Allozyme electrophoresis as a tool for distinguishing different zooxanthellae symbiotic with giant clams

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The taxonomy of zooxanthellae in marine invertebrate symbioses is not well understood owing mainly to their lack of reliable morphological differences. Nevertheless, previous work using protein and DNA electrophoreses has set the stage for advancing our taxonomic understanding of cnidarian zooxanthellae. Here we present the use of allozymes as genetic markers for distinguishing algal isolates from tridacnid hosts. Zooxanthellae from seven *Tridacna* and *Hippopus* species were isolated and maintained in axenic clonal cultures over many generations. Of 16 enzyme systems, α - and β -esterase (EST), esterase-F (EST-F), glucose phosphate isomerase (GPI), and malate dehydrogenase (MDH) were found suitable polymorphic markers of genetic differences among clonal cultures. Of 39 clonal isolates, 97% were found to be genetically distinguishable. This high extent of genetic variation in zooxanthellae within and between clam species was unexpected, and is difficult to explain based solely on the general notion of asexual reproduction in symbiotic zooxanthellae. Our results are also consistent with the occurrence of sexual reproduction in clam zooxanthellae. The close genetic similarity of the symbionts of *Tridacna gigas*, the largest and fastest-growing clam species, and the difficulty of initiating their clonal cultures in the given nutrient medium, compared with the symbionts of other clam species, are further indicative of possibly distinct algal symbionts in *T. gigas*. These findings are discussed in light of current taxonomic understanding of these organisms.

Keywords: allozyme; genetic marker; electrophoresis; zooxanthellae; tridacnid clam; symbiosis

1. INTRODUCTION

Tridacnid clams live in symbiotic association with photosynthetic dinoflagellate algae of the genus *Symbiodinium* (Taylor 1969; Trench & Blank 1987). These algal symbionts secrete photosynthetic products which support most energy and some structural demands of their animal host (Trench *et al.* 1981; Fisher *et al.* 1985; Griffiths & Streamer 1988; Klumpp *et al.* 1992). Indeed, the success and large size of giant clams in reef waters have been related, to some extent, to the presence of these algal symbionts, owing to the extra nourishment derived by the former from the latter (Klumpp *et al.* 1992; Lucas 1994).

Giant clams have long been used in the shell-craft industry (Juinio *et al.* 1989) and as a source of food in many Indo-Pacific countries (Juinio *et al.* 1989; Lucas 1994). However, pressure from overfishing and the destruction of reef environments have led to the localized extinction of some species (Lucas 1994) and pose a threat to the others. Mariculture and conservation efforts have therefore been underway for sometime now to ensure the

continued existence and supply of clams in the Indo-Pacific region (Lucas 1994).

Whilst the genetics of the clam host has been investigated to some extent (Benzie & Williams 1992, 1996; Munro 1993), that of its algal symbionts is virtually unexplored. The taxonomy of symbiotic dinoflagellates in general is not well understood, due to the paucity of their distinguishing morphological characteristics (Freudenthal 1962; see Schoenberg & Trench 1980b). Research into zooxanthellae genetics is also difficult due to the apparent lack of sexual reproduction in these organisms (see Schoenberg & Trench 1980a; Trench 1987; Rowan & Powers 1991a). In many studies of marine invertebrate symbiosis, zooxanthellae are referred to simply as *Symbiodinium* sp., since the specific taxonomy is still unknown.

There have been several taxonomic studies of zooxanthellae, mostly from cnidarian hosts. These studies have relied upon morphological comparisons (Schoenberg & Trench 1980b), host interactions (Schoenberg & Trench 1980c), and DNA-based methods (Rowan & Powers 1991a,b, 1992; Rowan & Knowlton 1995). The general picture that has emerged from these studies is that many taxa of symbiotic zooxanthellae occur in the marine environment, and that genetically similar symbionts are specific to a host species. However, genetically similar taxa may be common to a number of

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taxonomically distant hosts (Rowan & Powers 1991a,b; McNally *et al.* 1994). Under controlled conditions, some algal symbionts may infect other hosts (Fitt 1985), but this does not always occur readily and may result in reduced numbers of infecting symbionts in time (Schoenberg & Trench 1980c).

