SYMBIOSIS (2006) 42, 123–134 ISSN 0334-5114

Physiological and genetic properties of two fluorescent colour morphs of the coral *Montipora digitata*

Anke Klueter, William Loh, Ove Hoegh-Guldberg, and Sophie Dove*

Centre for Marine Studies, University of Queensland, St Lucia, QLD 4072, Australia, Tel. +61-7-3365-1156, Fax. +61-7-3365-4755, Emails. ankeklueter@gmx.de, w.loh@uq.edu.au, oveh@uq.edu.au, sophie@uq.edu.au

(Received February 15, 2006; Accepted December 16, 2006)

Abstract

A comparison of strongly, weakly or non-fluorescent colour morphs has the potential to provide an ideal model for investigating the possible ecological function of GFP-homologs in reef-building corals. It was the aim of this study to identify the physiological and genetic characteristics of two fluorescent colour morphs of *Montipora digitata*, both of which are abundant in the shallow waters of Heron Island Reef, GBR. Morphological and physiological differences among corals in response to photo-acclimation from the algal perspective have been the subject of numerous studies. However, corals themselves may also respond by varying the intensity and type of host pigmentation. Both colour morphs can be assigned to our current taxonomic understanding of *M. digitata*. The symbionts of both morphs belonged to the same genotype of *Symbiodinium* (clade C15) and as such is unlikely to be accountable for the ecological differences observed. Spectral characterisation of the purified fraction of GFP-like proteins revealed the presence of three different GFP-like proteins that were distinguishable by their excitation and emission spectra. Moreover, fluorescence intensities and the content of GFP-like proteins were found to be significantly higher in the strongly fluorescent colour morph. High concentrations of pigments in shallow water corals have been postulated to simulate a low-light environment for symbiotic dinoflagellates, which live beneath the pigment layer. In the strongly fluorescent colour morph, symbiotic dinoflagellates occurred in greater abundance, providing support for this hypothesis, which is further sustained by the occurrence of higher concentrations of chlorophyll *a*. While the differences in the parameters observed cannot offer sufficient explanation for underlying physiological mechanisms, the approach of this study can be further extended to include all colours associated with the expression of GFP-like proteins. The observed physiological flexibility of the coral host observed in this study further emphasizes the fundamental problem of focusing solely on the dinoflagellate symbiont to explain the variability in reef-building corals with respect to their tolerance to heat and light.

Keywords: Corals, symbiotic dinoflagellates, GFP, GFP-homologs, pocilloporin, fluorescent protein

1. Introduction

Coral reefs are characterised by a diversity of form and function within and among species. Individuals of a single species often consist of a range of distinctive morphs. Several studies have recognised the intraspecific variability in reef-building corals (Yonge, 1968; Foster, 1980; Veron and Pichon, 1982; Veron and Wallace, 1984; Willis, 1985; Willis and Ayre, 1985), yet the understanding of underlying physiological mechanisms and ecological consequences is still limited.

Different colour morphs for example, have been shown to display different ecological and physiological responses. The Caribbean reef-building coral *Porites astreoides* occurs as green or brown colour morphs. Both morphs differ not only in their expression of host-based pigments but also in their vertical distribution. The habitat of the brown morph extends to depths of 20 m, while the green morph is more abundant on the shallow fore-reef and has significantly greater concentrations of ultraviolet (UV) absorbing compounds known as Mycosporine-like Amino Acids (MAAs) (Gleason, 1993). The different physiological profile of the two colour morphs provides a logical explanation for the greater abundance of the green morph in the shallows.

*The author to whom correspondence should be sent.

It is likely that many other distinguishable species have similar physiological or other behavioural differences. The intraspecific variability of the ubiquitous coral *Pocillopora damicornis* probably provides one of the best-documented examples of this type of variability in reef-building corals. Colonies of this species demonstrate intensive intraspecific variations in branching morphology (Veron and Pichon, 1982; Lesser et al., 1994) and tissue colour (Dove et al., 1995; Takabayashi and Hoegh-Guldberg, 1995). In Hawaii, *P. damicornis* occurs in two differently pigmented morphs (yellow and brown). Besides colour, these two morphs can be distinguished by their branching morphology and behavioural traits, such as differing patterns of larval release and larval longevity (Richmond and Jokiel, 1984). Examination of pink and brown colour morphs of *P. damicornis* from the Great Barrier Reef (GBR) revealed that phenotypic colour differences in this species were due to a pigment called Pocilloporin (Dove et al., 1995). Further characterisation of the pigment identified it as a homolog of the Green Fluorescent Protein (GFP) (Dove et al., 2001) isolated from the Hydrozoan jellyfish *Aequorea victoria*.

The pink and brown colour morphs of *P. damicornis* have been shown to differ in their spatial distribution as well as their growth rates (Takabayashi and Hoegh-Guldberg, 1995). Corals that contained Pocilloporin were found to be more abundant in shallow waters and had lower growth rates compared to their brown counterpart. Furthermore, Takabayashi and Hoegh-Guldberg (1995) showed that the production of Pocilloporin in *P. damicornis* could be induced by photosynthetically active radiation (PAR), but not by UVR. Nonetheless, some coral colonies did not express quantifiable amounts of Pocilloporin and maintained their overall brown colouration, indicating that the expression of Pocilloporin was under both genetic and phenotypic control (Takabayashi and Hoegh-Guldberg, 1995).

Consequently, we expect to see many other coral species with similar pigment-related behavioural or physiological differences. Several recent studies have reported differences in spectral properties of coral pigments that are due to GFP-homologs (Mazel, 1995; Salih et al., 1998; Salih et al., 2000; Dove et al., 2001; Labas et al., 2002; Papina et al., 2002; Mazel et al., 2003). As previously shown (Salih et al., 2000), intraspecific differences of fluorescence intensities can often be found among corals that grow under similar environmental conditions. While intraspecific variability of tissue colour in reef-building corals has been documented in previous studies (Mazel, 1995; Takabayashi and Hoegh-Guldberg, 1995; Salih et al., 1998; Salih et al., 2000; Dove et al., 2001; Labas et al., 2002; Papina et al., 2002; Dove, 2004; Mazel et al., 2003), the physiological parameters that underlie or accompany these variations has not been studied.

On reefs near Heron Island, GBR, the reef-building coral *M. digitata* was found to occur in two distinct fluorescent colours which are also spatially distinguishable. The strongly fluorescent colour morph can only be found on the Inner Reef Flat (IRF) of Heron Island Reef, whereas the weakly fluorescent colour morph can be found only on the Outer Reef Flat (ORF). It is the aim of this study to gain a better understanding of the genetic and physiological mechanisms underlying the different fluorescent colour morphs of *M. digitata*. Intraspecific similarities and/or differences will be assessed in order to evaluate the suitability of colour morphs as an exploratory model to determine a photo-adaptive role of GFP-homologs.

2. Materials and Methods

Sample collection

Seven mature colonies of *Montipora digitata* (Acroporidae; Dana 1846) were randomly collected from the IRF and ORF, respectively, of Heron Island Reef flat (23°26.995'S, 151°55.961'E, WGS 84), GBR, Australia. Coral explants (branch tips) of 2–3 cm in length were excised from the upper parts of vertically orientated colonies, chilled (–70°C) and transported to the laboratory for processing. Subsequent confirmation of the IRF and ORF colony skeletons as *M. digitata* was undertaken by Dr. P. Arnold, Museum of Tropical Queensland, Townsville,

Spectral properties of two fluorescent colour morphs of Montipora digitata

GFP-homologs were extracted by immersing coral explants for 38 h at 4°C in a known volume of 0.06 M phosphate buffer (pH=6.65; Dove et al., 1995). The coarse extracts were through 0.45 µm syringe filters (Millex-HA MCE/MF) and absorption spectra were recorded on a Shimadzu UV 2450 UV/vis spectrophotometer. Excitation and emission maxima for GFP-homologs were determined using a Perkin-Elmer LS50B fluorescence spectrophotometer. GFP-homologs were purified by gel filtration on a FPLC column (Pharmacia, 12 HR 10/30; Dove et al., 2001). The injection volume was 200 µl and separation was accomplished using an aqueous 0.06 M phosphate buffer as a mobile phase at a flow rate of 0.5 $ml.min^{-1}$. Chromatograms were monitored for 485 nm excitation and 515 nm emission using a fluorescence detector (Model RF 10AXL, Shimadzu) and a UV detector (Model 490E Millipore-Waters Australia), set for 280 nm to monitor protein absorption (Dove et al., 1995). Quantification of sub-sets of GFP-homologs was achieved by integration and data were normalised to surface area, extraction and injection volume. Coral surface area was determined using the technique of (Stimson and Kinzie, 1991).

