterogeneity. The fact that close on 25% of the samples screened showed sequence heterogeneity seriously places the use of ITS1 in doubt for population studies on *P. verrucosa*.

Pocilloporin

Unlike Takabayashi (2000) who failed to amplify the pocilloporin gene from *Stylophora pistillata* and *Acropora nasuta*, positive amplifications for *P. verrucosa* were recorded. Thus, the primers designed to amplify a 514 bp product from the pocilloporin cDNA sequence (POC-F/POC-R) were used to amplify the 50 individuals of *P. verrucosa* from the 10 sample populations. No amplification was ever recorded using 'coral-free' zooxanthellae DNA despite consistent positive amplifications from *P. verrucosa* DNA. SSCP was used to screen the sequence variability of the 50 amplified samples and an example of an SSCP gel obtained is shown in Fig. 3b. No differences in banding patterns were detected across all 50 individuals. Six random individuals were sequenced to test whether the lack of SSCP variation was actually due to no sequence variation. All five sequences obtained per individual were identical, which has never previously been seen in any other scleractinian coral species (Dr. Sophie Dove, University of Queensland, pers. comm.). Sequence alignments of all the pocilloporin sequences obtained showed 100% sequence homology. Thus, as with COI, the lack of sequence variability across the populations of *P. verrucosa* prevents the use of the pocilloporin gene for population genetic studies.
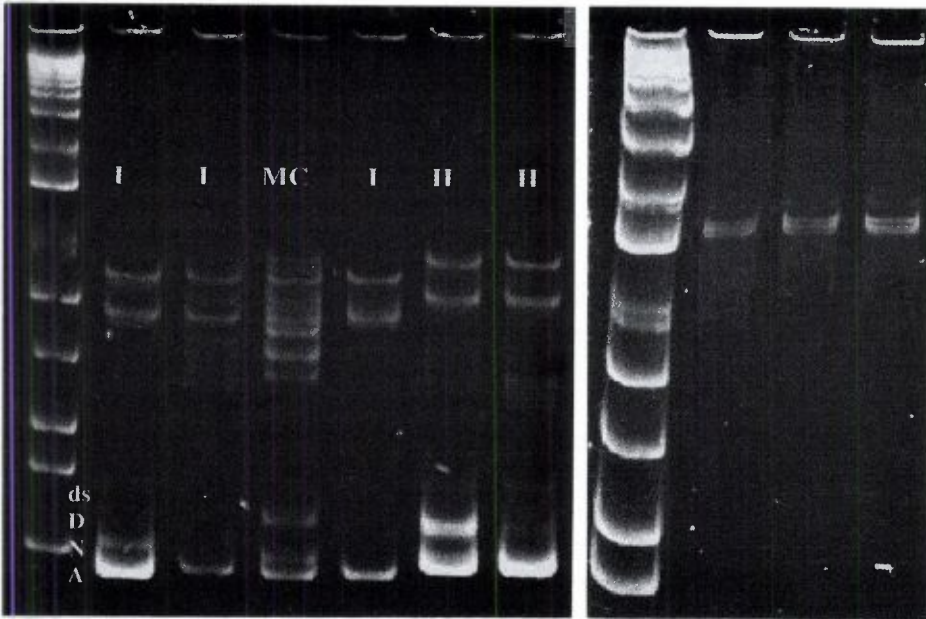
a   b

Figure 3.   SSCP gel for *Pocillopora verrucosa* samples showing a) the two different banding
patterns and an example of the multi-copy nature of the ITS1 region; b) the
homologous banding pattern of pocilloporin. I = Banding pattern I; II = Banding
pattern II; MC = multi-copy; ds DNA = double-stranded DNA.

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                    10          20          30          40          50          60
                     .           .           .           .           .           .
Type II   TACTACGAGTTCTTCTACCCTGGGAACCGGTGGAAAAAATTATCATTAGGGGTCGGCTGG
Type I    TACTACGAGTTCTTCTACCCTGGGAACCGGTGGAAAAAATTATCATTAGGGGTCGGCTGG
          ************************************************************

Type II   TTCCGTTAAAAGGAGAGGGCCGCGAGGCGTCGCTCGGACTGCCGTGCTAGGTGACTTTTG
Type I    TTCCGTTAAAAGGAGAGGGCCGCGAGGCGTCGCTCGGACTGCCGTGCTAGGTGACTTTTG
          ************************************************************

Type II   TGGAGATGGATGGATGGAGACTGCTCAGTGGGATCGTAGCGTGACCGTGCGGTGTGGCGT
Type I    -------------ATGGAGACTGCTCAGTGGGATCGTAGCGTGACCGTGCGGTGTGGCGT
                       *********************************************

Type II   AGGACGCTTCGTGCGCCGCGTCGGTGCCTGCCTACGTTTCTC
Type I    AGGACGCTTCGTGCGCCGCGTCGGTGCCTGCCTACGTTTCTC
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Figure 4.   Sequence alignment of the ITS1 sequences of *Pocillopora verrucosa* representative
of the three banding patterns. Type I = banding pattern I; Type II = banding
pattern II. Asterisks indicate sequence position is conserved throughout all
sequences.

