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Application of Denaturing Gradient Gel Electrophoresis (DGGE) Methods on Parent-Offspring Relationship of the Coral *Pocillopora damicornis*

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Abstract

DGGE (Denaturing Gradient Gel Electrophoresis) is the most powerful methods for mutation detection currently available. In DGGE, DNA fragments of the same length but with different sequences can be separated. The sensitivity of DGGE to slight sequence differences is high since single base changes could be observed. There has been a debate about whether planulae of the coral Pocillopora damicornis are produced sexually or asexually. If produced sexually, planulae are expected to be genetically different from each other and also from their parents. In order to detect possible genetic difference between planulae and their parents, DGGE analysis of ITS2 region of rDNA was used. If there are genetic differences, it is proved that planulae are produced sexually. A total 49 adult colonies and 78 planulae from 11 localities were used for analysis. However, only in 2 families (Bise01#02 and Bise02#03YL) showed different DGGE profile, suggesting genetic difference between parent and offspring. The attempt to detect genetic difference in planulae of P. damicornis and their parents using DGGE method was not completely success since DGGE method can not prove clearly the genetic difference between parent and offspring. However, the possibility that DGGE method is applicable for studying coral can be suggested. PCR-DGEE amplification may perform with new STR (short tandem repeat) polymorphic loci of P. damicornis that currently found to answer whether planulae are produced sexually or asexually.

Key words *: Pocillopora damicornis, planula, DGGE, sexual reproduction, coral*

Abstrak

DGGE (Denaturant Gradient Gel Electrophoresis) merupakan salah satu teknik akurat untuk mengetahui mutasi DNA. Sensivitas DGGE sangat tinggi, karena dengan menggunakan DGGE, fragmen DNA yang memiliki panjang basa yang sama dapat dipisahkan hanya berdasarkan perbedaan satu basa penyusunnya saja. Model reproduksi karang Pocillopora damicornis telah lama menimbulkan perdebatan. P. damicornis dilaporkan memproduksi planulae secara seksual dan aseksual. Jika diproduksi secara seksual, planula akan berbeda secara genetik dengan induknya. Analisa DGGE dikombinasikan marker ITS2 dari rDNa digunakan untuk mendeteksi perbedaan tersebut. Jika terdapat perbedaan genetik, dapat disimpulkan planulae diproduksi secara seksual. Analisa dilakukan terhadap 53 koloni dan 78 planulae dari 11 lokasi yang berbeda. Dua famili (Bise01#02 and Bise02#03YL) mempunyai profil DGGE berbeda antara planulae dan induknya. Hal ini menunjukkan adanya perbedaan susunan gen antara planulae dan induknya dan kemungkinan produksi planulae dilakukan secara seksual. Namun demikian, kombinasi analisa DGGE dan marker ITS2 dari rDNA kurang memberi hasil memuaskan. Saat ini telah ditemukan STR (short tandem repeat) spesifik untuk P. damicornis. Kombinasi penggunaan amplifikasi PCR-DGGE dengan STR diharapkan lebih akurat untuk menjawab permasalahan produksi planulae pada karang P. damicornis.

Kata kunci *: Pocillopora damicornis, planulae, DGGE, reproduksi seksual, karang*

Introduction

DGGE (Denaturant Gradient Gel Electrophoresis) is one of the most powerful methods for mutation detection currently available (Wu *et al.*, 1998). DGGE relies on polyacrylamide gels that have an increasing linear gradient of denaturants to separate double-stranded DNA fragments on the basis of sequence rather than size (Buchan *et al.*, 2001). In DGGE, DNA fragments of the same length but with different sequences can be separated. Separation is based on

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the decreased electrophoretic mobility of partially melted doubled stranded DNA molecule in polyacryamide gels containing a linear gradient of DNA denaturants (a mixture of urea and formamide) Muyzer & Smalla, 1998). The sensitivity of DGGE to slight sequence differences is high since single base changes could be observed. Therefore, DGGE has become a common and reliable tool for sequence variation analysis (Wu *et al.*, 1998). By applying DGGE, 50% of the sequence variants can be detected in DNA fragments up to 500 base pair (Myers *et al.*, 1985). Through PCR using GC-clamped primers attach to one side of DNA fragment, the percentage could be increased to nearly 100% (Sheffield *et al.*, 1989).