Analysis of Mycosporine-like Amino Acids (MAAs)

Coral explants were immersed successively in three volumes of 100% methanol. Extractions were carried out in darkness at 4°C for 60 min. Individual MAAs were separated by reverse-phase, isocratic High -Performance Liquid Chromatography (HPLC), as described by Dunlap and Chalker (1986). Separation was accomplished using a RP-C8-column and guard column (Phenomenex-Phenosphere 5 µ C8 80 Å, 250 × 4.5 nm and 30 × 4.5 nm, Phenomenex, Australia Pty Ltd.). The mobile phase consisted of 40% aqueous methanol and 0.1% acetic acid (v:v) in water and was run at a flow rate of 0.8 $ml.min^{-1}$. Chromatograms were detected by ultraviolet absorbance at 313 nm and 340 nm (Model SPD-M10A_{VP}, Shimadzu). Identification of MAAs was attained by co-chromatography with verified standards of palythine, palythinol, porphyra-334, shinorine, asterina-330 and palythene (standards and calibration courtesy W.C. Dunlap).

Biomass parameters

Coral tissue was removed with a jet (WaterPik™) of recirculated filtered seawater (Whatman Glass Microfibre filters GF/C and 0.45 µm membrane filters) (Johannes and Wiebe, 1970). The slurry produced from the tissue-removal process was homogenized in a blender (ProMix duo, Krups Electronic Silver) for 45 s and the volume of the homogenate was recorded. Four aliquots of 10 ml were taken from the homogenate. One aliquot was used to count symbiotic dinoflagellates; the other three aliquots were used to determine chlorophyll contents. Aliquots to determine the density of symbiotic dinoflagellates were preserved by adding 1 ml of 37% formaldehyde and stored at 4°C. Aliquots used to determine chlorophyll contents were kept frozen at –20°C until analysed.

Densities of symbiotic dinoflagellates. Symbiont cells were counted using 8 replicate counts in a Neubauer Improved hemocytometer (0.1 mm^3, 0.1 mm depth). The total number of symbiotic dinoflagellates per sample was determined by multiplication by the total homogenate volume and expressed as a number per unit surface area (cm^2).

Analysis of chlorophyll. Aliquots of homogenate were centrifuged at 3,500 *g* for 15 min at 4°C. The supernatant was discarded and the pellet re-suspended in 100% acetone. Chlorophyll was extracted for 24 h under dark conditions at 4°C. After 24 h samples were centrifuged at 3,500 *g* for 10 min at 4°C. Optical densities of the supernatant were measured at 630 nm and 663 nm on a Perkin Elmer spectrophotometer MBA 2000. Chlorophyll concentrations were calculated using the equations of Jeffrey et al. (1997). Chlorophyll contents were normalised to surface area and single symbiont cells.

Identification of algal clades from two fluorescent colour morphs of Montipora digitata

DNA extraction and PCR amplification. Approximately 3–4 cm^2 of tissue was removed from the coral skeleton using directed jets of high-pressure air. The tissue slurry was mixed with an equal volume of 20% DMSO in 0.25 M EDTA, saturated with NaCl (pH 8.0) for the preservation of DNA (Seutin et al., 1991). DNA was extracted and quantified using the method described by Loh et al. (2001). Partial 28S nuclear ribosomal (nr) DNA of *Symbiodinium* (D1/D2 domains) was amplified using the dinoflagellate-specific primers; Forward 5'-CCC GCT GAA TTT AAG CAT ATA AGT AAG CGG-3' and Reverse 5'-CTT AGA CTC CTT GGT CCG TGT TTC AAG A-3' (Zaradoya et al., 1995). The PCR protocol is described by Loh et al. (2001).

Single Stranded Conformational Polymorphism analysis. Single Stranded Conformational Polymorphism (SSCP) analysis was performed using 10 µl of PCR product mixed with an equal volume of loading buffer (95% formamide 10 mM NaOH, 0.25% bromophenol blue and 0.25% xylene cyanol) and denatured for 5 min at 95°C. After denaturing, the mixed aliquots were immediately chilled on crushed ice and run in a polyacrylamide MDE gel (FMC Bioproducts, Maine) in 0.6 x TBE at 160 v for 13 h at room temperature. The SSCP patterns were stained with ethidium bromide and visualized with UV transillumination.

Cloning and sequencing. PCR products were ligated into PGEM-T vectors (Promega, USA) according to the manufacturer's instructions. The plasmids were transformed into competent *Escherichia coli* cells (TOPO, Invitrogen, USA). Selected clones were picked from plates and directly PCR amplified following the aforementioned PCR protocols. Products of this amplification were purified for sequencing using GFX™ columns (Amersham Pharmacia, USA). DNA sequences were determined in the forward direction using the dye terminator automatic sequencing facility at the Australian Genome Research Facility at the University of Queensland, AU.

Phylogenetic analysis. Cloned *Symbiodinium* sequences from two IRF (strongly fluorescent) and three ORF (weakly fluorescent) *M. digitiata* colonies were used for phylogenetic analyses. All sequences were aligned by using Clustal X (Thompson et al., 1994). We follow the clade systematics reviewed by Baker (2003) where many sequences currently denoted clade E in GenBank (sequences marked with an asterisk below) should belong to clade D. Clade E has been assigned to *Symbiodinium* (*Gymnodinium*) *varians* and other genotypes from temperate waters (Baker, 2003). The reference symbiont sequences included here are clade A (accession numbers AF427453, AY596824, DQ060729), clade B (AF427457, AF427459, AY596825), clade C (AJ308892, AJ620935,

AY239387), clade D (AF396626*, AF396628*, AF170149), clade E (AY684264, *S. varians* AF060899), clade F (AJ621142, AJ621144, AJ621146), clade G (AJ291536), clade H (AJ621129, AJ621149, AJ621131). The tree was rooted using *Gymnodinium beii* (AF060900). Clade C reference sequences were chosen after BLASTN (Altschul et al., 1997) searches, on the basis of showing the highest identity with our sequences. Trees were constructed using Bayesian, maximum parsimony (MP) and maximum likelihood (ML) methods. Prior to these analyses, MODELTEST version 3.7 (Posada and Crandall, 1998) was used to determine the most appropriate substitution model for the data for the ML and Bayesian analyses. Using the AIC and BIC test, this was found to be the (TrN+G) for the *Symbiodinium* 28S nuclear ribosomal (nr) DNA. ML and MP analyses were conducted using the PAUP beta version 4.0b10 (Swofford, 2002). All characters were given equal weight and were unordered. For ML, a heuristic search was carried out with 100 random additions of taxa, followed by TBR branch-swapping. ML clade support was assessed with 100 bootstrap replicates, using the same model as the heuristic searches. The MP analysis was also carried out by the heuristic search method, with 100 random additions of taxa, each followed by TBR branch swapping. Starting-trees were obtained by stepwise-addition using simple addition sequence. MP clades were assessed with 1000 bootstrap replicates (excluding uninformative characters), with 100 random additions of taxa for each replicate. The Bayesian analysis was implemented in MrBayes (Ronquist and Huelsenbeck, 2003) and were based on the models selected by Modeltest above. Starting from random trees, four Markov chains (with three heated chains) were run simultaneously to sample trees using the Markov Chain Monte Carlo (MCMC) principle. After the burn-in phase (the first 12,900 generations was discarded), every 100th tree out of 10^6 was considered. The phylogenetic trees generated in all analyses were visualized using TREEVIEW version 1.6.5 (Page, 1996).

