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Evidence for changing symbiotic algae in juvenile tridacnids

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Abstract

This study investigated the effects of different clonal strains of *Symbiodinium* sp. (zooxanthellae) on clam growth and survival, while monitoring the persistence of the induced symbioses in outdoor tanks and in the field using allozyme and random amplified polymorphic DNA (RAPD) analyses. Aposymbiotic clam larvae that were inoculated with homologous zooxanthellae (cultured or freshly isolated from the same host species) or heterologous zooxanthellae (cultured from different host species) had significantly-different survival rates at harvest (3 months post-spawning) with small growth differences. The improved survival rates in juvenile *Hippopus hippopus* (heterologous infection) and *Tridacna gigas* (homologous infection) were maintained through 3 months onshore and 3 months offshore. However, isozyme and RAPD analyses of re-isolated zooxanthellae after 3, 6, and 9 months revealed a high genetic diversity of symbionts (ca. 99% variation in 200 re-isolates) from individual hosts, within and between treatments. Furthermore, the genetic patterns of the re-isolated algae following clonal culture were different from those of the introduced clones, which, in contrast, retained their unique genetic patterns over many culture generations in the laboratory. These results demonstrate that the subsequently-established symbiont populations in juvenile clams were not clonal. The allozyme and RAPD techniques detected individual genetic differences in clam symbionts, but not differences between algal taxa. The presence of significant survival trends suggests possible differences between subsequently-established dominant symbiont taxa, which were uncultureable or undetectable using these genetic markers. The implications of this symbiont diversity in giant clams are discussed.

Keywords: Zooxanthella; Dinoflagellate; Diversity; Genetic marker; Isozyme; RAPD; Tridacnid clam; Survival

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1. Introduction

The majority of symbiotic dinoflagellates (zooxanthellae) occurring in marine invertebrates, specifically those harboured by many cnidarians and tridacnid clams, have been placed in the genus *Symbiodinium* due to their lack of distinguishing morphological characteristics and elusive sexual reproduction (see Freudenthal, 1962; Schoenberg and Trench, 1980a,b; Trench, 1987; Rowan and Powers, 1991a). Recently, however, the use of restriction fragment length polymorphism (RFLP) of the 18S rRNA gene has revealed the existence of high genetic diversity and several major clades among cnidarian zooxanthellae (Rowan and Powers, 1991a,b, 1992; Rowan and Knowlton, 1995). The increasing use of molecular genetic techniques has significantly advanced our understanding and appreciation of the role of these algal symbionts, whose precise taxonomic status is still unresolved.

For some time now it has been realised that single hosts may contain more than one taxon in residence at any one time (Rowan and Knowlton, 1995). Recent work on corals has shown that the greater resistance to high temperatures and irradiation of certain zooxanthellae taxa is indicative that resistance to bleaching may be a property of the zooxanthellae and not the animal (Fitt and Warner, 1995). Thus, the particular strain of zooxanthellae establishing symbiosis with the animal host could have a major influence on the stability and properties of the intact association.

This raises important questions relating to clam production. If zooxanthellae strains have different efficiencies in terms of photosynthesis and ability to transfer photosynthates to the host (see Iglesias-Prieto and Trench, 1994), then there is likely to be a relationship between the infecting strain and clam performance. Previous work on induced symbiosis of giant clams with zooxanthellae from various sources (different host species or fast-growing hosts) demonstrated differences in growth and survival rates in the laboratory (Fitt and Trench, 1981; Fitt, 1985) and outdoor culture tanks (Molea and Munro, 1994). Indeed, the need to evaluate the different ‘strains’ of zooxanthellae in tridacnids was underscored as among the research priorities at the previous international Giant Clam Genetics Workshop (Macaranas, 1993).

The present study carries previous efforts a step further by investigating the effects of homologous (from the same host species) or heterologous (from different host species) clonal strains of zooxanthellae on the growth and survival of clams in onshore tanks and in the field. It is not known whether initial zooxanthellae populations in tridacnid juveniles persist or are replaced by other competitive strains in the long run. For this reason, the persistence of zooxanthellae infection was monitored through symbiont re-isolation, clonal culture, and re-characterisation after periods of in hospite growth onshore and offshore. The genetic similarity between symbiont re-isolates and introduced clones was assessed by comparing both their allozyme patterns, resolved by polyacrylamide gel electrophoresis, and the patterns of their random amplified polymorphic DNA (RAPD), obtained from polymerase chain reactions (PCR). The results have important implications for the symbiotic partnership and how it adapts to its changing environment.