In coral reef studies, DGGE method was used to resolve taxonomic complexity of zooxanthellae. Started with the work conducted by Belda-Baillie *et al.* (2002), they successfully used DGGE analysis to investigate the specificity of cnidarian-zooxanthellae association. La Jeunesse (2002) successfully applied DGGE method coupled with sequencing of ITS2 region to classify *Symbiodinium* diversity from Caribbean coral reefs. Similar method was used to describe the community from southern Great Barrier Reef corals which lead them to conclude that *Symbiodinium* from those areas consist of lower diversity than those from Caribbean (La Jeunesse *et al.*, 2003). DGGE method was widely used to reveal bacteria community associate with corals as reported for *Montastraea franksi* from Caribbean (Rohwer *et al.*, 2001) and *Pocillopora damicornis* from Great Barrier Reef (Bourne and Munn, 2005). The bacterial consortium associated with black band disease has been successfully characterized using DGGE method (Cooney *et al.*, 2002) as well as the composition of microbial biofilms which affect metamorphosis of coral (Webster *et al.*, 2004).

The brooding coral *Pocillopora damicornis* is one of the most widespread and well studied corals. The coral is highly variable not only in morphology (Veron & Pichon, 1976) but also in the reproductive mode (see Harrison & Wallace, 1990; Glynn *et al.*, 1991). Within some areas, *P. damicornis* has been claimed to consist of two distinct growth forms with asynchronous planulation time (Richmond and Jokiel, 1984). In particular, the coral, is well known for its ability to produce both sexual and asexual (i.e. parthenogenic) larvae to different degrees across its range. The coral has been considered to produce planula larvae asexually in Western Australia and Hawaii (Stoddart, 1983; Ayre & Miller, 2004). However, available evidence from other regions points to sexual reproduction. For example, the coral displays monthly maturation pattern of gonads (Stoddart & Black, 1985; Permata *et al.*, 2000). Population genetic surveys on Great Barrier Reef by Benzie *et al.* (1995) and Ayre *et al.*(1997) of adult populations showed no evidence of localized asexual recruitment in reef crest or reef-lagoon sites. Almost every colony displayed distinct multi-locus allozyme genotype and genotype frequencies matched either the expectation for random mating or inbreeding populations with limited dispersal of sexual propagules. Similarly, in Japan a tight correlation of planulae development and the disappearance of mature sperm have been observed in histological studies and sexual production of planulae had been suggested (Permata *et al.*, 2000; Permata & Hidaka, 2006).

Interestingly, despite the range and extent of studies of *P. damicornis*, the reproductive mode of the species is not fully understood. Such widely reproductive option in a coral is unparalleled. Indeed, genetic assessment of the differences between offspring and their broodparent is needed. Parentage analyses using molecular assessments involve linking an offspring to its biological mother and/or father when parents are uncertain from other evidence (Avise, 1994). Hyper-variable markers are still necessary to determine male fertility within individuals or populations because paternity of sperm can only be assigned with high probability of loci, even in brooding corals, as *P. damicornis* (Carlon, 1999).

In this study, DGGE analysis of ITS2 region of rDNA was used to detect the genetic difference between planulae and their parent of the coral *P. damicornis*. This technique successfully produced high-resolution banding profiles for zooxanthellae studies to resolve diversity and community structure of zooxanthellae (La Jeunesse, 2002; La Jeunesse *et al.*, 2003). DGGE might be suitable for surveying possible genetic differences between many parent-offspring pairs by identified one base pair substitution.

Materials and Methods

Collection of corals and planulae

Colonies of *P. damicornis* (approximately 10 cm in diameter) were collected during 2002-2003 from two sites, Bise (PB and PBK) and Motobu (MT), off northern Okinawa Island, and two sites, Zamami Island (ZR) and Tokashiki Island (TS), in the Kerama Islands region (Figure 1). Colonies were transported to Sesoko Station, Tropical Biosphere Research Center, University of the Ryukyus, Okinawa, Japan, and kept individually in 2-L plastic buckets supplied with unfiltered running seawater. Planulae were collected around the first quarter-moon by filtering overflowing seawater

through planula collectors made of 60- or 180-mm nylon mesh (Hidaka, 1985). Samples of adult colonies collected from Thailand and Hawaii on 1999 were added to compare the genetic difference between adult colonies from different regions.