More recently, Molea & Munro (1994) investigated the effects of zooxanthellae taken from various clam sources on the growth of *Tridacna gigas* juveniles. They found that algae taken from fast-growing *T. gigas* had a significant positive effect on the survival and growth rates of juvenile clams. This observation and suggestions made by a number of researchers (Fitt 1985; Macaranas 1993) have prompted us to investigate the identity and properties of zooxanthellae isolates from different tridacnid clams.

In this study, we have analysed the same allozyme markers investigated by Schoenberg and Trench (1980a), with emphasis on the use of polyacrylamide gel electrophoresis (PAGE). Allozyme analysis is particularly well suited to the analysis of genetic diversity under field conditions, unlike DNA-based methods, which require sensitive, expensive equipment, and a high degree of operator skill. The technique allows large numbers of samples to be processed quickly and cheaply, making it an ideal choice for identifying clonal isolates with only minimal equipment and culture facilities, a situation common to giant clam mariculture facilities in the Indo-Pacific region.

The ability to identify and characterize genetically different zooxanthellal isolates from giant clams, in addition to its obvious scientific interest, is a necessary first step in evaluating zooxanthellae for the potential improvement of the mariculture of these bivalves. Enhanced mariculture techniques, in turn, should lead to decreased fishing pressure on natural populations, with increased production of juvenile clams for commercial purposes and conservation programmes.

2. MATERIALS AND METHODS

(a) Isolation and culture of zooxanthellae

T. gigas, *T. derasa*, *T. squamosa*, *T. maxima*, *T. crocea*, *Hippopus hippopus* and *H. porcellanus* were obtained from cultured and captive wild stocks being held in the ocean nurseries of the University of the Philippines Marine Science Institute's (UPMSI) Bolinao Marine laboratory in Pangasinan, Philippines (16°25'N, 119°55'E). The wild clams originally came from north-eastern, north-western, and western Philippines. As larvae, the cultured clams were initially infected with zooxanthellae extracted from parent clams that originally came from central Philippines and the Solomon Islands. Mantle clippings (<1 cm²) from each clam were homogenized in 0.45 µm Millipore-filtered sea water (MFSW) using a blender, and filtered on a double layer of cheese cloth to remove animal mucus and debris (Belda *et al.* 1993). Zooxanthellae were collected and washed several times in MFSW by centrifugation at 1000 *g* for 5 min to produce freshly isolated zooxanthellae (FIZ). These were then analysed by electrophoresis or brought into crude culture in f/2 media (Guillard & 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 & Yellowlees 1995). After a few days of

incubation, freshly pulled Pasteur pipettes and an inverted microscope were used to isolate single motile cells from serial dilutions of a drop of zooxanthellae suspension placed in a Petri dish. Single cells were then incubated for at least a month in individual test tubes containing f/2 media, under the above conditions of growth. Viable clones were 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 carried out periodically to check for bacterial contamination. Uncontaminated cultures were subcultured into 50-ml cultures and routinely transferred every two weeks. Subsequently, the clonal cultures were further purified by re-isolating a single algal cell from each parent culture, and growing into a 50-ml culture or a one-litre culture in Fernbach flasks, under the conditions mentioned above.

(b) Harvest of zooxanthellae and extraction of algal proteins

After 2–3 weeks of culture (log phase), zooxanthellae were harvested by centrifugation at 1000 *g* for 5 min. Zooxanthellae pellets were washed once in MFSW, twice in distilled water, and then recovered by further centrifugation after each wash. Clean pellets were resuspended in two volumes of an extraction buffer modified from Schoenberg and Trench (1980a), i.e. 100 mM Tris-HCl, 5 mM ethylenediaminetetraacetic acid, 15 mM 2-β-mercaptoethanol, 50 µM β-nicotinamide adenine dinucleotide phosphate, 50% (w/v) glycerol, and 0.01% (w/v) bromophenol blue, at pH 7.1, and then sonicated for 1 min on ice. Extracts were stored at –20 °C until ready for use. Extracts were later centrifuged at 15 000 *g* for 2 min, and the supernatant loaded onto electrophoresis gels.