2. Materials and methods

2.1. Establishment and maintenance of clonal zooxanthellae cultures

Zooxanthellae were isolated from different species of giant clams held in culture or captivity at the University of the Philippines Marine Science Institute's (UPMSI) Bolinao Marine Laboratory in northwestern Philippines. Clippings of the clam mantle were rinsed in sterile 0.45 μm Millipore-filtered sea water (MFSW). The zooxanthellae were scraped out into a petri dish, resuspended in MFSW, and washed by centrifugation at 4000 rpm for 5 min, until easily resuspended in MFSW. These were brought into crude culture in f/2 medium (Guillard and Ryther, 1962) at a constant temperature of 28°C, irradiance level of 150 μE m^{-2} s^{-1}, and photoperiod of 12 h:12 h (Belda and Yellowlees, 1995). After a few days of incubation, single motile cells from serial dilutions were isolated under an inverted microscope using freshly-pulled sterile pasteur pipettes. Single-cell isolations were incubated in test tubes for at least a month. Viable clones were aseptically transferred, then treated with a triple-antibiotic solution of penicillin G (125 g l^{-1}), streptomycin (62.5 g l^{-1}), and chloramphenicol (12.5 g l^{-1}) for 24 h (Belda, 1994). Sterility tests in nutrient broth were made periodically to check for bacterial contamination. Uncontaminated cultures were subcultured into 50 ml cultures and routinely transferred every 2 weeks. Clonal cultures were purified further by re-isolating a single cell from each parent culture.

2.2. Production of clam larvae

Two separate groups of 30 *Hippopus hippopus* (22–30 cm shell length) and 40 *T. gigas* (54–63 cm shell length) were transported on consecutive occasions from the ocean nurseries off Silaqui Island (16°25′N, 119°55′E) to the outdoor hatchery of the marine laboratory. These clams were brushed free of epiphytes, left exposed to the air and sun for 1 h, and placed in a spawning tank containing UV-treated, 0.2 μm filtered sea water (UV-FSW). Representatives of each group were injected in the gonad with 2–4 ml of 2 mM serotonin via the anterior side of the exhalent siphon. Needles were rinsed in methylated spirits and in distilled water between use to minimise infection. Six clams of each species released sperm immediately after serotonin injection, while a couple of clams of each species released eggs half an hour later. Released sperm was scooped out into separate buckets and kept under shade. Egg-releasing clams were rinsed with UV-FSW, and allowed to release more eggs in separate containers of UV-FSW. For the large *T. gigas*, sperm and eggs were separately caught in plastic bags upon forceful expulsion from the clams' exhalent siphon. Eggs (ca. 8×10^6 for *H. hippopus* and 65×10^6 for *T. gigas*) were immediately fertilised with a mixture of sperm (ca. 1 ml dense sperm suspension per litre egg suspension) from conspecific clams which did not release eggs. Fertilised eggs of *H. hippopus* and *T. gigas* were transferred at stocking rates of 5 and 20 eggs ml^{-1} into three and six hatching tanks, respectively, containing 500 l of gently aerated UV-FSW with 10 ppm streptomycin. Every other day, larval

cultures were siphoned from 5 cm off the bottom of the tanks onto a series of 53 μm filter sieves to retrieve swimming veligers and replace the sea water. Beginning on day 3 and after each water change, the larvae were fed on cultured *Tetraselmis* sp. (2000–7000 cells ml^{-1} larval medium).

2.3. Design and maintenance of experiments

Two similar experiments were conducted in parallel for 9 months. Eight-day-old pediveliger larvae of *H. hippopus* and *T. gigas* were stocked at 58,400 and 55,000 larvae bin^{-1}, respectively (i.e., 26 and 25 pediveligers cm^{-2} of bin bottom, respectively), into 60-l plastic bins containing gently aerated UV-FSW and a slab of roughened cement substrate for larval settlement. On day 9, cultured (CZ) or freshly-isolated zooxanthellae (FIZ) were harvested or extracted, washed, and introduced into each larval bin at a density of ca. 200 cells $larva^{-1}$. *H. hippopus* bins were randomly allocated into one of four treatments in triplicate bins: CZ originally isolated from (1) *T. derasa* (PHMS TD1A), (2) *T. squamosa* (PHBO TS3A), (3) *H. hippopus* (PHMS HH1A), and (4) FIZ extracted from a parent *H. hippopus* (conspecific control). For *T. gigas* larvae, there were three treatments in four replicate bins: (1) a mixture of CZ from *T. gigas* (PHSO TG1A), *T. derasa* (PHMS TD1A), *H. hippopus* (PHMS HH1A), and *H. porcellanus* (PHSU HP1A), (2) CZ from *T. gigas* alone, and (3) FIZ from a parent *T. gigas*. Zooxanthellae infection was repeated on day 13 to ensure intake of the algal symbionts by the metamorphosing juveniles. Flow-through but serially-filtered (50, 10, 1 μm) sea water was used during the third and fourth weeks to keep out any 'wild' zooxanthellae and potential clam predators. All bins were partially immersed in a flow-through water bath, revolved weekly, and covered with a plastic net to control temperature, light, and algal fouling, respectively. Temperature (30 ± 1°C, $n=29$), salinity (34 ± 1‰, $n=29$), and irradiance level (250 ± 125 μE m^{-2} s^{-1}, $n=29$) were monitored twice weekly. At 1 month post-spawning, supply of unfiltered sea water was started. At 2 months post-spawning, juvenile clams (1–2 mm) were visible, but left undisturbed until they reached a size amenable to handling at harvest.