3. Results

Fluorescence properties

Fluorescent properties of both colour morphs show the presence of three excitation and emission pairs (Fig. 2). For the strongly fluorescent morph, the first excitation maximum is situated in the UV-A range (λ_{max}=360 nm) and emits in the violet-blue band of the spectrum (λ_{max}=450 nm) (Fig. 2A). The second excitation maximum is situated in the violet-blue range (λ_{max}=468 nm) with an emission maximum in the blue range (λ_{max}=485 nm). This emission maximum is not shown in Fig. 2B as it is too close to the excitation maximum of the dominant GFP-homolog and therefore overshadowed by the third excitation/emission pair that has an excitation maximum in the blue (λ_{max}=485 nm) and an emission maximum in the green range (λ_{max}=515 nm) of the spectrum (Fig. 2C). The weakly fluorescent morph also shows the presence of three different excitation and emission pairs that are positioned in similar colour ranges to the pairs of the strongly fluorescent morph. However, excitation and emission pairs of the weakly fluorescent morph differ in their maxima and their intensity. The first excitation/emission pair has their λ_{max} at 368 nm and 444 nm, the second pair has a λ_{max} of 464 nm and 480 nm and the third pair has a λ_{max} of 483 nm and 513 nm. The fluorescent intensities of those three excitation and emission pairs are increasing in both morphs from the first pair to the third pair (Figs. 2A–C). Whilst fluorescent intensities of both morphs are similar for the excitation/emission pair that is located in the UV-A range of the spectrum, fluorescent intensities of the second and third pair are lower in the weakly fluorescent morph of *M. digitata*.

GFP-homologs

Fig. 3A shows gel-filtration chromatograms of the aqueous soluble GFP-homologs monitored at Ex 485 nm / Em 515 nm, where maximum green fluorescence occurs in *M. digitata*. The strongly fluorescent morph shows a much stronger intensity of green fluorescence than the weakly fluorescent morph. The significance of this difference is indicated by 95% confidence intervals of the gel-filtration chromatograms (Fig. 3A). This difference of fluorescent properties between the colour morphs was statistically analysed for the GFP-homolog fraction (24–29.5 min). Those data were analysed using two factorial ANOVA. The analysis confirmed (Mo, $F_{1,24}$=61.3, $p<0.001$) the significant difference between both colour morphs that was revealed by gel-filtration chromatography (Fig. 3A). Fig. 3B also shows gel-filtration chromatograms of the same GFP-homologs monitored at 280 nm as an estimation of protein. Correspondingly these chromatograms show that the strongly fluorescent morph contains higher amounts of GFP-homologs. The GFP-homolog fraction of the A_{280} detection was also analysed using two factorial ANOVA and found to be significantly different (Mo, $F_{1,24}$=32.8, $p<0.001$).

Mycosporine-like amino acids (MAA)

Both colour morphs of *M. digitata* contain quantifiable amounts of mycosporine-glycine, palythine, asterina-330 and palythinol (Fig. 4). Concentrations of MAAs in both fluorescent morphs were analysed using three factorial ANOVA. The two predominant MAAs in both morphs were mycosporine-glycine and palythine. In the strongly fluorescent morph the mean concentration of mycosporine-glycine is 72.33 nmol mg protein^{-1} ± 22.46, while the

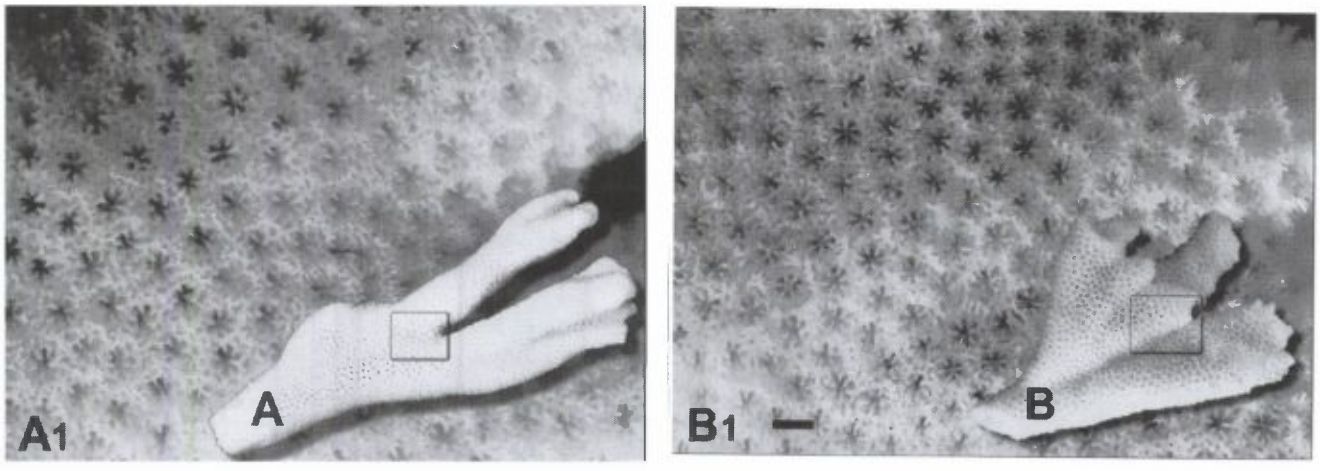

Figure 1. Skeletons of *Montipora digitata* from two different locations on Heron Island reef flat, GBR. A: General growth form of a single branch taken from colonies growing on the Inner Reef Flat; A1: Detailed view of corallites (IRF). B: General growth form of a single branch taken from colonies growing on the Outer Reef Flat; B: Detailed view of corallites (ORF); Scale is 5 mm and applies to skeletons shown in A and B. *See cover illustration.*

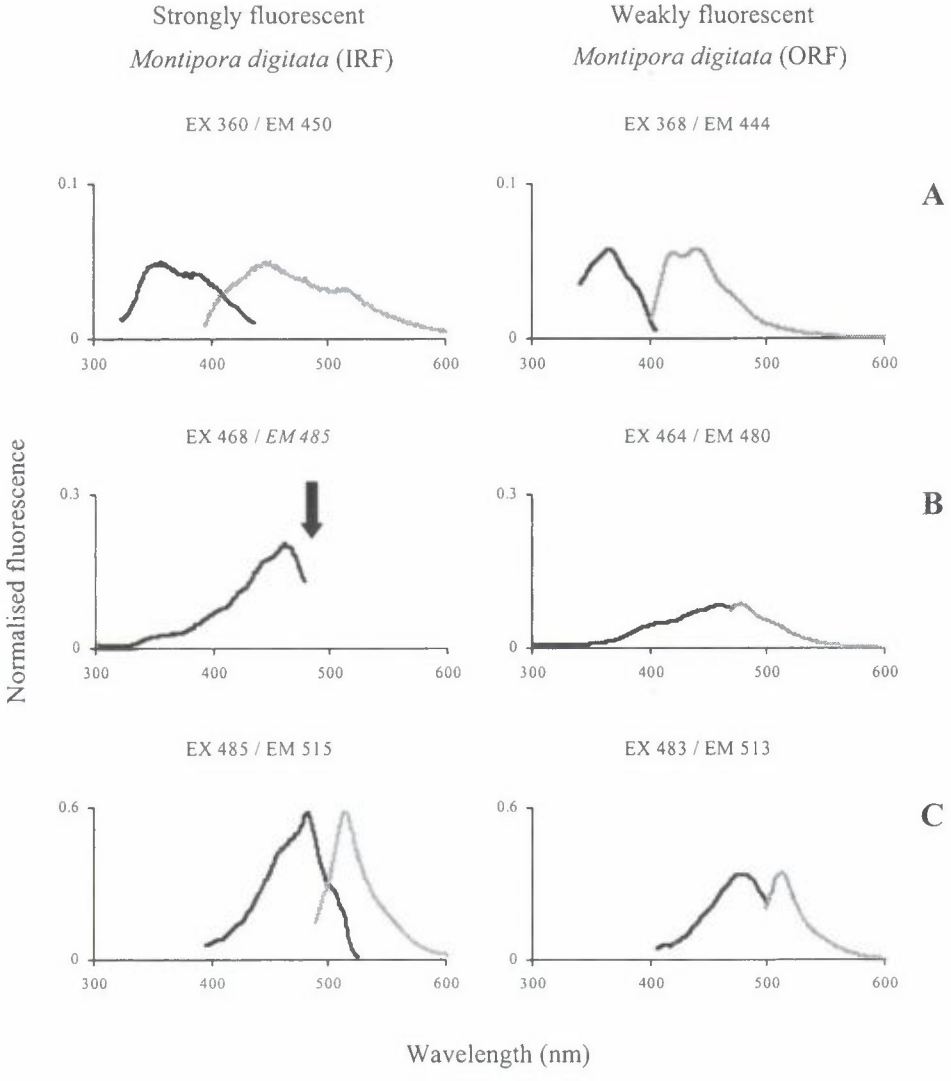

Figure 2. Excitation (black line) and emission (grey line) spectra of the GFP-homolog fraction (elution at 25–26.5 min) extracted from two fluorescent colour morphs of *Montipora digitata* (IRF and ORF), collected from the Heron Island reef flat. Measurements were carried out in phosphate buffer. Separation of aqueous soluble protein was achieved by high performance liquid chromatography. Data are normalised to surface area, extraction and injection volume. A–C: 3 different excitation and emission pairs.