Samples from branches were collected by cutting branches into small pieces using cutting pliers and put into a 2ml vial. Planulae were kept in 2 ml vial. Both of samples were preserved with DNA extraction buffer (CHAOS: 4M Guanidine thiocyanate, 0.5% Sarkosyl, 2.5mM Tris-HCl (pH 8.0) and 0.1 M ^{2}-mercaptoethanol) or 95% Ethanol. Planulae were kept individually when preserved in CHAOS extraction buffer, each contained ~100-300 ¼l, but when preserved in 95% ethanol 5-10 planulae were stacked together. Isoporan acropora (*Acropora palifera*) were used as additional samples since the coral was reported as brooder which derived azooxanthellate planula sexually (Kojis, 1986). Both samples of tissue and planulae were collected. Preservation of samples was done as mentioned for *P. damicornis*. All samples were kept in 4^{o}C until used (Table 1).

DGGE studies

DNA extraction

Small amount (300-500 µL of adult samples and 100-300 µL of planulae) that preserved in CHAOS buffer were taken and put into 1.5 ml microcentrifuge tube. Equal volume of PCI (Phenol:chloroform:isoamyl alcohol=25:24:1) followed by CI (chloroform:isoamyl alcohol=24:1) were added to extract DNA. DNA was then precipitated with 1/10 volume of 3M Sodium acetate (pH 5.2) and equal volume of cold isopropanol, and rinsed in cold 70% ethanol. The extracted DNA was dried overnight at room temperature and resuspended in 50 µL millique. The resuspended DNA was then purified using DNeasy Tissue Kit (QIAGEN, K.K) with RNase (DNase free, Boehringer Mannheim) according to the manufacturer's protocols. Total DNA was then visually quantified using 1% -1.5% agarose gel staining with ethidium bromide.

PCR amplification

PCR amplification was conducted using universal primer (White *et al.,* 1990) as follows. The ITS1-5.8S-ITS2 rDNA region together with a partial sequence of the flanking small subunit rDNA and large subunit rDNA genes were amplified with the universal primer ITS4 and ITS5. Amplification was performed in a 100ml volume of a solution consisted of 10 µL of 10x reaction buffer, 10 µL of 10x dNTP (2mM), 6 µL $MgCl_2$ (25mM), 0.4 µL of each primer, 2 µL of template DNA, 0.5 µL (U/µL) *Taq* polymerase (Takara).

Amplifications were carried out on a Perkin-Elmer 9600 thermocycler with the following profile: 1 cycle of 4 min 94^{o}C; 30 cycles of 1 min 94^{o}C, 2 min 50^{o}C, 3 min 72^{o}C; and 1 cycle of 10 min 72^{o}C. Amplifications were visually quantified on ethidium-bromide-stained 1.5% TAE agarose gels according to standard methods. If PCR product was not sufficient, the remained PCR product was purified using GFXÔ Purification Kit. The purified product was resuspended in 50 µL millique.

For DGGE analysis, PCR products containing the ITS2 were amplified using primer published by La Jeunesse (2002).Two different amplification procedures were applied to amplify the ITS2 region; a 'touchdown' and a routine PCR amplification. A 'touchdown' amplification was done with annealing conditions 10 ^{o}C above the final annealing temperature (52^{o}C). Every 2 cycles, the annealing temperature was decreased by 1^{o}C; after 20 cycles, the annealing temperatures were maintained at 52^{o}C for another 15 cycles. For routine PCR amplification annealing temperature was set at 55-58^{o}C. Amplifications were visually quantified on ethidium-bromide-stained 1.5% TAE agarose gels according to standard methods. PCR products (about 500 bp) were purified with standard method of ethanol purification. The purified PCR products were digested by cutting restriction enzyme (ApaL1) to confirm its size.

Re-amplification was later performed using the "ITSintfor2" forward and the conserved 3'flanking primer, ITS reverse with GC clamp. Amplification protocol was similar to that described above except that annealing temperature was modified at 55^{o}C-60^{o}C until a sharp and single band was obtained for DGGE samples.