2.4. Monitoring of clam growth and survival

At 3 months post-spawning, juvenile clams were harvested by carefully cutting their byssi off the cement substrates. Total numbers of clams were recorded, and shell lengths of 30 haphazardly-collected clams bin^{-1} were measured to the nearest mm using calipers. A few clams bin^{-1} were set aside for zooxanthellae re-isolation, culture, and subsequent re-characterisation using genetic markers. Bins and substrates were brushed clean before re-stocking with equal numbers of clams ($N=500$ for *H. hippopus* and $N=400$ for *T. gigas*) for continued monitoring during the onshore phase of culture. After 3 months onshore, equal numbers of clams ($N=100$ for *H. hippopus* and $N=150$ for *T. gigas*) were transferred into 12 perforated plastic trays ($0.5\times0.3\times0.1$ m) containing cement substrates, and allowed to re-attach overnight. The next day, the clam trays were deployed to the subtidal ocean nursery and placed separately in 12 elevated

PVC-framed, polyethylene cages (1.2×0.6×0.3 m) for a 3-month field monitoring. In the *T. gigas* setup, each clam tray was further covered with a 0.5 cm mesh net while inside the 2.5 cm mesh cage to keep out small gastropod and hermit crab predators to which *Tridacna* spp. are susceptible. The trays were revolved weekly among the cages for light control. Temperature, salinity, and irradiance levels in the ocean nursery ranged from 27 to 32°C, from 30 to 34‰, and from 220 to 790 μE m^{-2} s^{-1}, respectively. Every month during the onshore and offshore culture, clams were counted and measured ($n = 30$). At the end of each culture phase, zooxanthellae were re-isolated from 2 clams bin^{-1} or $tray^{-1}$ and cultured for subsequent re-characterisation using genetic markers.

2.5. Molecular genetic characterisation of introduced and re-isolated zooxanthellae

To compare the genetic identities of introduced and re-isolated zooxanthellae, clonal cultures (over at least 10 and three generations, respectively) were maintained in uniform standard conditions, and log-phase cultures were harvested by centrifugation. Both RAPD and allozyme patterns were then analysed and compared between algal isolates. Isolates were considered indistinguishable if all the reproducible bands generated by allozyme and RAPD analyses were identical between isolates being compared.

Details of the allozyme analysis were reported previously (Baillie et al., 1998). Briefly, algal pellets were sonicated on ice for 3 min in two volumes of extraction buffer (100 mM Tris–HCl, pH 7.2; 15 mM mercaptoethanol; 5 mM EDTA; 50 mM NADP; 50% w/v glycerol; 0.02% w/v bromophenol blue). Cellular debris was removed by centrifugation (10,000 *g*, 1 min), and the supernatant was analysed by PAGE on 15% (w/v) polyacrylamide gels (Laemmli, 1970). Polyacrylamide gels were stained for the presence of α- and β-esterase (EST) isozymes, the most polymorphic and stable of 17 isozymes previously screened (Baillie et al., 1998), using the enzyme-staining protocols from Schoenberg and Trench (1980a) and Macaranas (1991).

For RAPD analysis, algal DNA was extracted using a modification of Rowan and Powers' (1991a,b) method. Each algal pellet was washed with 1 ml of DNA Isolation Buffer (DNAB: 0.4 M NaCl; 50 mM EDTA, pH 8.0), resuspended in DNAB with SDS (1% v/v final concentration), and heated to 65°C for 1 h. Proteinase K was added to the samples (0.5 mg ml^{-1} final concentration), which were then incubated at 45°C overnight. Crude DNA extracts were boiled for 10 min to inactivate Proteinase K. Extracted DNA was PCR-amplified using RAPD primer #18 of primer set #1 purchased from the Nucleic Acid/Protein Service Unit of the University of British Columbia (NAPS UBC). RAPD primer #18 is a 10-mer primer (GGG CCG TTT A) that yielded the highest number of distinct and reproducible polymorphic bands out of 100 primers screened and purchased from NAPS UBC (B.K. Baillie, C.A. Belda-Baillie, M. Sison, E.D. Gomez, V. Silvestre, V. Monje, unpublished data). The PCR thermal profile consisted of the following: 90 s initial denaturation at 94°C; 10 cycles of 5 s denaturation at 94°C, 10 s annealing at 37°C, and 120 s elongation at 72°C; another 30 cycles of 5 s denaturation at 94°C, 10 s annealing at 40°C, and 120 s elongation at 72°C; and a final 240 s elongation at 72°C. Amplified DNA was separated on a 2% agarose gel and visualized by ethidium-bromide staining. Duplicate RAPD analyses were made for all of the samples to ensure that only reproducible bands were considered.