(c) Gel electrophoresis

Electrophoresis was used to resolve the allozymes present in the protein extracts from harvested algae. Both starch gel electrophoresis (SGE) and polyacrylamide gel electrophoresis (PAGE) were initially used for this purpose.

For SGE analysis, 12% (w/v) starch gels (15 cm wide × 20 cm long) were run at 35 mA and 4 °C for 4 h. The buffers used were those outlined by Schoenberg & Trench (1980a). PAGE analysis of samples was carried out using the method of Laemmli (1970), without the addition of SDS to gels or buffers. The gel dimensions were 5 × 10 cm, with a resolving gel concentration of 15% acrylamide, 1.4% bis-acrylamide and a stacking-gel concentration of 3.9% acrylamide, 0.34% bis-acrylamide. Gels were run at 15 mA and 4 °C until the bromophenol blue dye-front reached the bottom of the resolving gel.

(d) Enzyme staining

The methods of Schoenberg and Trench (1980a) were used to detect α- and β-esterase (EST), tetrazolium oxidase (TO), glucose phosphate isomerase (GPI), and malate dehydrogenase (MDH). EST staining involved the simultaneous detection of both α- and β-specific enzymes. Acid phosphatase (ACP), alkaline phosphatase (AKP), catalase (CAT), esterase-F (EST-F), glucose-6-phosphate dehydrogenase (G6PDH), glycerol-3-phosphate dehydrogenase (G3PDH), superoxide dismutase (SOD), diaphorase (DIA), glutamate dehydrogenase (GDH), lactate dehydrogenase (LDH), alcohol dehydrogenase (ADH), and peroxidase (PER) staining were performed according to the methods described by May (1992).

(e) *Analysis of allozyme patterns*

To ensure the accuracy of allozyme patterns, all EST results were run in triplicate, and all others at least in duplicate. Patterns that could not be confirmed by duplicate or triplicate analyses were repeated until a consensus was reached. Replicates consisted of batches of algae grown at different times. Both Fernbach flask (one-litre) and flask (50-ml) cultures were analysed for most isolates. Repurified clones of most isolates were analysed once to confirm that the parent cultures were clonal.

The analytical method described by Schoenberg & Trench (1980*a*) was adopted. This involved scoring each allozyme band as a morphological character, then calculating the Jaccard similarity coefficient (Sneath & Sokal 1973) for each symbiont, and finally presenting the results as a pairwise dendrogram.

(f) *Scoring of allozymes and analysis of data*

After staining, the gels were photographed and prints were made for scoring. Comparisons between the allozyme patterns allowed each unique allozyme band to be identified. The presence (1) or absence (0) of each allozyme band was noted for every isolate. Jaccard similarity coefficients (S_j) (Sneath & Sokal 1973) were calculated from the data collected. The data were then displayed graphically in the form of dendrograms created using the unweighted average linkage method of clustering analysis (Sneath & Sokal 1973).

3. RESULTS

(a) *Culture of zooxanthellae*

On average, 20% (two out of ten replicate clones) of the clonal zooxanthellae cultures initiated from the FIZ of *T. derasa*, *T. squamosa*, *T. maxima*, *T. crocea*, *H. hippopus*, and *H. porcellanus* were viable. Cloning attempts on FIZ from *T. gigas* were not at all successful. Zooxanthellae isolated from *T. gigas* had to be first brought into crude culture for the cloning attempt to be successful, if at all. On the other hand, the success rate of establishing repurified clonal cultures from previously cloned zooxanthellae was *ca.* 50%. It generally took 3–4 weeks to detect visible growth of zooxanthellae clones in test tubes. The 39 clonal cultures that were subsequently analysed consisted of one to ten clones from each of one to two individual hosts of each of the seven clam species.

