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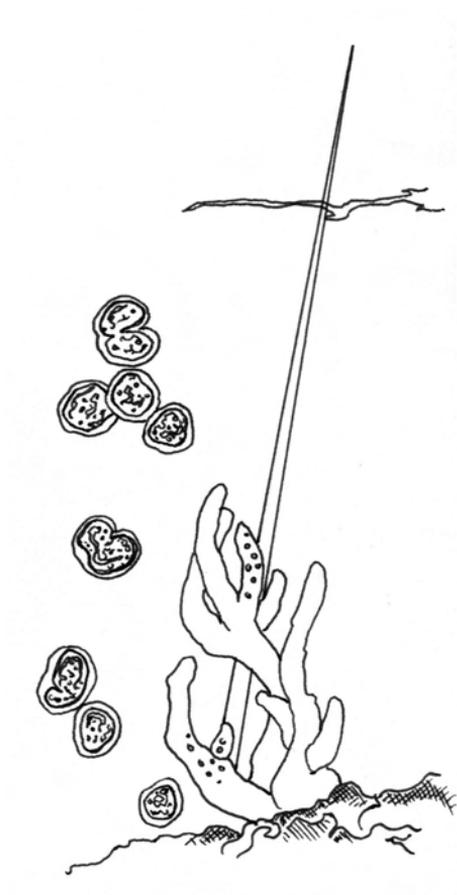
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CHAPTER 2

Variation in symbiont distribution between closely related coral species over large depth ranges

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ABSTRACT

Symbiotic algae in coral species distributed over a large depth range are confronted with major differences in light conditions. We studied the genetic variation of *Symbiodinium* in the coral genus *Madracis* over depth (5–40 m) and at two different colony surface positions. Using polymerase chain reaction–denaturing gradient gel electrophoresis ITS2 nuclear ribosomal DNA analyses, we consistently identified three symbiont genotypes with distributions that reveal patterns of host specificity and depth-based zonation. ITS2 type B7 *Symbiodinium* is the generalist type, occurring in all zooxanthellate *Madracis* corals and at all depths. Type B13 is restricted to the shallow water specialist *Madracis mirabilis*. Type B15 is typical of deep reef environments and replaces B7 in the depth generalist *Madracis pharensis*. Contrasting with variation over depth, we found strong functional within-colony uniformity in symbiont diversity. Relating symbiont distributions to measured physical factors (irradiance, light spectral distribution, temperature), suggests depth-based ecological function and host specificity for *Symbiodinium* ITS2 types, even among closely related coral species.

INTRODUCTION

Tropical reef-building corals maintain mutualistic symbioses with phototrophic dinoflagellates of the genus *Symbiodinium* Freudenthal (1962). The symbiotic association is credited for the success of these corals in nutrient-poor environments (Muscatine and Porter 1977). Originally thought to be a monotypic genus (Yonge 1973), *Symbiodinium* was revealed to be a taxonomically diverse group (reviews by Trench 1993; Rowan 1998). This was first shown by biochemical (Trench 1971b), morphological (Trench and Blank 1987) and physiological studies (Chang *et al.* 1983; Iglesias-Prieto and Trench 1994), and lately by molecular genetics (reviews by Baker 2003; Coffroth and Santos 2005). Based upon nuclear ribosomal DNA (rDNA) gene polymorphisms, the genus *Symbiodinium* is currently recognised to comprise at least eight highly divergent phylogenetic clades (A to H) (Rowan and Powers 1991a; Baker and Rowan 1997; Pochon *et al.* 2004), a distinction that finds support in other recently used markers (see, e.g. Santos *et al.* 2002; Takabayashi *et al.* 2004; Barbrook *et al.* 2006; Pochon *et al.* 2006). Each of these subgeneric clades includes an unknown number of types or “species”, first defined based on sequence variation of the faster evolving rDNA internal transcribed spacer (ITS) region (Hunter *et al.* 1997; LaJeunesse and Trench 2000; LaJeunesse 2001). The ITS-rDNA analyses strongly support the few *Symbiodinium* species formally described (LaJeunesse 2001) and generate phylogenies partially confirmed by other markers (e.g. chloroplast rDNA, Santos *et al.* 2003a). As opposed to the clade level distinction, which poorly correlates with physiological or ecological properties (LaJeunesse 2001; Tchernov *et al.* 2004), the ITS types approximate physiological distinct populations and are widely accepted as representing ecologically meaningful diversity (Santos *et al.* 2001; van Oppen *et al.* 2001a; LaJeunesse 2002; LaJeunesse *et al.* 2003; Thornhill *et al.* 2006; Warner *et al.* 2006).