2.6. Statistical analysis of data

To test the null hypothesis that different zooxanthellae and sampling times had no effect on clam growth and survival at $\alpha = 0.05$, data were analysed using ANOVA for two orthogonally-arranged fixed factors (treatment and sampling time), with a third random factor (bin or tray) nested within each treatment combination (Underwood, 1981). Statistical analyses were performed using Statistix 3.0 (Analytical Software, 1985). Data on *T. gigas* survival were arcsine-transformed to conform to the assumption of variance homogeneity (Cochran's test, $P > 0.05$). Where the ANOVA results were significant, Tukey's test was used to compare treatment means (Zar, 1984).

3. Results

3.1. Survival and growth of clams

Initial survival rates at harvest of *H. hippopus* juveniles infected with CZ from heterologous sources (*T. derasa*, *T. squamosa*) were three-fold significantly higher than those infected with homologous CZ or FIZ (i.e., 2.6±0.4% versus 0.8±0.2%, $n = 6$) (ANOVA, $P < 0.001$; Tukey, $P < 0.05$) (Fig. 1). Subsequent survival rates onshore were relatively unchanged at 2.4±0.4% ($n = 6$) for those with heterologous infection, and 0.8±0.2% ($n = 6$) for those with homologous infection. This trend continued offshore.

For *T. gigas* a reverse trend was evident. Initial survival rates were three-fold significantly higher for clams infected with homologous CZ or FIZ than with heterologous CZ (from a mixture of *Tridacna* and *Hippopus* sources) (i.e., 2.0±0.2% versus 0.6±0.1%, $n = 8$ and $n = 4$, respectively) (ANOVA, $P < 0.001$; Tukey, $P < 0.05$) (Fig. 1). Subsequent survival rates onshore changed little at 1.7±0.2% ($n = 8$) for those with homologous infection, and 0.3±0.1% ($n = 4$) for those with heterologous infection. This trend also continued offshore.

Differences in growth rates were smaller than those for survival. At harvest, *H. hippopus* with heterologous infection were slightly, but not significantly, bigger than those with homologous infection (i.e., 6.0±0.2 mm versus 5.0±0.1 mm, $n = 180$) (ANOVA, $P > 0.05$) (Fig. 2). This trend continued after 3 months onshore (29.0±0.4 mm versus 27.0±0.5 mm, $n = 180$). After 3 months offshore, clams infected with heterologous CZ from *T. derasa* were significantly smaller (45.0±1.8 mm, $n = 90$) than those infected with heterologous CZ from *T. squamosa* or homologous CZ or FIZ (50.0±0.4 mm, $n = 270$) (Tukey, $P < 0.05$).

For *T. gigas*, those with homologous infection were significantly bigger at harvest than those with heterologous infection (i.e., 3.0±0.1 mm versus 2.0±0.1 mm, $n = 240$ and 120, respectively) (ANOVA, $P < 0.05$; Tukey, $P < 0.05$) (Fig. 2). Clams infected with homologous CZ were the biggest of all (3.2±0.1 mm, $n = 120$) (Tukey, $P < 0.05$). This trend continued after 3 months onshore, where clams infected with homologous CZ were significantly the biggest (22.0±0.5 mm, $n = 120$), followed by those infected with homologous FIZ (20.0±0.8 mm, $n = 120$), while those with heterologous infection were significantly the smallest (19±0.7 mm, $n = 120$) (Tukey, $P < 0.05$). After 3 months