(b) *Allozyme patterns*

Clones PHMS TD1A, PHMS TM4A, PHBO TS3A, PHMS HH1A, and PHSU HPIA (Culture codes: clone no. [A] from clam no. 1, 3, or 4 [numerals] of the clam species *T. derasa* [TD], *T. maxima* [TM], *T. squamosa* [TS], *H. hippopus* [HH] or *H. porcellanus* [HP] that came from an area in the Philippines [PH] such as Bolinao [BO], or were UPMST's [MS] or Silliman University Marine Laboratory's [SU] cultured clams from wild parent stocks) were used to determine the usefulness of different allozyme systems in determining the genetic difference among the algal isolates. SGE analysis of the cultures produced polymorphic allozyme patterns. However, it was difficult to determine the patterns for most of the cultures due to poor resolution and staining (results not shown). PAGE analysis of the same samples demonstrated clear polymorphic patterns with good resolution of allo-

zymes. For this reason PAGE analysis was used to investigate all allozyme markers.

After allozyme analysis of these algal cultures, EST, EST-F, MDH, and GPI were found to be polymorphic, with considerable differences detected in the allozyme patterns between isolates. ACP, AKP, G6PDH, and G3PDH were polymorphic but difficult to detect. CAT, SOD, TO, and PER were monomorphic and therefore unsuitable for use in distinguishing genetic differences. DIA, GDH, LDH, and ADH could not be detected.

Due to their polymorphic nature, stability, and ease of detection, EST, EST-F, MDH, and GPI allozymes were used for the analysis of all algal isolates. Extracts could be stored for up to two weeks at -20°C before some of these allozyme patterns became unstable. MDH and GPI allozymes were particularly sensitive to degradation; whereas, EST and EST-F were, in general, very stable. Of the four allozyme systems, EST was the most informative with respect to the number of unique scoreable bands (22). By comparison EST-F (12), GPI (12), and MDH (7) were less informative. The allozyme patterns of the 39 clonal isolates obtained for these four enzyme systems are given in figure 1 (*a–d*).

(c) *Analysis of allozyme patterns*

The presence or absence of each allozyme band was recorded for all cultures and the results entered into an array of 53 characters (scorable allozyme bands) by 39 species (data not shown). The pattern for each species was compared with all other species using the coefficient S_j (Sneath & Sokal 1973). The results of the unweighted average linkage method of clustering analysis of the calculations of S_j are displayed in figure 2.

From the S_j dendrogram (figure 2), 11 out of 12 TG (*T. gigas*) clones form a cluster. PHMS TC2B, PHMS TC2C, and PHMS TC2D (*T. crocea*) clones also form a cluster; however, this cluster is by no means as clear as that for the TG clones. There appears to be at least four major clusters distinguishable at the level where TG clones cluster. Similar trends were observed from a graphical display of calculated Jaccard genetic identity coefficients, I_j (Sneath & Sokal 1973) (data not shown).

The α - and β -EST patterns of FIZ from *T. gigas* juveniles were also investigated (figure 1*a*). The patterns did not match those of the Tg cultures. Both FIZ and cultured zooxanthellae (CZ) contained allozymes which were absent in the other. Likewise, the MDH and GPI allozyme bands, which were distinctive for different clonal cultures in the HH CZ, could not be detected in the HH FIZ. Only bands of GPI activity present in all CZ clones could be detected easily in the FIZ (figure 1*d*).

4. DISCUSSION

(a) *Asexual versus sexual reproduction in symbiotic zooxanthellae*

Individual zooxanthellae isolated from giant clams were virtually all genetically distinguishable from each other, based on their allozyme patterns. This degree of variation in clonal isolates from the same host species (and individual) has not as yet been reported.