4. Discussion

The extent of genetic variability within a species depends on a number of factors, and for scleractinian corals some difficulty has been experienced in determining which of these factors are responsible for the large intraspecific genetic variation typically observed (Takabayashi, 2000). The difficulties tend to arise from the range of reproductive and life history strategies exhibited by scleractinian corals, and these differences are thought to drive the population genetic structures both between and within species (Ayre and Hughes, 2000). Previous studies on marine invertebrates have highlighted the relative importance of the factors creating this intraspecific genetic variation at different geographical scales (Hellberg, 1994; Palumbi, 1996a; Bohonak, 1999). However, the extent of genetic variation within scleractinian coral species has not been well studied. The limited studies that have been undertaken have largely been based on allozyme electrophoresis. As the precision of allozymes is not as comprehensive as the newer molecular based approaches, there is a need for the development and application of molecular markers to the study of corals. According to Avise (1994), the process of choosing a molecular genetic marker that is likely to be appropriate is perhaps the most critical step in any molecular analyses. Prior to this study, very few scleractinian genetic markers were available, with population level analyses only recently being assessed using ITS (Takabayashi, 2000; Rodriguez-Lanetty and Hoegh-Guldberg, 2002). Thus, considerable effort was placed in assessing the applicability of molecular markers to the intraspecific genetic variability within *P. verrucosa* along the southeast African coastline. These included the analysis of nucleotide variation in COI, ITS1, and the pocilloporin gene.

Cytochrome c oxidase subunit I (COI)

Mitochondrial DNA (mtDNA) sequences are often used as population-level genetic markers, but have also been used to estimate divergence times between closely related taxa. Within the mitochondrial genome, different regions evolve at different rates (Avise, 1994). In general, the control region (D-loop) is the most rapidly evolving region, with the two rRNAs and the 22 tRNAs evolving at the slowest rate (Avise, 1994). Most of the mitochondrial protein-coding genes, which accumulate base substitutions at an intermediate rate, have been used in phylogenetic or population genetic studies (see Avise, 1994). However, prior to this study, only the 16S (Romano and Palumbi, 1996), cytochrome b (van Oppen et al., 1999), and COI (Medina et al., 1999) subunits had been studied for scleractinian corals. All three studies indicated that the mitochondrial genome of scleractinian corals appeared to evolve at an incredibly slow rate. Nonetheless, COI sequences have been shown to reveal

genetic structure in populations of other marine invertebrates such as copepods (Burton and Lee, 1994) and sea urchins (Palumbi, 1996a), and the applicability of COI was therefore tested in this study. However, the results obtained only further confirmed the unusually slow rate of evolution of mitochondrial DNA in scleractinian corals. No differences in the sequences of the mitochondrial COI fragment (710 bp) were detected within the *P. verrucosa* sequences from the WIO, or between the WIO *P. verrucosa* sequences and the *P. damicornis* sequences from the GBR. Medina et al. (1999) followed a similar approach, using COI as a molecular marker to increase the resolution offered by allozyme electrophoresis in an attempt to differentiate *Montastrea* species throughout their geographic and depth ranges. The sequence of the COI fragment was identical for all three *Montastrea* species studied, leading Medina et al. (1999) to conclude that evolution rates are too slow to detect speciation events in closely related taxa of scleractinian corals. More recently, Fukami et al. (2000) used the mitochondrial genes cytochrome b and ATPase 6 to infer the phylogenetic relationships within the Acroporidae, and once again very low levels of divergence were found. Thus, it appears that the mitochondrial genome in scleractinian corals evolves at an unusually slow rate, and unlike most other animal groups, the use of mtDNA for population level studies is not feasible for scleractinian corals.

Internal transcribed spacer region (ITS)

The ITS regions of the nuclear rDNA have been used to study relationships at the population and species levels of a variety of taxa (Vogler and DeSalle, 1994; Tang et al., 1996). More specifically, ITS regions have been successfully used in corals (Lopez and Knowlton, 1997; Odorico and Miller, 1997; Medina et al., 1999; Takabayashi, 2000; Diekmann et al., 2001; Rodriguez-Lanetty and Hoegh-Guldberg, 2002; van Oppen et al., 2002), and it has been shown that the extent of ITS variability differs greatly among different scleractinian coral species. For example, ITS1 sequence variation is ~29% in *Acropora valida* (Odorico and Miller, 1997) and as low as ~1% among the closely related species within the *Montastrea annularis* species complex (Lopez and Knowlton, 1997). Until now, ITS studies on scleractinian corals have focussed largely on species boundary resolution in the *Montastrea* (Lopez and Knowlton, 1997; Medina et al., 1999), *Porites* (Hunter et al., 1997), *Acropora* (van Oppen et al., 2000), and *Madracis* (Diekmann et al., 2001) genera. However, both Takabayashi (2000) and Rodriguez-Lanetty and Hoegh-Guldberg (2002) assessed the applicability of ITS sequence variability to the study of coral population genetics. Takabayashi (2000) used nucleotide sequence analysis of the ITS1 to analyse the population structure of *Stylophora pistillata* at small (local) and large

(regional) scales, whereas Rodriguez-Lanetty and Hoegh-Guldberg (2002) assessed the phylogeography and connectivity of *Plesiastrea versipora* throughout the Western Pacific Ocean using both ITS1 and ITS2. Interestingly, both studies concluded that the use of ITS sequences were applicable to coral population analyses in that the general connectivity patterns observed for both *S. pistillata* and *P. versipora* agreed with previously suggested patterns based on allozyme electrophoresis of the same or similar species.