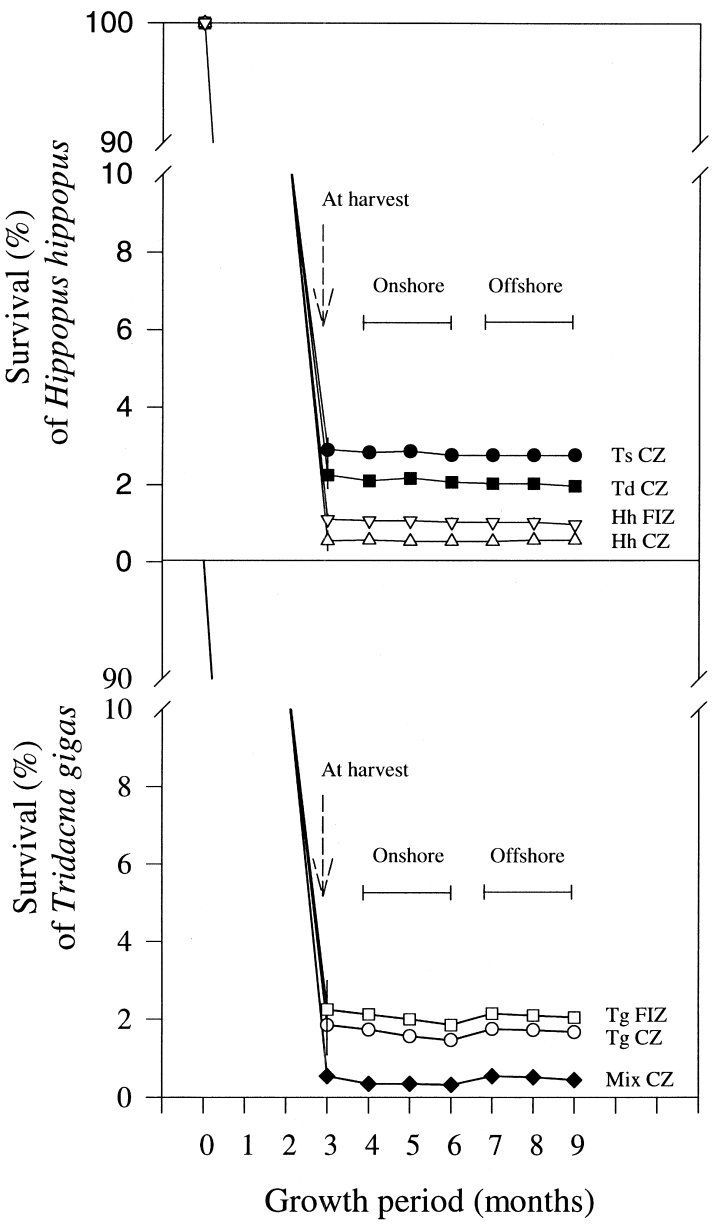

Fig. 1. Mean survival (%±SE) of *Hippopus hippopus* and *Tridacna gigas* over a 9-month growth period. Clam larvae were infected with different cultured (CZ) or freshly-isolated zooxanthellae (FIZ). Mixed CZ were originally from *T. gigas*, *T. derasa*, *H. hippopus*, and *H. porcellanus*. Ts, *T. squamosa*; Td, *T. derasa*; Hh, *H. hippopus*; Tg, *T. gigas*.