DGGE analysis was performed in D-Code universal mutation detection system (Bio-Rad). Purified PCR products (25ml) were loaded with an equal volume of 2x loading dye on a parallel DGGE and resolved on a 6% acrylamide gel (acrylamide/bis 37.5:1) contained 40-60% denaturant gradient for 5h at 58^{o}C and a constant voltage of 150V. Gels were stained with Ethidium Bromide under dark condition for 30 min and washed in deionized water for 2-5 min and visualized using a UV transilluminator.

Results and Discussion

One single band of PCR product was obtained from *Pocillopora damicornis* samples (both planula and adult) using ITS4-ITS5 primer (universal primer). The size of the band was ~1000bp (1kb). Since the genomic DNA of *P. damicornis* was extracted without removal of its endosymbiont we expected to obtain

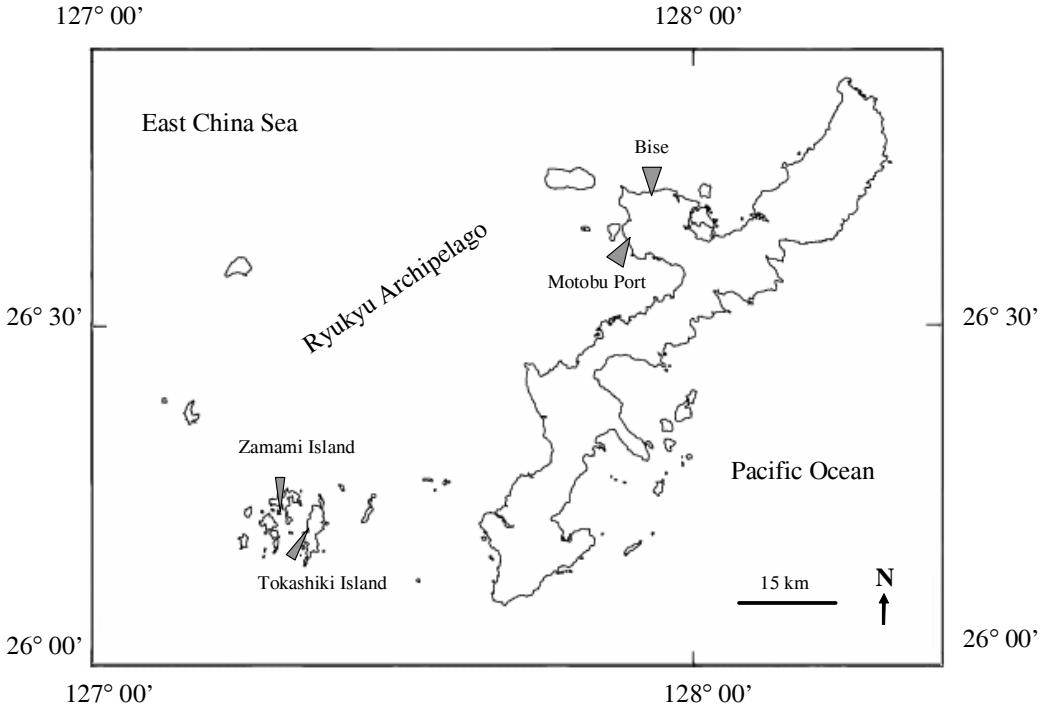


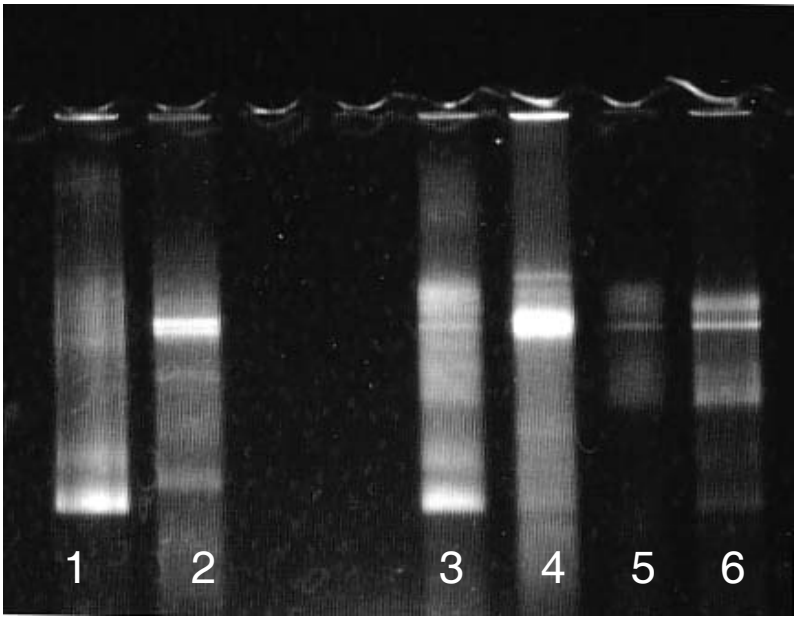
Figure 1. Ryukyu Archipelago, showing locations of four sampling sites of *Pocillopora damicornis.*