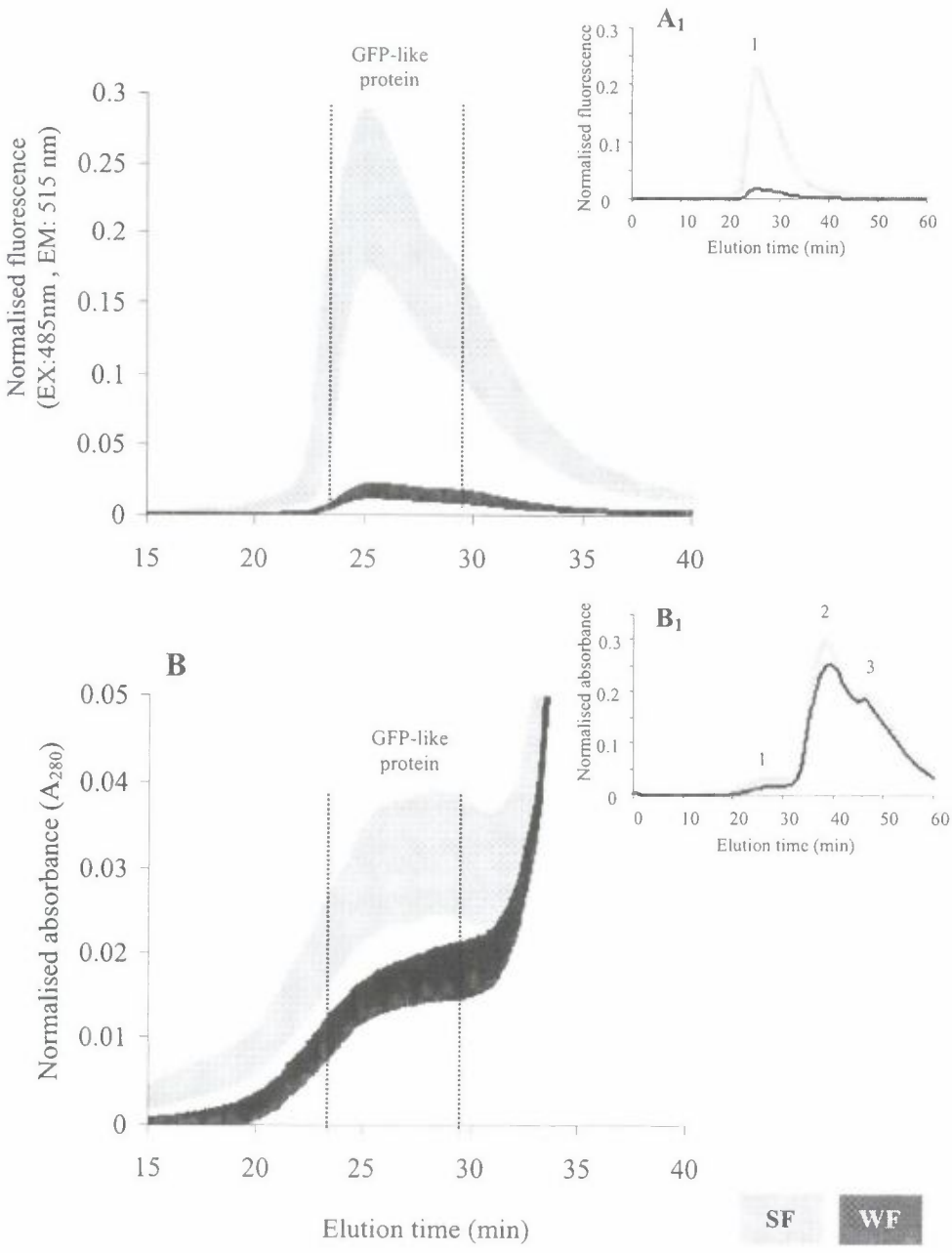

Figure 3. Gel-filtration chromatograms of aqueous soluble proteins from two fluorescent colour morphs of *Montipora digitata* normalised to surface area, extraction and injection volume (grey = strongly fluorescent colour morph, black = weakly fluorescent colour morph). A: GFP-homolog fraction for normalised fluorescence, B: GFP-homolog fraction for normalised absorbance (A_{280}). Shown are 95% confidence intervals for N=16. A1 and B1 show equivalent chromatograms to A and B for the mean and 60 min. 1: GFP-homolog fraction (24–29.5 min), 2 and 3: not identified.

concentration of palythine is 193.04 nmol mg protein^{-1} ± 41.48. In the weakly fluorescent morph the mean concentrations of mycosporine-glycine is 50.74 nmol mg protein^{-1} ± 11.09, while the concentration of palythine is 175.76 nmol mg protein^{-1} ± 33.77. Asterina-330 and palythinol are also present in both fluorescent morphs, but in much lower concentrations than mycosporine-glycine and palythine. Asterina-330 in the strongly fluorescent morph is present in a concentration of 5.97 nmol mg protein^{-1} ± 3.76 and palythinol with 15.58 nmol mg protein^{-1} ± 7.28. The SNK test revealed that concentrations of asterina-330 and palythinol are significantly lower (MAA × Mo, $F_{4,20}$=120, p=0.09) in the weakly fluorescent morph than they are in the strongly fluorescent morph, with values of 0.82 nmol mg protein^{-1} ± 0.62 for asterina-330 and 2.18 nmol mg protein^{-1} ± 1.32 for palythinol (Fig. 4). However, concentrations of the total pool of MAAs showed no difference between both the strongly fluorescent colour morph (286.92 nmol mg protein^{-1} ± 55.92) and the weakly fluorescent colour morph (229.50 nmol mg protein^{-1} ± 35.03).

Density of symbiotic dinoflagellates

Densities of symbiotic dinoflagellates extracted from the tissues of both fluorescent colour morphs were analysed

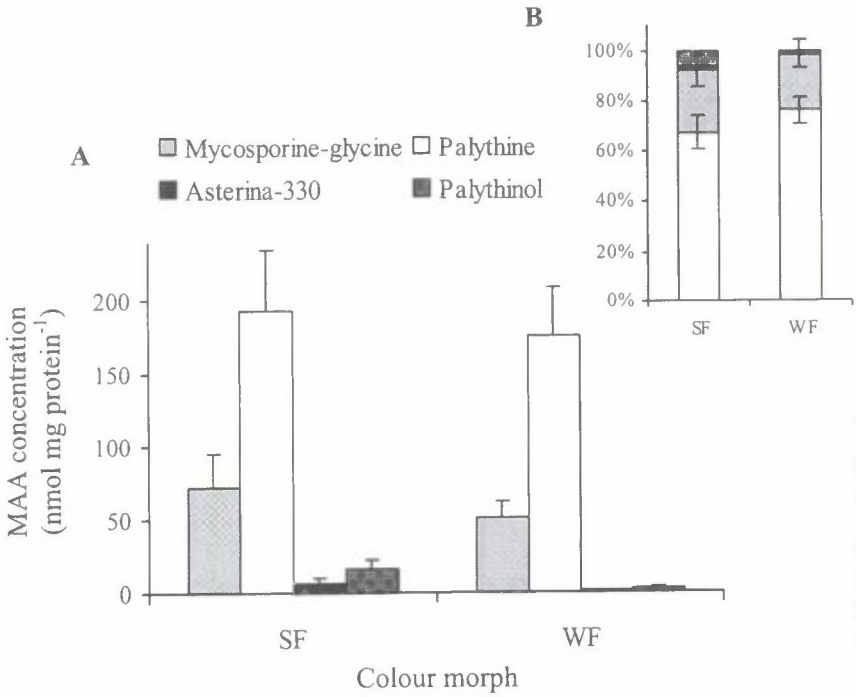

Figure 4. A: Mean concentration of mycosporine-like amino acids (+SE) extracted from the tissue of two fluorescent colour morphs of *Montipora digitata* (SF = strongly fluorescent colour morph, WF = weakly fluorescent colour morph), collected from Heron Island reef flat, GBR. B: Percentage contribution of individual MAAs to the total MAA pool, error bars indicate standard errors only for palythine and mycosporine-glycine. N=16.