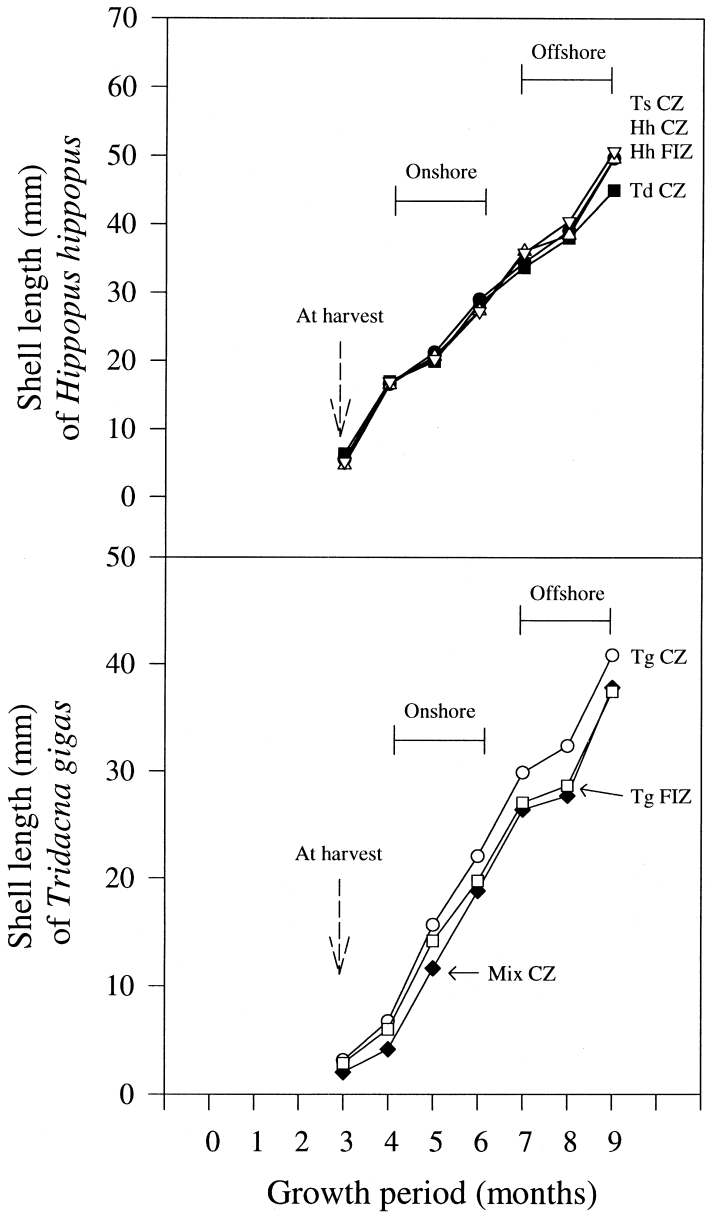

Fig. 2. Mean growth (mm±SE) of *Hippopus hippopus* and *Tridacna gigas* over a 9-month growth period. Clam larvae were infected with different cultured (CZ) or freshly-isolated zooxanthellae (FIZ). Mixed CZ were originally from *T. gigas*, *T. derasa*, *H. hippopus*, and *H. porcellanus*. Ts, *T. squamosa*; Td, *T. derasa*; Hh, *H. hippopus*; Tg, *T. gigas*.

Table 1
Comparison of α+β-EST isozyme and RAPD[a] electrophoretic patterns of introduced and re-isolated zooxanthellae

Experimental host	Source of introduced zooxanthellae	EST and RAPD electrophoretic patterns	
		Introduced zooxanthellae	Re-isolated zooxanthellae
Hippopus hippopus	*H. hippopus*	HH1A	Unlike HH1A[b]
	T. derasa	TD1A	Unlike TD1A[b]
	T. squamosa	TS1A	Unlike TS1A[b]
Tridacna gigas	*T. gigas*	TG1A	Unlike TG1A[b]
	T. gigas, *T. derasa*, *H. hippopus* and *H. porcellanus*	nd[c]	nd
		nd	nd

[a] RAPD primer #18 (GGG CCG TTT A) of primer set #1 from Nucleic Acid/Protein Service Unit of the University of British Columbia.
[b] All 200 re-isolated, then cultured zooxanthellae after 3, 6, and 9 months had patterns different from those of introduced zooxanthellae.
[c] nd, no data since introduced zooxanthellae were a mixture of different clonal strains.

offshore, clams infected with homologous CZ remained significantly bigger (41±0.8 mm, $n = 120$) than those infected with homologous FIZ or heterologous zooxanthellae, which by then had comparable sizes (38.0±0.6 mm, $n = 240$) (Tukey, $P < 0.05$).

3.2. Molecular genetic characterisation of introduced and re-isolated zooxanthellae

All zooxanthellae re-isolated at every stage of the experiments were found to have α+β-EST isozyme and RAPD band patterns different from those of the clonal cultures initially introduced to the clam larvae (Table 1). In contrast, the genetic patterns of the introduced zooxanthellae remained unchanged over many culture generations in the laboratory (Fig. 3). Also, the band patterns of the zooxanthellae re-isolates were different within individual hosts, within and among treatments (ca. 99% variation in 200 re-isolates).

4. Discussion

4.1. Genetically-diverse symbiont populations in giant clams

This study has clearly demonstrated that the subsequently-established symbiont populations in juvenile *Hippopus* and *Tridacna* were not clonal (cf. Schoenberg and Trench, 1980a), as opposed to the clonal strains of zooxanthellae initially introduced to the aposymbiotic clam larvae. About 99% of a total of 200 re-isolated, then cultured zooxanthellae from individual test hosts, within and between treatments, had isozyme and RAPD electrophoretic patterns different from one another. This confirms previous