Such variation may arise from a number of processes, which include accumulated mutations during asexual

Figure 2. Dendrogram demonstrating the genetic relationships among the clonal isolates of zooxanthellae from giant clams based on Jaccards similarity coefficient (Sneath & Sokal 1973). Culture codes: clones no. 1–10 [A–J] from clams no. 1–4 [numerals] of the clam species *T. gigas* [TG], *T. derasa* [TD], *T. squamosa* [TS], *T. maxima* (TM), *T. crocea* (TC), *H. hippopus* [HH], or *H. porcellanus* [HP] that came from an area in the Philippines [PH] such as Bolinao [BO], or were cultured clams from wild parent stocks at UPMSI, Philippines [MS], Silliman University Marine Laboratory, Philippines [SU], or ICLARM Coastal Aquaculture Centre, Solomon Islands [SO].

reproduction, as well as sexual recombination. Some of the variation observed in this study was almost certainly caused by mutation during clonal growth. However, it is difficult to imagine that the large variation seen between our clonal isolates, based on comparisons of a small number of alleles, was caused solely by clonal mutation. A large part of this variation may well be caused by sexual recombination.

Sexual reproduction has been reported in dinoflagellates (Pfiester 1987), but so far there is little evidence of this in *Symbiodinium* sp. Suggestions of the absence of sexual reproduction in zooxanthellae have been based largely on observations of cultured algae (see Schoenberg & Trench 1980a; Trench 1987). Whilst it has been assumed that sexual reproduction is lacking in zooxanthellae symbiotic with cnidarians (Schoenberg & Trench 1980a; Trench 1987; Rowan & Powers 1991a), suggestions of sexual reproduction have been made by Freudenthal (1962) and Taylor (1974). However, the life cycles reported by these researchers are not in agreement (see also Trench 1997).

Examination of the coccoid and mastigote stages of *Symbiodinium* sp. (Blank & Trench 1985; Trench 1997) suggests that both stages are haploid. Hence, meiosis

would not occur in the coccoid cells. Trench (1997) suggests that if sexual reproduction does occur in these organisms, infrequent events of sexual recombination may take place in the 'free-living' state. If such is the case, then it seems likely that clams are able to acquire their genetically diverse symbionts from the water column. Giant clams initially obtain their complement of algal symbionts during their larval and early juvenile stages of development (Fitt & Trench 1981). It is possible then that as clams grow, they are able to obtain new zooxanthellae from their surroundings through filter feeding, or even exchange their symbionts, thereby accounting for their highly diverse algal population.

That only haploid forms have been seen *in hospite* does suggest that sexual reproduction, if it occurs in these organisms, occurs in the 'free-living' form. However, although the work of Trench & Blank (1985) has been used to imply that both the coccoid and mastigote stages are haploid (Trench 1997), later evidence suggests that 'chromosome' counting may not be as reliable as was first thought, since condensed DNA regions (CDRs) may not represent whole chromosomes (Udy & Hinde 1993). If the coccoid cells are diploid or haploid, and if sexual reproduction occurs *in hospite*, this would imply that

zooxanthellae have a number of cryptic life stages of possibly short duration and infrequent occurrence, which occur *in hospite*. As these life stages have not been observed to date, they could probably be morphologically similar to the dominant coccoid form and/or infrequent in occurrence.

It is interesting that the ability of other dinoflagellates to reproduce sexually is induced by nutrient limitation (Chesnick & Cox 1987, 1989), while zooxanthellae numbers within hosts are thought to be controlled by nutrient limitation (Muscatine & Pool 1979; Trench 1987; Belda *et al.* 1993; Belda & Yellowlees 1995; Belda-Baillie *et al.* 1998). It might therefore be possible that nutrient limitation triggers the sexual stage of the zooxanthellae life cycle. Further work should investigate this interesting possibility.

Acquisition or proliferation of different algal taxa has been suggested as a means by which a symbiotic association adapts to varying environmental factors (Rowan *et al.* 1997). If sexual reproduction does occur, this might mean that environmental adaptations may also rely, to some extent, on the proliferation of adaptive alleles within the resident zooxanthellae population.