using two factorial ANOVA. The strongly fluorescent morph from the IRF showed significantly (Mo, $F_{1,22}$=8.17<0.05) higher densities of symbiotic dinoflagellates (3.07 × 10^6 cell cm^{-2} ± 0.74) than the weakly fluorescent morph that occurs on the ORF (2.12 × 10^6 cell cm^{-2} ± 0.50; Fig. 5A).

Chlorophyll content

Concentrations of chlorophyll *a* and c_2 were normalised to surface area as well as per single symbiont cell and analysed using a two factorial ANOVA. These different expressions showed variation in the significance of chlorophyll contents of symbionts from both colour morphs. The content of chlorophyll *a* per surface area is significantly higher (Mo, $F_{1,22}$=18.58, p<0.01) in the strongly fluorescent morph (13.46 µg cm^{-2} ± 1.90) than it is in the weakly fluorescent morph (9.68 µg cm^{-2} ± 1.52; (Fig. 5B). The same significant difference (Mo, $F_{1,22}$=4.58 p<0.01) between both morphs was found when the chlorophyll *a* content was calculated for a single symbiont cell, with higher values for the strongly fluorescent morph (2.71 pg 10^4 cell^{-1} ± 0.38) and lower values for the weakly fluorescent morph (1.96 pg 10^4 cell^{-1} ± 0.31; Fig. 6B). Concentrations of chlorophyll c_2 normalised to surface area showed no significant difference between the strongly fluorescent morph and the weakly fluorescent morph (2.58 µg cm^{-2} ± 0.86 and 2.59 µg cm^{-2} ± 0.47; Fig. 5B). Similar, non-significant results between the strongly fluorescent morph and the weakly fluorescent morph were found for the chlorophyll c_2 content calculated for a single symbiont cell (1.48 pg 10^4 cell^{-1} ± 0.21 and 1.56 pg 10^4 cell^{-1} ± 0.33; Fig. 5C).

Clades of symbiotic dinoflagellates

PCR amplifications of the 28S rDNA from symbiotic dinoflagellates isolated from 10 strongly and 10 weakly fluorescent colour morphs of *M. digitata*, produced an amplicon of approximately 650 bp. SSCP analysis of the amplified symbiotic dinoflagellate 28S rDNA of all colour morphs yielded a single uniform pattern of 4 bands (Fig. 6).

The number of bands in all SSCP patterns suggested that populations of symbiotic dinoflagellates from the strongly and weakly fluorescent morph were heterogenous (the denaturation of double stranded DNA into single strands produces two distinct fragments, therefore heterogenous populations will produce more than 2 distinct fragments based on sequence heterogeneity (Loh et al., 2002)). The uniformity of all SSCP patterns detected showed that symbiont populations were likely to consist of the same genotypes, within and among colonies of the strongly and weakly fluorescent morphs. Bayesian, Maximum Parsimony and Maximum Likelihood phylogenetic analyses using the amplified DNA sequences of *Symbiodinium* from representative strongly and weakly fluorescent morphs and all known *Symbiodinium* clades, showed that they grouped with Clade C. Since all trees shared similar topology, only the Bayesian tree is shown.

Fig. 7 shows the Bayesian tree with bootstrap values of all three phylogenetic trees shown at the nodes. The phylogenetic analyses show that *Symbiodinium* from strongly and weakly fluorescent colour morphs of *M. digitata* most likely belong to subclades C15 and C90.

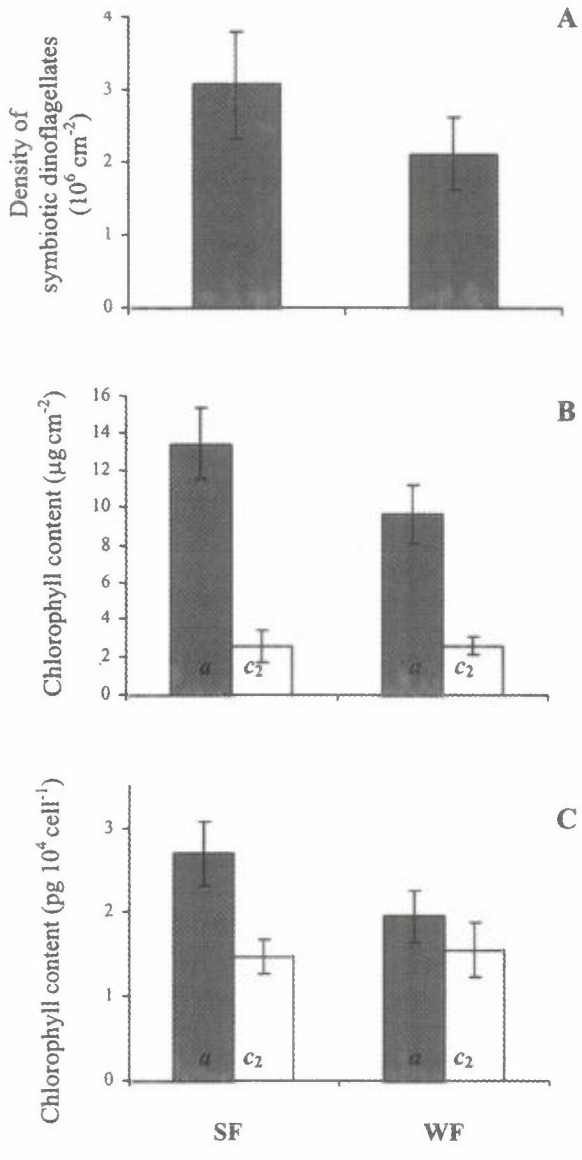

Figure 5. A: Mean density of *Symbiodinium* cells (+SE) in the tissue of two fluorescent colour morphs of *Montipora digitata* (SF = strongly fluorescent colour morph, WF = weakly fluorescent colour morph). N=15; B: Mean variation (+SE) of chlorophyll contents (chlorophyll *a* and c_2) in *Symbiodinium* of two fluorescent colour morphs of *Montipora digitata* (SF = strongly fluorescent colour morph, WF = weakly fluorescent colour morph), normalised to surface area (cm^{-2}), C: chlorophyll contents normalised to single symbiont cells, N=15.

4. Discussion

Fluorescent GFP-homologs in Montipora digitata

Spectral analysis of the purified GFP-homolog fraction showed the presence of three different fluorescent pigments in both colour morphs of *M. digitata*. All three pigment types were shown to differ visibly in their fluorescence intensities and to some extent in their excitation and emission pairs (Figs. 2A–C). The lowest fluorescence intensities were found in the UVA range. Medium and maximum fluorescence intensities occurred in the blue and green range of the visible spectrum. The gradient from low to maximum intensities of fluorescence is similar for both colour morphs, although having lower intensities in the blue and green range in the weakly fluorescent colour morph. While further research is needed to explain the presence of different types of GFP-homologs, the present study showed that fluorescence intensities were noticeably lower in the weakly fluorescent colour morph (Figs. 2B and C). It further demonstrated that a significant difference between fluorescence intensities (detected for the peak excitation and emission pair of 485/515 nm of both colour morphs) as well as for the estimate of GFP-homologs (detection at 280 nm).