two distinct band of PCR product. To check whether the band is a true single band, PCR product was subjected to electrophoresis on 2% agarose gel and was loaded in a series of decreasing amount (from 4 µl to 1.25µl). The result showed that all samples had only a single band even at the smallest amount of samples.

It has been suggested that, in DGGE analysis, polymorphism appears as either additional bands or a shift in position of an existing band (Sheffield *et al.*, 1990). Our results showed that the DGGE profiles of adults and planulae usually characterized by two distinctive bands, sometimes accompanied by faint or smear background. One (PB1ad and PB1pla) out of five pairs of planulae and their parents showed different positions of bands (Figure 2A). However, it was difficult to interpret whether base substitutions in the ITS2 sequence did occur as the signatures band between adult and planulae were similar. To check whether the signatures band obtained from DGGE are typical bands of adult and planula of *P. damicornis*, PCR product isolated from adult tissue of *Acropora palifera* and their offspring were added as DGGE samples. *A. palifera* was reported as brooder (Kojis, 1986). The coral spawned azooxanthellate planulae which derived sexually (Kojis, 1986; Benzie *et al.*, 1995). However, the signatures band obtained from DGGE profiles are similar to *P. damicornis* (Figure 2B).

The ITSintfor2 forward and the conserved 3'flanking primer, ITS reverse with GC clamp were actually designed to classify *Symbiodinium* clade of various corals (La Jeunesse, 2002). The possibility that those primers are specific to zooxanthellae could not be ruled out. Amplification of PCR product using those primers yielded in two distinctive bands both from adult tissue and their planulae. Two excised gel were obtained, one is from coral (adult or planulae) and the other are from their endosymbiont. We then applied this endosymbiont as DGGE samples after purification of the gel. Figure 2B shows the DGGE profiles which involving the endosymbiont PCR product as the samples. The results show a similar profile as in adult or planulae of *P. damicornis*.

The studies using DGGE methods could not reveal genetic difference between planulae of *P. damicornis* and their parents. DGGE signatures showed similar bands between planulae and parent colonies (Figure 2A). The similar result was obtained when parent tissue of *A. palifera* and their offspring were used as DGGE samples. It seems the primers that were used for DGGE studies were not suitable for typing ITS region of planulae of *P. damicornis* and their parents since the primer was designed for typing zooxanthellae diversity in various corals. It seems primers that had been used for DGGE studies might be work specifically for algal typing.