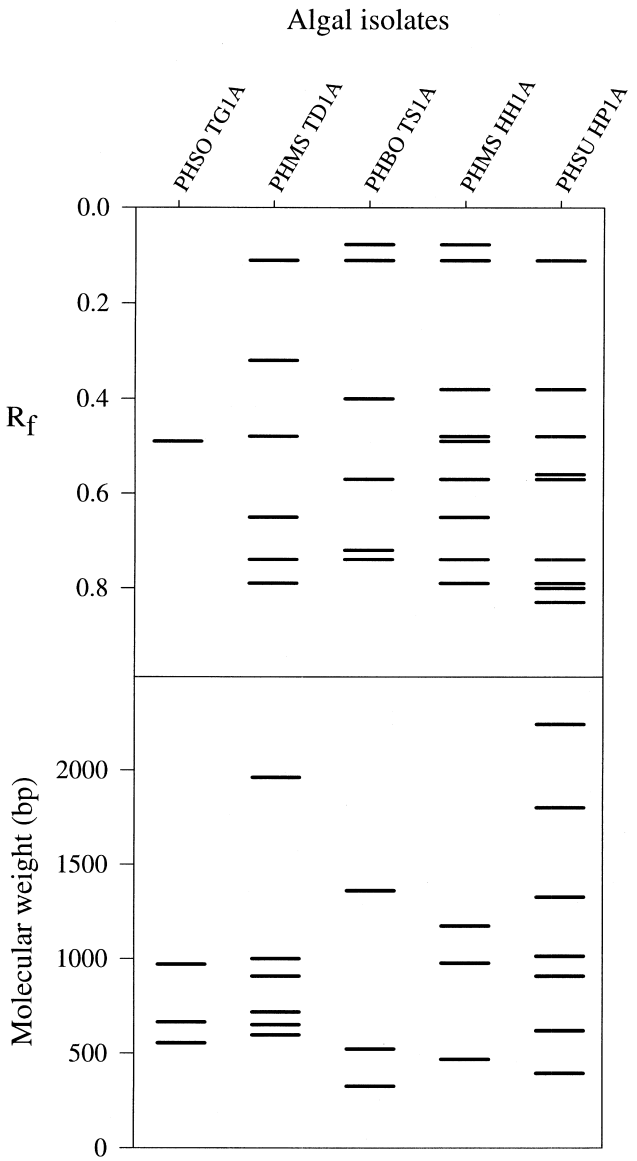

Fig. 3. (top) α+β-EST isozyme and (bottom) RAPD (UBC primer #18) electrophoretic patterns of the original clonal stocks of zooxanthellae used for the infection trials. Cultured zooxanthellae were originally isolated from *T. derasa* (PHMS TD1A), *T. squamosa* (PHBO TS3A), *H. hippopus* (PHMS HH1A), *T. gigas* (PHSO TG1A), and *H. porcellanus* (PHSU HP1A) (Rf, relative mobility; bp, base pairs).

findings that individual zooxanthellae isolated from seven giant clams species (adult or juvenile) had distinguishable isozyme and RAPD patterns (Baillie et al., 1998). Recently, analyses of the complete 18S rDNA sequences of FIZ and CZ from members

of the giant clam family indicate that cultureable zooxanthellae, which belong to Clade A, comprise only a fraction of the total zooxanthellal population (Carlos et al., 1999). Furthermore, zooxanthellae from *T. gigas* have been previously observed to be predominantly type C with a lesser amount of type A based on RFLP analysis of the 18S rRNA gene (Rowan et al., 1996). This suggests that the symbiont diversity found in this study represents only a subset of the total genetic diversity present in clams and in the natural environment.

The potential sources of such symbiont genetic diversity in clam hosts have been discussed previously (Baillie et al., 1998), and may derive from a number of sources that include acquisition of new genotypes from the water column during filter feeding, mutation during clonal growth, and sexual reproduction. The fact that few, if any, of the zooxanthellal isolates were genetically indistinguishable strongly indicates that clonal growth is not the only process of reproduction in clam zooxanthellae. The high diversity of the zooxanthellae populations may be important in allowing the giant clam association to adapt to changing environmental conditions, including variations in solar radiation, temperature, and nutrients.

4.2. Molecular tools for monitoring of symbiont populations

This study has also demonstrated that allozyme and RAPD electrophoreses are very sensitive techniques that detect individual genetic differences in clam symbionts but not differences between algal taxa. On the other hand, ongoing analysis of the internal transcribed spacer (ITS) region of cultured zooxanthellae from giant clams shows that they all belong to Clade A, distributed into two subgroups that differ from one another by only one nucleotide (B.K. Baillie and T. Maruyama, unpublished data). RFLP and sequence analyses of the 18S rRNA gene have previously been unable to further resolve Clade A of the genus *Symbiodinium* from clams (Rowan and Powers, 1991a,b; Carlos et al., 1999).

The choice of isozyme and RAPD as genetic markers used in this study was dictated by the types of facilities available at the time. Use of these techniques, in turn, necessitated the culture of a large number of zooxanthellae samples under identical conditions and over several generations for a meaningful comparison of their band patterns. For these reasons, only a couple of the most useful genetic markers [$\alpha+\beta$-EST and RAPD UBC primer #18 (Baillie et al., 1998)] were used to efficiently check the genetic characteristics of the re-isolated zooxanthellae from the experimental clam hosts. While these techniques have been useful in confirming the non-clonal nature of symbionts in giant clams (see also Baillie et al., 1998), they are not suitable for monitoring taxonomic changes in symbiont populations over time. On the other hand, recent use of temperature-gradient gel electrophoresis (TGGE) of the PCR-amplified 18S rDNA hypervariable region has shown that giant clams normally associate with up to four different dominant symbiont taxa, one or two at a time, along with a few minor ones (A.A. Carlos, C.A. Belda-Baillie, B.K. Baillie and T. Maruyama, unpublished data). This technique is potentially more useful than RFLP and sequence analyses of the 18S rRNA gene (Rowan and Powers, 1991a,b; Carlos et al., 1999) in distinguishing closely-related symbiont taxa.