This positive correlation between the fluorescence intensities and GFP-homolog content is suggestive of the fact that the weakly fluorescent colour morph has less of the fluorescent GFP-homologs as opposed to the alternative explanation that it contains a variant of GFP-homologs that shows reduced quantum yields. Similar findings were also described in a study that compared physiological properties of three colour morphs (due to GFP-homologs) of *Acropora aspera* (Dove, 2004). While differences between the two colour morphs of *M. digitata* are evident, the actual cause for differing expressions of GFP-homologs remains speculative (Mazel et al., 2003). Colonies of the two colour morphs used in this investigation lived in the unshaded shallow waters of the Heron Island intertidal reef flat, where physical factors are likely to influence the expression of fluorescent pigments. The strongly fluorescent colour morph from the IRF has a wider branching growth form and is infrequently surrounded by other coral colonies.

On the other hand, the weakly fluorescent colour morph from the ORF is frequently surrounded (partly shaded) by dense colonies of other corals (pers. observations). Changes in light intensities on a very small scale has recently been shown to influence physiological responses of photosynthetic corals with a similar importance as light differences related to depth affect (Vermeij and Bak, 2002; Anthony and Hoegh-Guldberg, 2003; Enriquez et al., 2005,). Although these factors have not been accounted for by this study, it outlines the importance of considering physical factors when differences in colour morphs are assessed. In a similar vein, the expression of the GFP-homolog pocilloporin in two colour morph (pink and brown) of *Pocillopora damicornis* were shown to be influenced by environmental factors such as photosynthetically active radiation (PAR) as well as genetic factors (Takabayashi and Hoegh-Guldberg, 1995). These findings were further confirmed in a seasonal study, which

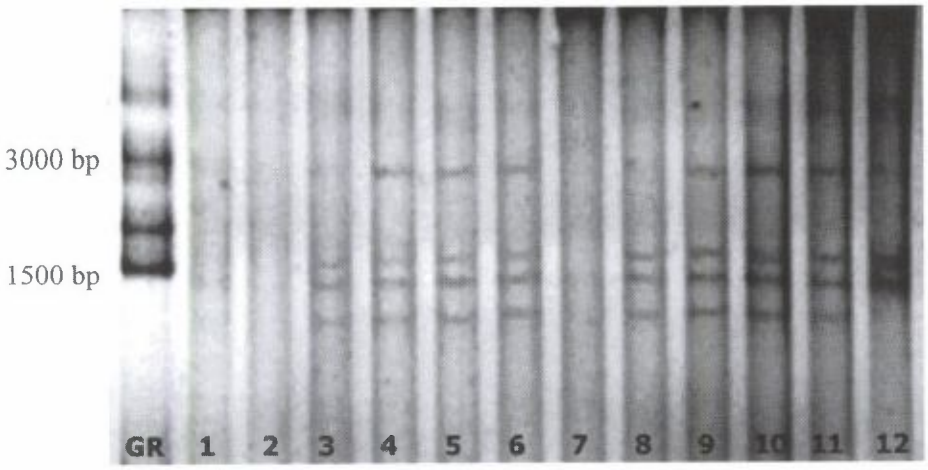

Figure 6. SSCP patterns derived from the PCR amplified *Symbiodinium* 28S RNA gene from two different fluorescent colour morphs of *Montipora digitata*. GR: Gene Ruler™ (1 kb DNA ladder, MBI Fermentas, 0.5 mg DNA ml^{-1}), Lanes 1–6: strongly fluorescent *M. digitata* collected from the Inner Reef Flat (IRF); Lanes 7–12: weakly fluorescent *M. digitata* collected from the Outer Reef Flat (ORF).

showed that colonies of the pink morph of *P. damicornis* always expressed greater amounts of pocilloporin than its brown counterpart. These findings led Takabayashi et al. (2003) to confirm the hypothesis that the difference in the expression of pocilloporin is genetically fixed (Takabayashi and Hoegh-Guldberg, 1995). Whether these findings also apply to the two fluorescent colour morphs of *M. digitata* requires further investigation.

Differences of biomass parameters such as mycosporine-like amino acids, densities of Symbiodinium *and photosynthetic pigments*

Both fluorescent colour morphs of *M. digitata* were shown to contain the same composition of four individual mycosporine-like amino acids (MAAs). The predominant MAA in *M. digitata* is palythine, which contributes equally to the total MAA pool of the two colour morphs (Fig. 4B). While mycosporine-glycine was shown to be the second dominant MAA and concentrations of this compound were also similar in both colour morphs. Concentrations of asterina-330 and palythinol on the other hand, were higher in the strongly fluorescent colour morph. Absorption maxima of asterina-330 and palythinol are located in the UV-A range (λ_{max}=330 nm and λ_{max}=332 nm), which is "red-shifted" from the more predominant MAA compounds palythine (λ_{max}=320 nm) and mycosporine-glycine (λ_{max}=310 nm). This suggests that the strongly fluorescent colour morph acquires additional protection against damaging effects of UV-A radiation.

The mean density of *Symbiodinium* cells was significantly higher in the strongly fluorescent colour morph of *M. digitata*. These findings are in agreement with a study conducted by Dove (2004) that investigated three colour morphs of *Acropora aspera* (blue, pale blue and cream). The blue colour morph was found to contain the highest concentrations of GFP-homologs and the greatest density of symbiotic dinoflagellates. Dove (2004) hypothesised that greater densities of symbiotic dinoflagellates were a consequence of the shading effect provided by the GFP-homologs. This hypothesis is further supported by a study of Brown et al. (2002), who found that colony parts of *Goniastrea aspera* that were exposed to high levels of solar radiation showed greater numbers of symbiotic dinoflagellates densities than colony parts that were exposed to lower light intensities. At the same time the light exposed colony parts were also found to show "abundant areas of fluorescent pigments". Although measurements of fluorescence intensities in *G. aspera* were not assessed quantitatively, they did show a similar association to densities of symbiotic dinoflagellates as Dove (2004) and the present study.

Photosynthetic pigments, such as chlorophyll *a* and c_2 showed the same trend between both fluorescent colour morphs regardless of its normalisation factor. The strongly fluorescent morph had significantly higher concentrations of chlorophyll *a* than its weakly fluorescent counterpart, but the same content of chlorophyll c_2. Similarly, the content of chlorophyll *a* calculated for a single symbiont cell was also higher in the strongly fluorescent morph, suggesting a shade-adaptive behaviour of this morph (Falkowski, 1990). This result is important, considering that GFP-homologs might act as a "shade" for symbiotic dinoflagellates under conditions of high levels of irradiance and it would further underline the assumption that the symbionts monitored in this study appear to be self-shaded physiologically. Intracellular measurements of light levels, however, are lacking from this study and hence remarks on light environment remain speculative.

It would be worthwhile to incorporate investigations of physiological processes, such as measurements of intracellular light levels, oxygen or photosynthetic rates. This would greatly benefit the examination of the possible shading effect by fluorescent pigments.