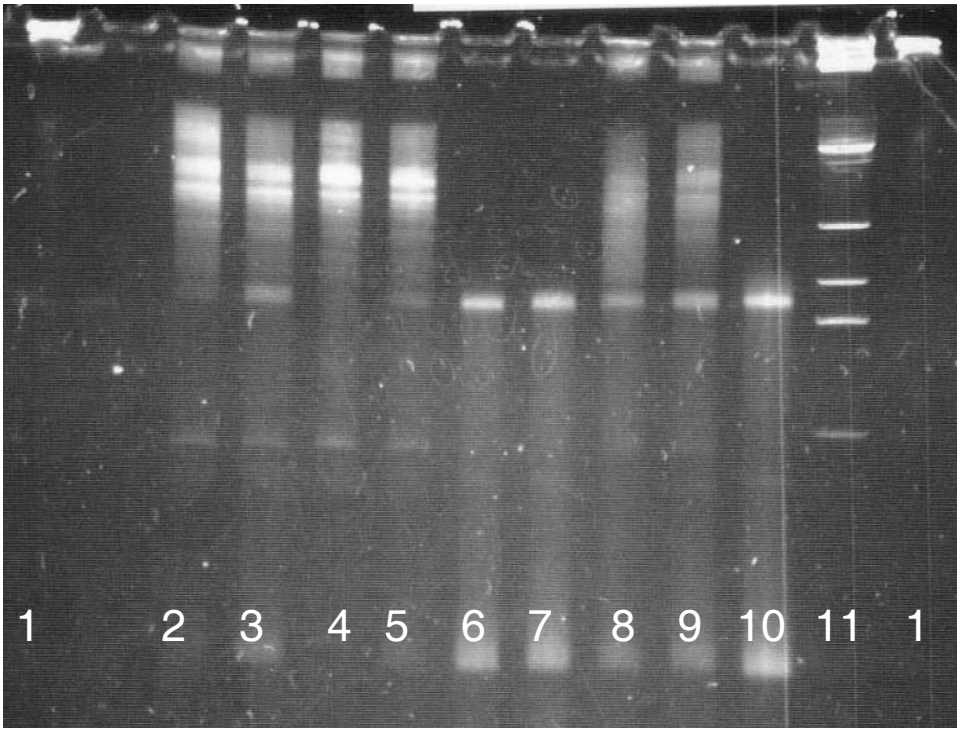


Figure. 2. **A**. PCR-DGGE analysis of the ITS2 (300-500 bp) of *Pocillopora damicornis* (P) and *Acropora palifera* (A); (1) PB5pla; (2) PB1pla; (3) AB2pla; (4) AB6pla; (5) PB1ad; (6) PB5ad;
B, Bise; comparison between adult (ad) and offspring (pla); B. PCR-DGGE analysis of the ITS2 of *P. damicornis* (P) and *A. palifera* (A); comparison between Host (H) and Zooxanthellae (Z), respectively. Number represent name of samples as follows: (1) Lambda (±) DNA; (2) PB1pla H; (3) PB1ad H; (4) PB5pla H; (5) PB5ad H;(6) PB5ad Z;(7) PB4ad Z; (8) PB4ad H; (9) AB6pla H ; (10) AB6ad H; (11) Lambda (±) marker; (12) Lambda (±) DNA

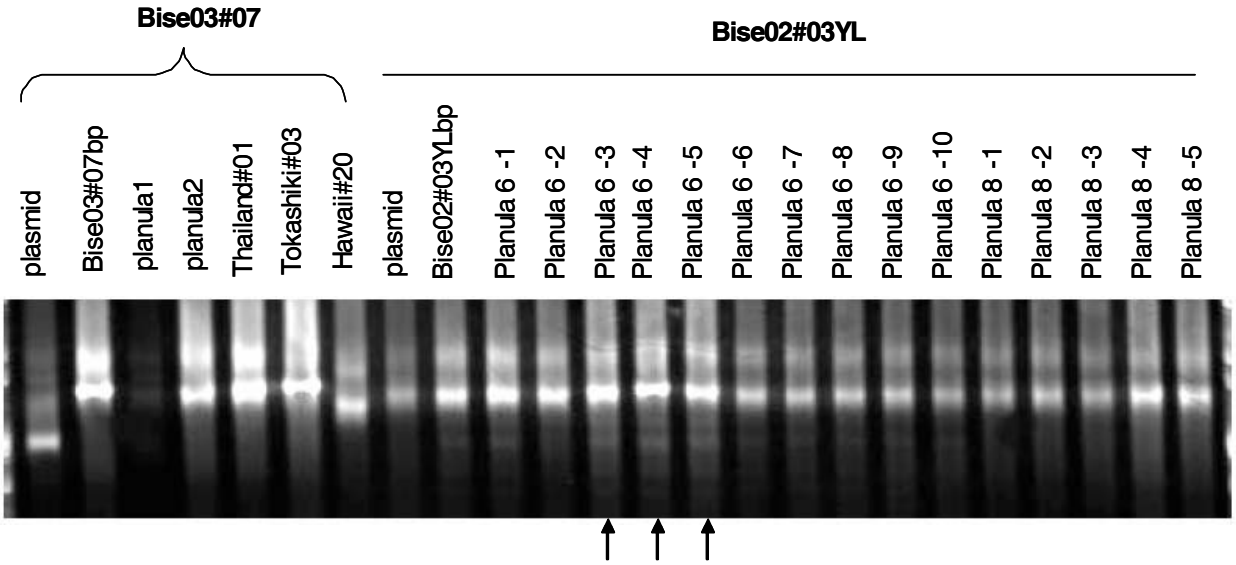


Figure 3. PCR-DGGE analysis of the ITS2 (300-500 bp) of *Pocillopora damicornis*. Loaded samples were 2 families consisted of parents collected from Bise (Bise03#07 and Bise02#03YL). Adult samples from Thailand, Tokashiki, and Hawaii are also shown. Profiles are typically characterized by one distinctive band. Arrows indicate the planulae which showed bands different from the displayed by other sibling planulae, bp beside the families name indicate "parent".