4.3. Survival trends in clams versus introduced zooxanthellae

In view of the foregoing discussion, the observed differences in clam growth and survival cannot be attributed directly to the clonal strains of zooxanthellae initially introduced to the aposymbiotic clam larvae. The presence of such significant trends, however, suggests possible differences between subsequently-established dominant symbiont taxa, which were uncultureable or undetectable using the allozyme and RAPD techniques.

Symbiosis between giant clams and zooxanthellae starts soon after metamorphosis of the clam larvae (Fitt, 1985) at 1 to 2 weeks post-spawning, during which heavy mortalities occur (Lucas, 1994). In this study, all clams were maintained undisturbed in serially-filtered sea water (50, 10, 1 μm) during the first month, after which flow of unfiltered sea water into the experimental setups was commenced. Previously, Fitt (1985) convincingly demonstrated that all zooxanthellae 'strains' from giant clams, sea anemone, and jellyfish, which were offered to *Hippopus hippopus* and *Tridacna squamosa* veliger larvae under controlled laboratory conditions, were observed to pass through the clams' digestive gland region, take up residence in the ''haemal sinuses'' of the metamorphosed clams, and divide for at least 1 week after metamorphosis, indicating healthy algae.

In this study, no attempt was made to re-isolate zooxanthellae from post-metamorphic clams prior to harvest (3 months post-spawning) in order to minimise clam stress and mortalities due to handling. This would ensure that early survival rates (a major variable under investigation) of juveniles that were amenable to harvest after 3 months (3–5 mm shell length) would be unaffected. It may be safe to assume that post-metamorphic clams initially established symbiosis with the introduced zooxanthellae during the first month when 'wild' zooxanthellae were excluded. This would be consistent with previous work on giant clams (Fitt, 1985), jellyfishes (Fitt, 1985), and sea anemones (Kinzie and Chee, 1979; Schoenberg and Trench, 1980b; Davy et al., 1997). However, given that the allozyme and RAPD techniques detected individual genetic differences, but not differences between algal taxa, we can only speculate on what happened immediately after initial establishment of symbiosis, and especially so after the clams were exposed to 'wild' zooxanthellae at 1-month post-spawning. Furthermore, inasmuch as the established symbionts in the harvested juveniles were found to be different and non-clonal, as opposed to the introduced clonal strains, it matters little that no symbiont re-isolation and re-characterisation were made earlier using the same molecular tools.

At 3, 6, and 9 months post-spawning, all the re-isolated zooxanthellae were found to have genetic patterns different from those of the introduced clonal strains of zooxanthellae (Table 1). The original stocks of infecting zooxanthellae, on the other hand, maintained their genetic patterns over many culture generations in the laboratory (Fig. 3). Given that the cultureable zooxanthellae are only a fraction of the total symbiont populations in giant clams (Carlos et al., 1999), it is probably not surprising that the introduced genotypes could no longer be detected after exposure of the juveniles to an alternative source of zooxanthellae.

The significant trends in clam survival and growth are interesting, particularly for *T. gigas*. Three-month-old *T. gigas* infected with homologous zooxanthellae had three-fold

higher survival rates and better growth rates than those infected with heterologous zooxanthellae (Fig. 1b). Comparable preliminary results were obtained in big raceways, where *T. gigas* infected with homologous CZ or FIZ had significantly higher survival and growth rates than those infected with a mixture of heterologous zooxanthellae from *Hippopus* spp. (C.A. Belda-Baillie, unpublished data). Previously, it has been shown that most re-isolated zooxanthellae from *T. gigas* clustered together as a group based on their allozyme patterns (Baillie et al., 1998) and RAPD patterns (B.K. Baillie, C.A. Belda-Baillie, E.D. Gomez, M. Sison, V. Silvestre and V. Monje, unpublished data). Based on these data, there appears to be some specificity in symbiosis between the largest and fastest-growing *T. gigas* species and a distinct group or taxon of symbionts in forming a successful symbiotic association. More manipulative studies on symbiosis specificity are clearly desirable.

5. Conclusions

The diversity of symbionts in giant clams raises the question of source of such genetic variation, and whether the same is true for most symbiotic reef organisms. The potential sources of such diversity (see Baillie et al., 1998) need to be elucidated to better understand the dynamics of zooxanthellae populations, and how these symbiotic associations adapt to their changing environment. Indeed, the increasing problem of widespread coral bleaching warrants more investigation on the extent to which zooxanthellae populations are involved in the bleaching phenomena. The giant clam–zooxanthellae symbiosis is a very useful system for investigating the mechanism of bleaching since they also suffer from it (Norton et al., 1995), host similar zooxanthellae taxa as corals (Rowan et al., 1996), are potentially sensitive indicators of disturbances in the environment (see Belda-Baillie et al., 1998), and can supply large quantities of both host tissue and zooxanthellae for analysis.

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