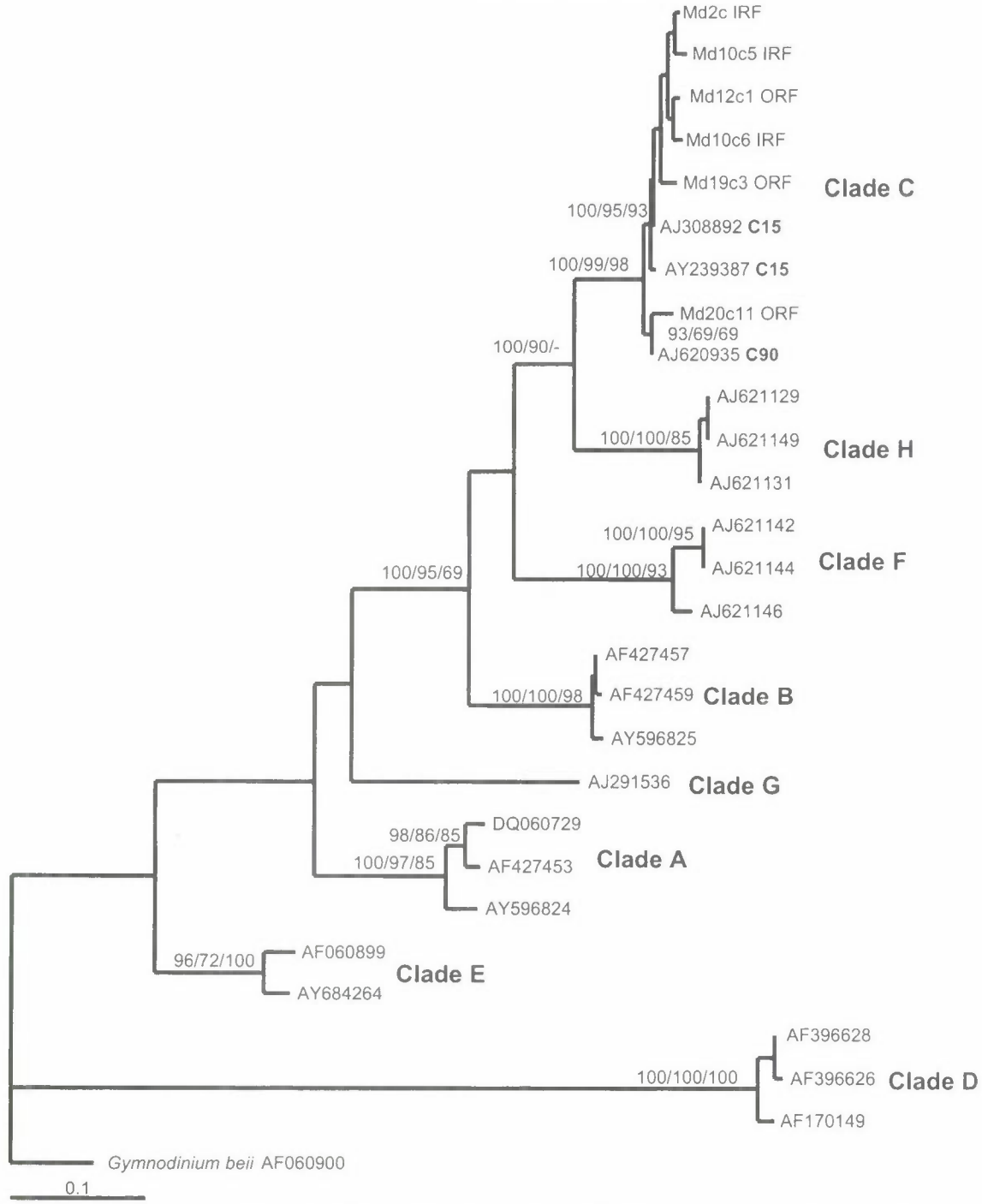

Figure 7. Bayesian tree derived from the PCR amplified *Symbiodinium* 28S RNA gene sequences from two fluorescent colour morphs of *Montipora digitata*. The strongly fluorescent morph was collected from the Inner Reef Flat (IRF), the weakly fluorescent morph from the Outer Reef Flat (ORF), at Heron Island, GBR. Clade controls (A, B, C, D, E, F, and G) and an outgroup organism (*Gymnodinium beii*) are included in the analysis (accession codes shown in figure). Bayesian posterior possibilities /maximum parsimony/maximum likelihood bootstrap percentages from are shown at nodes. Only bootstrap values >95% are shown unless otherwise denoted. Distance represents the number of substitutions per 100 bases.

Do both fluorescent colour morphs of Montipora digitata *host the same* Symbiodinium *genotypes?*

Physiological properties of symbiotic corals appear to be closely related to the symbiotic dinoflagellates that are associated with the coral host (Fitt and Warner, 1995). Subsequently, the physiology of a specific symbiont type is likely to influence the physiological mechanisms of the

host. It may also determine the degree to which a coral can adapt to environmental stresses such as high intensities of solar radiation or high temperatures (Rowan et al., 1997). Critically then, it is also important to verify that the symbiont like the host represents a single species.

The results of the present study clearly show that strongly and weakly fluorescent colour morphs of *M. digitata* contained identical *Symbiodinium* of clade C (subclades 15 and 90). These findings corroborate those of previous studies that revealed the prevalence of clade C symbionts in eastern Pacific corals (Loh et al., 1998; Baker, 1999; LaJeunesse et al., 2001). There is some evidence that different levels of irradiance influence the distribution of different types of *Symbiodinium* clades. For example, in reef corals of the Caribbean, clades A and B were found to predominate in shallow water habitats that are characterised by high irradiance, or in exposed parts of colonies that receive high levels of irradiance. Clade C is associated with habitats that experience low levels of irradiance or more shaded parts of colonies (Rowan and Knowlton, 1995). Clade C however is highly diverse and dominates all light regimes experienced by corals in the Pacific. The present study highlights the point that the shading of endosymbiotic dinoflagellates can be achieved by increasing host and/or symbiont pigmentation.

It has been suggested that recombination of different algal partners and selection of host populations can result in better adapted symbionts, and therefore create communities of symbiotic corals that are better suited for developing environments (Buddemeier and Fautin, 1993). The potential adaptability of corals via these mechanisms to environmental changes has received great attention over the last decade (Buddemeier and Fautin, 1993; Rowan et al., 1997; Baker, 2001; Hoegh-Guldberg et al., 2002). Differences in physiological responses of shallow water corals from Heron Island reef flat were recently investigated with a view to assessing differences in bleaching susceptibility among coral taxa (O. Hoegh-Guldberg et al., unpublished data). These investigations showed that the thermal tolerance of C15 in *Porites cylindrica* was found to be considerably greater than types C8a and C1, which are present in *Stylophora pistillata*. Similarly, *M. digitata* is known to demonstrate a greater resistance to thermal/irradiance bleaching (O. Hoegh-Guldberg and R.J. Jones, unpublished data) and was also shown to contain the algal type C15 (LaJeunesse et al., 2001).

In the present study, the *Symbiodinium* type C15 was found in both the strongly and weakly fluorescent colour morphs of *M. digitata*, and uniform SSCP pattern indicates that this *Symbiodinium* type is representative for all *M. digitata* colonies studied. This study showed that strongly and weakly fluorescent colour morphs of *M. digitata* do not differ in their algal type, and that therefore, differences in their ecological behaviour are likely to be attributable to physiological processes that are not caused or influenced by the genetic nature of the symbiotic algae. This observation can be extended to the majority of corals in the Indo-Pacific and would suggest that attempts to explain the thermal tolerance of corals as being due to the genotype of the symbiotic dinoflagellates alone dismiss the huge flexibility and importance of the coral host.

Acknowledgements

The authors like to thank Dr. W. Dunlap from the Australian Institute of Marine Science for his assistance with MAA standards as well as J. Nielson and N. Wilson for valuable comments on the manuscript. The permit was provided by GBRMPA and the work was supported by the Australian Research Council Centre for Excellence in Reef Studies at the University of Queensland (funding to SD and OHG), and the Coral Reef Targeted Research Project (www.gefcoral.org).

REFERENCES

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D.J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25**: 3389–3402.

Anthony, K.R.N. and Hoegh-Guldberg, O. 2003. Variation in coral photosynthesis, respiration and growth characteristics in contrasting light microhabitats: an analogue to plants in forest gaps and understoreys? *Functional Ecology* **17**: 246–259.

Baker, A.C. 1999. The symbiosis ecology of reef-building corals, University of Miami, FL, 120 pp.

Baker, A.C. 2003. Flexibility and specificity in coral-algal symbiosis: diversity, ecology and biogeography of *Symbiodinium*. *Annual Review of Ecology, Evolution & Systematics* **34**: 661–689.

Brown, B.E. and Downs, C.A. 2002. Exploring the basis of thermotolerance in the reef coral *Goniastrea aspera*. *Marine Ecology Progress Series* **242**: 119–129.

Buddemeier, R.W. and Fautin, D.G. 1993. Coral bleaching as an adaptive mechanism – a testable hypothesis. *Bioscience* **43**: 320–326.

Dove, S. 2004. Scleractinian corals with photoprotective host pigments are hypersensitive to thermal bleaching. *Marine Ecology Progress Series* **272**: 99–116.

Dove, S.G., Hoegh-Guldberg, O., and Ranganathan. S. 2001. Major colour patterns of reef-building corals are due to a family of GFP-like proteins. *Coral Reefs* **19**: 197–204.

Dove, S.G., Takabayashi, M., and Hoegh-Guldberg, O. 1995. Isolation and partial characterization of the pink and blue pigments of pocilloporid and acroporid corals. *Biological Bulletin Woods Hole* **189**: 288–297.