This study attempted to follow on from the work of Takabayashi (2000) and Rodriguez-Lanetty and Hoegh-Guldberg (2002) and assess the applicability of ITS1 to the population genetics and patterns of connectivity of *P. verrucosa* populations from South Africa and Mozambique. However, of the 50 sequences that were screened, 12 showed sequence heterogeneity indicating that different multiple copies of the sequence exist within individuals. Also, of the 38 sequences that were homogenous, only two different sequence types were found amongst samples covering approximately 1,200 km of coastline. This low level of variation in *P. verrucosa* was unexpected given that Rodriguez-Lanetty and Hoegh-Guldberg (2002) found up to 25 sequence types in *P. versipora* along a similar distance of the southeast Australian coastline.

According to Quijada et al. (1997), the strength of variable repetitive markers such as rDNA ITS regions lie in the fact that they are additively combined in hybrids. However, these same repetitive markers have the disadvantage in that they provide data that cannot be analysed using standard population genetic approaches, such as F-statistics (van Oppen et al., 2002). Although these repetitive markers may show Mendelian inheritance, it is usually not possible to determine whether individuals are homozygous or heterozygous due to the effects of concerted evolution. Thus, given the high percentage (24%) of multi-copy sequences, the apparent low variability of the ITS1, and the fact that ITS sequence data do not fit conventional population models, it was concluded that ITS was not a suitable marker to address the patterns of gene flow of *P. verrucosa* in this study.

Pocilloporin

While ITS sequences are useful in evolutionary genetic, and possibly population genetic studies of scleractinian corals, there is a real need for the development of single-copy nuclear markers. According to van Oppen et al. (2002), data from such single-copy markers will not be confounded by the homogenisation process of concerted evolution acting on repetitive markers. Due to the simple pattern of inheritance of single-copy markers, quantitative estimates of gene flow, inbreeding, sexual versus asexual reproduction, and population demography can be obtained from single-locus DNA sequence data.

A small number of variable nuclear single-copy markers (i.e., introns) have been used in phylogenetic studies in *Acropora*. Hatta et al. (1999) used the mini-collagen second intron to examine interspecific hybridisation in five *Acropora* species, whereas van Oppen et al. (2001) performed a phylogeny of 28 *Acropora* species based on the sequences of the PaxC 46/47 intron. Given the unsuitability of COI and ITS to the study of *P. verrucosa* in this study, it was hoped to address the issues of population connectivity by using a single-copy nuclear marker.

According to Dr. Masayuki Hatta (National Institute of Genetics, Japan, pers. comm.), the primers used to amplify the mini-collagen second intron (Hatta et al., 1999) were *Acropora*-specific, preventing the use of this intron region in the present study. As no sequences for members of the genus *Pocillopora* were available on GenBank (besides the partial 16S sequence for *P. damicornis,* Romano and Palumbi, 1996), it was decided to screen the pocilloporin cDNA sequence of *P. damicornis* for possible introns. Pocilloporin in *P. verrucosa* contains no introns, therefore the pocilloporin gene was screened for 50 individuals, but no variation was detected along the 1,200 km of coastline studied. The lack of introns and the lack of variation in the pocilloporin gene of *P. verrucosa* therefore render it unsuitable for the population level analyses of this study.

Thus, given the lack of scleractinian coral sequence information and the apparent lack of applicability of the COI mitochondrial gene, ITS1, and the pocilloporin gene to the population genetic analysis of *P. verrucosa*, it appears that sequencing individual genes may be less efficient than the use of approaches that screen broadly across the genome. Fingerprinting methods, such as random amplified polymorphic DNA (RAPD, Williams et al., 1990), DNA amplification fingerprinting (DAF, Caetano-Anollés et al., 1991), and amplified fragment length polymorphism (AFLP, Vos et al., 1995) meet these requirements for broad screening of genomes. Moreover, these methods are based on the amplification of random genomic DNA fragments by arbitrarily selected PCR primers, thereby generating DNA fragment patterns without prior sequence knowledge. These random primers anneal with some probability in any given genome, and by screening a large number of primers, it may be possible, by chance, to find some that produce useful products (Palumbi, 1996b). Thus, the application of random amplification techniques to rapidly screen the coral genome in the search for novel coral population level markers is an approach that definitely warrants attention in future studies.

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