Such symbiont diversity might be useful to the clams in allowing them to establish associations with zooxanthellae suitable to the nutrient, temperature, and light conditions of their environment. Zooxanthellae, which thrive well under prevailing conditions, may outgrow pre-existing symbiont populations, and maintain the host's food supply. Variations in resident zooxanthellae taxa and numbers within a clam species might also be expected to occur from season to season, or between different depths and different light conditions. Observations of habitat-specific symbioses in some Caribbean corals (Rowan & Knowlton 1995) and the varying responses of different *Symbiodinium* species to changes in photon-flux densities (Iglesias-Prieto & Trench 1994, 1997) suggest that the algae may contribute to photoadaptation in symbiotic associations.

It should be noted that our allozyme analysis was limited to those zooxanthellae that grew in f/2 media, and therefore may represent a fraction of the total population within the host. It is likely that our analysis only uncovered a small portion of the total genetic variation in these populations. Indeed, our preliminary analysis of the same clonal cultures using random amplification of polymorphic DNA (RAPD) has demonstrated that all but a few clonal isolates are genetically distinguished by a single primer analysis (B. K. Baillie, V. Monje, V. Silvestre, M. Sison and C. A. Belda-Bailey, unpublished data). Such data, in addition to the present results, clearly demonstrate that algae within giant clams are genetically diverse. Further research should establish whether the source of this high genetic variation was clonal mutation or sexual recombination. If clonal mutation was indeed responsible for the variations detected here, symbiotic zooxanthellae probably have little need for sexual reproduction since the degree of genetic variation afforded by asexual reproduction is so large. Further study should also determine if the degree of variation detected in this study represents different algal taxa or individuals within a taxon.

Schoenberg & Trench (1980a) reported no detectable differences in the allozyme patterns of clonal isolates from

the same host species (i.e. various cnidarian host species, with one *T. gigas* host individual). The reason for this may be that the PAGE method used in our study was more sensitive and allowed the resolution and detection of more allozyme bands, which were not easily resolved or detected by SGE. Our initial difficulties in resolving and detecting allozymes on starch gels, in contrast to PAGE, supports this premise. Separation by PAGE is on the basis of differences in charge, molecular weight, and shape (Hames 1990); whereas, separation of allozymes on starch gels is primarily due to charge differences (May 1992). Our EST allozyme patterns (figure 1a) demonstrate an average of seven bands per isolate, whereas Schoenberg & Trench (1980a) reported an average of three for SGE. This is presumably due to the higher resolution available from PAGE.

(b) *Current taxonomic groupings of symbiotic zooxanthellae*

Rowan & Knowlton (1995) have reported that more than one taxa of symbionts can occupy the same host. Our research has shown that it is also possible (at least for giant clams) that there is genetic variation between individuals within the same taxa.

Based on the 18S-rDNA sequences of various algal symbionts of cnidarian hosts, Rowan & Powers (1991a) demonstrated that symbiotic dinoflagellates fall into three clades: A, B, and C. Zooxanthellae from tridacnid clams apparently fall within clade A of this scheme (Rowan's unpublished data cited in Baker & Rowan (1997)). This clade also contains some symbionts from hard corals. Earlier work has indicated that zooxanthellae from clams fall within clades A and C (unpublished data mentioned in Rowan *et al.* (1996)).

It seems likely that our algal isolates fall within clade A. Our results demonstrate that clade A has a discernible sub-structure based on allozyme comparisons. Whilst 18S-rDNA sequence comparisons and RFLP of 18S-rDNA are able to distinguish symbiont taxa into three groups with some degree of substructure in clades B and C, it appears limited in its ability to resolve finer details in clade A. We would expect, based on our present results and as more data are accumulated from 24S-rDNA, internal transcribed spacer (ITS) sequences, and protein-coding genes, that clade A (and the others) will resolve into finer details, revealing some of the broader details detected in our study.