Dunlap, W.C. and Chalker, B.E. 1986. Identification and quantification of near-UV absorbing compounds (S-320) in a hermatypic scleractinian. *Coral Reefs* **5**: 155–159.

Enriquez, S., Mendez, E.R. et al. 2005. Multiple scattering on coral skeleton enhances light absorption by symbiotic algae. *Limnology and Oceanography* **50**: 1025–1032.

Falkowski, P.G. 1990. Irradiance and corals. In: *Ecosystems of the World: Coral Reefs*. Dubinsky, Z., ed. Elsevier, Netherlands. **25:** 89–107.

Fitt, W.K. and Warner, M.E. 1995. Bleaching patterns of four species of Caribbean reef corals. *Biological Bulletin* **189**: 298–307.

Foster, A.B. 1980. Environmental variation in skeletal morphology within the Caribbean reef corals *Montastrea annularis* and *Siderastrea siderea*. *Bulletin of Marine Science* **30**: 678–709.

Gleason, D.F. 1993. Differential effects of ultraviolet radiation on green and brown morphs of the Caribbean coral *Porites astreoids*. *Limnology and Oceanography* **38**: 1452–1463.

Hoegh-Guldberg, O., Jones, R.J., Ward, S., and Loh, W.K. 2002. Is coral bleaching really adaptive? *Nature* **415**: 601–602.

Jeffrey, S.W. and Mantoura, R.C.F. 1997. *Phytoplankton Pigments in Oceanography: Guidelines to Modern Methods*. Paris.

Johannes, R.E. and Wiebe, W.J. 1970. Method for determination of coral tissue biomass and composition. *Limnology and Oceanography* **15**: 822–824.

Labas, Y.A., Gurskaya, N.G., Yanushevich, Y.G., Fradkov, A.F., Lukyanov, K.A., Lukyanov, S.A., and Matz, M.V. 2002. Diversity and evolution of the green fluorescent protein family. *Proceedings of the National Academy of Sciences of the USA* **99**: 4256–4261.

LaJeunesse, T.C. 2001. Investigating the biodiversity, ecology, and phylogeny of endosymbiotic dinoflagellates in the genus *Symbiodinium* using the ITS region: In search of a "species" level marker. *Journal of Phycology* **37**: 866–880.

Lesser, M.P., Weis, V.M., Patterson, M.R., and Jokiel, P.L. 1994. Effects of morphology and water motion on carbon delivery and productivity in the reef coral *Pocillopora damicornis* (Linnaeus): Diffusion barriers, inorganic carbon limitation, and biochemical plasticity. *Journal of Experimental Marine Biology and Ecology* **178**: 153–179.

Loh, W., Hidaka, M., Hirose, M., and Titlyanov, E.A. 2002. Genotypic diversity of symbiotic dinoflagellates associated with hermatypic corals from a fringing reef at Sesoko Island, Okinawa. *Galaxea* **4**: 1–9.

Loh, W.K. and Carter, D. 1998. Diversity of zooxanthellae from scleractinian corals of One Tree Island (Great Barrier Reef). Australian Coral Reef Society 75th Anniversary Conference, Heron Island, University of Queensland.

Loh, W.K.W. and Loi, T. 2001. Genetic variability of the symbiotic dinoflagellates from the wide ranging coral species *Seriatopora hystix* and *Acropora longicyathus* in the Indo-West Pacific. *Marine Ecology Progress Series* **222**: 97–107.

Matz, M.V., Fradkov, A.F., Labas, Y.A., Savitsky, A.P., Zaraisky, A.G., Markelov, M.L., and Lukyanov, S.A. 1999. Fluorescent proteins from nonbioluminescent Anthozoa species. *Nature Biotechnology* **17**: 969–973.

Mazel, C.H. 1995. Spectral measurements of fluorescence emission in Caribbean cnidarians. *Marine Ecology Progress Series* **120**: 185–191.

Mazel, C.H., Lesser, M.P., Gorbunov, M.Y., Barry, T.M., Farrell, J.H., Wyman, K.D., and Falkowski, P.G. 2003. Green-fluorescent proteins in Caribbean corals. *Limnology and Oceanography* **48**: 402–411.

Page, R.D.M. 1996. TREEVIEW: An application to display phylogenetic trees on personal computers. *Computer Applications in the Bioscience* **12**: 357–358.

Papina, M., Sakihama, Y., et al. 2002. Separation of highly fluorescent proteins by SDS-PAGE in Acroporidae corals. *Comparative Biochemistry and Physiology* **131**: 767–774.

Posada, D. and Crandall, K.L. 1998. MODELTEST: Testing the model of DNA substitution. *Bioinformatics* **14**: 817–818.

Richmond, R.H. and Jokiel, P.L. 1984. Lunar periodicity in larval release in the reef coral *Pocillopora damicornis* at Enewetak and Hawaii. *Marine Biology* **34**: 280–287.

Ronquist, F. and Huelsenbeck, J.P. 2003. MRBAYES 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**: 1572–1574.

Rowan, R. and Knowlton, N. 1995. Intraspecific diversity and ecological zonation in coral-algal symbiosis. *Proceedings of the National Academy of Science of the USA* **92**: 2850–2853.

Rowan, R., Knowlton, N., et al. 1997. Landscape ecology of algal symbionts creates variation in episodes of coral bleaching. *Nature* **388**: 265–269.

Salih, A., Hoegh-Guldberg, O., and Cox, G. 1998. Photoprotection of symbiotic dinoflagellates by fluorescent pigments in reef corals. *Australian Coral Reef Society 75th Anniversary Conference*, Heron Island.

Salih, A., Larkum, A.W.D., Cox, G., Kuehl, M., and Hoegh-Guldberg, O. 2000. Fluorescent pigments in corals are photoprotective. *Nature* **408**: 850–853.

Seutin, G. and White, B.N. 1991. Preservation of Avian Blood and Tissue Samples for DNA Analyses. *Canadian Journal of Zoology–Revue Canadienne De Zoologie* **69**: 82–90.

Stimson, J. and Kinzie, R.A. 1991. The temporal pattern and rate of release of zooxanthellae from the reef coral *Pocillopora damicornis* (Linnaeus) under nitrogen-enrichment and control conditions. *Journal of Experimental Marine Biology and Ecology* **153**: 63–74.

Takabayashi, M. and Hoegh-Guldberg, O. 1995. Ecological and physiological differences between two colour morphs of the coral *Pocillopora damicornis*. *Marine Biology* **123**: 705–714.

Takabayashi, M., Carter, D.A., Lopez, J.V., and Hoegh-Guldberg, O. 2003. Genetic variation of the scleractinian coral *Stylophora pistillata*, from the western Pacific reefs. *Coral Reefs* **22**: 17–22.

Thompson, J.D. and Higgins, D.G. 1994. CLAUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acid Research* **22**: 4673–4680.

Vermeij, M.J.A. and Bak, R.P.M. 2002. How are coral populations structured by light? Marine light regimes and the distribution of *Madracis*. *Marine Ecology Progress Series* **233**: 105–116.

Veron, J.E.C. and Wallace, C. 1984. *Scleractinia of Eastern Australia*. Canberra, Australian Government Publishing.

Veron, J.E.N. and Pichon, M. 1982. *Scleractinia of Eastern Australia*. Canberra, Australian Government Publishing.

Willis, B.L. 1985. Phenotypic plasticity versus phenotypic stability in the reef corals *Turbinaria mesenterina* and *Pavona cactus*. *Proceedings of the Fifth International Coral Reef Symposium*, Moorea, French Polynesia, Antenne Museum-EPHE.

Willis, B.L. and Ayre, D.J. 1985. Asexual reproduction and genetic determination of growth form in the coral *Pavona cactus*: biochemical genetic and immunogenetic evidence. *Oecologia* **65**: 516–525.

Yonge, C.M. 1968. Living corals. *Proceedings Royal Society* **169**: 329–344.

Zardoya, R. and Costas, E. 1995. Revised dinoflagellate phylogeny inferred from molecular analysis of large-subunit ribosomal-RNA gene-sequences." *Journal of Molecular Evolution* **41**: 637–645.