New primer specific for *P. damicornis* were then designed (Hirose *et al.*, 2006) and PCR condition was optimized. The results showed, in Bise02#03YL family, 3 out of 15 planulae tested, displayed band at different position of their parent and the rest of their sibling planulae (Figure 3). This suggests the occurrence of polymorphisms, hence may imply that planulae were not merely the clones of their parent. When similar samples were used for contact reaction studies between planulae and their parents, similar results were obtained. Only few genetic differences were detected between planulae and their parents after 9 months experiments (Permata and Hidaka, 2006). Though few, a genetic difference between planulae and their parents did occur.

There are several explanations as to why only few genetic difference were detected between planulae and their parents though the primer of DGGE was designed specific for *P. damicornis*. First, indeed, planulae might be derived asexually as previously suggested by Stoddart (1983) and recently by Ayre & Miller (2004). There has been reluctance to accept the idea that broods larvae of mature cnidarians are generated asexually as suggested for sea anemone, *Actinia tenebrosa* (Black and Johnson, 1979) or azooxanthellate coral, *Tubastrea diaphana* and *T. coccinea* (Ayre & Resing, 1986). However, on the contrary, the coral displays a monthly maturation pattern of gonads (Stoddart & Black, 1985; Permata *et al.*, 2000) and early embryonic stages within polyps of *P. damicornis* were observed which suggested that planulae are produced sexually, at least in Okinawan populations (Permata *et al.*, 2000).

It has been proposed that *P. damicornis* might be fit with suggestion that the coral display a mixed mode of reproduction. The possibility that *P. damicornis* might be a species complex has been also suggested (Knowlton & Jackson, 1994; Ayre *et al.*, 1997). The dispersal potential of its brooded larvae appear to fit the expectation that asexual reproduction is operated to maintain populations of locally adapted clones (Stoddart 1984a, b), while sexual reproduction is used to generate genetically diverse colonist.

Second, it is also likely that the genotypic diversity of *P. damicornis* has declined because of a mass coral bleaching event in 1998, during which branching corals, including *P. damicornis*, suffered a high mortality (Loya *et al.*, 2001).

Third, this might be due that *P. damicornis* is a brooder. It has been suggested that the dispersal potential of brooded larvae are limited (Harrison & Wallace, 1990). Most larvae of *P. damicornis* settled soon upon release (Miller & Ayre, 2004; personal observation), though competency experiments have demonstrated that *P. damicornis* planulae are capable to settle and metamorphosis successfully for periods up to 103 days after release and as the consequent it dispersal potential may be high (Richmond, 1987).

The attempt to detect genetic difference in planulae of *P. damicornis* and their parents using DGGE method

was not completely success. However, the possibility that DGGE method is applicable for studying coral other than their zooxanthellae only can be suggested. PCR-DGEE amplification may combine with new STR (short tandem repeat) polymorphic loci of *P. damicornis* that recently found (Starger *et al.*, 2008) to detect a genetic difference between planulae and their parents to answer the question whether planulae of *P. damicornis* are derived sexual or asexually.

Conclusions

A total 49 adult colonies and 78 planulae from 11 localities were analysed to detect possible genetic difference between planulae and their parents using DGGE analysis of ITS2 region of rDNA. If there are genetic differences, it is proved that planulae are produced sexually. Results revealed that only 2 families (Bise01#02 and Bise02#03YL) showed different DGGE profile, suggesting genetic difference between parent and offspring. The attempt to detect genetic difference in planulae of *P. damicornis* and their parents using DGGE method was not completely success since DGGE method can not prove clearly the genetic difference between parent and offspring. However, the possibility that DGGE method is applicable for studying coral can be suggested. PCR-DGEE amplification may perform with new STR (short tandem repeat) polymorphic loci of *P. damicornis* that currently found to answer whether planulae are produced sexually or asexually.

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