If a significant proportion of the genetic variation in zooxanthellae is undetectable via 18S-rDNA, then the idea that there are only a small number of resident taxa may need to be extended to recognize the fact that the resident algal populations are genetically diverse. This may have implications for the ecological zonation reported in corals (Rowan & Knowlton 1995). RFLP analysis of 18S-rDNA may resolve only broad zonation effects which occur in these symbioses.

The limitations of 18S-rDNA sequence and RFLP analysis to resolve clade A is presumably a function of the rate at which this gene evolves and the similarity of the algae involved. A similar problem is seen in the identification of bacterial species based on 16S-rDNA sequence analysis, where different organisms may share 16S-rDNA sequence identity (Fox *et al.* 1992). This problem in

Symbiodinium sp. identification has been acknowledged previously (Rowan & Powers 1991a; Trench 1997).

Alternative methods for the analysis of *Symbiodinium* isolates appears to be the answer to this problem. In the case of 16S-rDNA, new methods using protein-coding genes such as the B subunit of DNA gyrase are being used successfully (e.g. Yamamoto & Harayama 1996). For *Symbiodinium* isolates, analysis of the LSU-rDNA (Wilcox 1998; see Baker & Rowan 1997) and the ITS region (e.g. Hunter *et al.* 1997) seem to be possible alternatives. The finest details revealed by our allozyme analysis, however, will probably not be completely resolved by such methods since they appear to represent differences between individual algae within a host.

(c) *Clam mariculture applications*

The dendrogram demonstrating the genetic similarities between clonal isolates shows several major branches, some of which may roughly correspond to species or strains. Such a tree is useful as it allows a logical choice of algal isolates to be made for use in infection trials. Choice of organisms for field trials is simplified, to some degree, by choosing organisms that are distantly related, hence ensuring the widest possible scope for the search for optimal symbionts. Once a suitable symbiont has been identified, other algae sharing the same clade may also have similar beneficial properties to host survival and growth.

For *T. gigas*, the success rate of growing clonal isolates in f/2 medium was much lower than that of any other species. This is circumstantial evidence suggesting that most of the algal symbionts of *T. gigas* may have nutrient requirements that are different from those of the symbionts of other clam species. Furthermore, analysis of the allozyme patterns revealed that the algal symbionts of *T. gigas* are more closely related to each other than to the symbionts of the other clam species. This observation is consistent with trends based on our preliminary RAPD analysis (B. K. Baillie, V. Monje, V. Silvestre, M. Sison and C. A. Belda-Bailey, unpublished data). We infer from these observations that the population of zooxanthellae symbiotic with *T. gigas* may be different and represent distinct zooxanthellae symbionts in giant clams. It would be interesting to test if symbionts from the fast-growing *T. gigas* would significantly improve the growth rates of slow-growing clam species (see Molea & Munro 1994).

The allozyme patterns from the FIZ of *T. gigas* are different from any of the 12 clonal isolates analysed (cf. Schoenberg & Trench 1980a). This is probably due to the fact that our study was only able to investigate a small fraction of zooxanthellae from this clam species that would grow in f/2 medium. Therefore, it is possible that the bulk of the FIZ have allozyme patterns different from those of the clonal cultures. In addition, some differences are likely due to the expression of different zooxanthellal allozymes *in situ*.

Most of the clonal cultures analysed were resolved based solely on their EST patterns. A similar situation was reported by Schoenberg & Trench (1980a) for algae isolated from a diverse range of host taxa. The simplicity of EST staining and the PAGE method make these methods useful for routine analysis of clonal isolates.

We are continuing our investigation of zooxanthellae from giant clams using RAPD and ITS sequence analyses

of the algal isolates to complement recent information from allozyme analysis. Work is also under way with zooxanthellae infection trials on clam larvae, which involves monitoring of juvenile growth and survival through short-term land- and ocean-based phases of clam culture. Among the interesting questions we further seek to address are: (i) do particular zooxanthellae improve clam performance; and (ii) do giant clams exchange or obtain new symbionts following initial infection? Results are forthcoming and will be reported later.

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