Host-symbiont associations are also nonrandom with respect to partners involved (Rowan and Powers 1991a) and therefore exhibit specificity (Trench 1997), for which there is evidence in varying degree among both hosts and symbionts (Baker 2003). Curiously, several authors (e.g. LaJeunesse *et al.* 2004) propose the existence of a relation between symbiont diversity and transmission mode but so far, not much evidence has been found (van Oppen 2004). Examples of scleractinian (stony coral) species hosting multiple *Symbiodinium* lineages, sometimes even over the surface of individual colonies (Rowan and Knowlton 1995; Rowan *et al.* 1997; LaJeunesse and Trench 2000; van Oppen *et al.* 2001a; Ulstrup and van Oppen 2003), show that there are ecological factors driving the distribution and zonation of *Symbiodinium* strains among coral colonies. This evidence for ecological zonation, together with a broad physiological diversity measured in endosymbionts isolated from different cnidarian hosts (Chang *et al.* 1983; Iglesias-Prieto and Trench 1994, 1997b,a; Robison and Warner 2006), favours the argument that certain symbionts have adaptations to particular light regimes. This inference is supported by additional *in vitro* evidence, such as the mycosporine-like amino acid production (Banaszak *et al.* 2000). The presence of specific *Symbiodinium* types adapted to different light regimes was hypothesised to constitute an important axis for niche diversification in reef corals (Iglesias-Prieto and Trench 1994, 1997a). Iglesias-Prieto *et al.* (2004) showed in a unique genetic and *in situ* physiological study, that this hypothesis can explain the vertical distribution patterns of dominant coral species. Research on *Symbiodinium* functional diversity will help to reveal the underlying mechanisms shaping coral vertical distribution. The main question presently posed is “Do different *Symbiodinium* genotypes relate to the distribution of

their coral hosts over depth suggesting different ecological functions?”

Although symbiotic corals are distributed over the entire euphotic zone, most species have limited vertical distributions (Veron 2000) along the gradient that is created by the vertical attenuation of light with depth. This attenuation in light intensity is linked to spectral distribution changes. In addition, the structural complexity of reefs generates further spatial variation in light availability (Anthony and Hoegh-Guldberg 2003), also severely affected at the colony level by skeletal morphology (Anthony *et al.* 2005; Enriquez *et al.* 2005), host pigments (Dove *et al.* 2006) or even polyp behaviour (Hoegh-Guldberg and Jones 1999). Among other depth-related environmental clines shaping coral reef ecology (see, e.g. Baird and Atkinson 1997), temperature differences correlate with the distribution of specific host-*Symbiodinium* associations, as shown by thermal tolerance studies (Glynn *et al.* 2001; Rowan 2004; Tchernov *et al.* 2004; Berkelmans and van Oppen 2006).

We study the genetic variation of *Symbiodinium* in corals with large vertical distributions. With a few rare exceptions (Toller *et al.* 2001; Warner *et al.* 2006), there are no robust data on *Symbiodinium* bathymetric variation below depths of 10-15 m and large vertical coral-symbiont distributions constitute an overlooked subject in need of research.

The coral genus *Madracis* Milne Edwards and Haime 1849 (Scleractinia) has a wide depth distribution, ranging from *c.* 2 to > 100 m on Caribbean reefs (Wells 1973a; Vermeij and Bak 2002). Despite the large depth range, all zooxanthellate specimens sampled to date harbour clade B *Symbiodinium* only (Diekmann *et al.* 2002). In a first step to unravel possible underlying genetic variation, three clade B variants (types B7, B13 and B15) were described from ITS-rDNA sequence analysis (Diekmann *et al.* 2003).

Here, we follow up on that initial research and report on a survey of *Symbiodinium* diversity for *Madracis* species. We are interested in variation with depth and over coral colony surfaces. Our final objective is to understand the role of *Symbiodinium* functional diversity in the acclimatisation, adaptation and distribution patterns of corals with large depth ranges. Combining the data on symbiont genetic variation and environmental factors, we show that symbiont ITS2 types have different distributions that suggest depth-based ecological function and host specificity.

MATERIALS AND METHODS

Study area

Research was conducted from July to October 2005 on Curaçao (Figure 2.1), southern Caribbean (12°05' N, 69°00' W). A shallow terrace (50-100 m wide), a drop-off at 8-12 m, and a steep slope extending to 50-60 m typically characterize these reefs (Bak 1977). The Buoy One reef location (Bak 1977; Bak *et al.* 2005; Vermeij *et al.* 2007b), situated 500 m west of the Caribbean Research and Management of Biodiversity (CARMABI) Foundation, was surveyed over a *c.* 2000 m² section.

The coral genus *Madracis*

Currently comprising six zooxanthellate species on Caribbean reefs (Wells 1973a,b; Vermeij *et al.* 2003a), the genus *Madracis* is characterised by morphological diversity between species

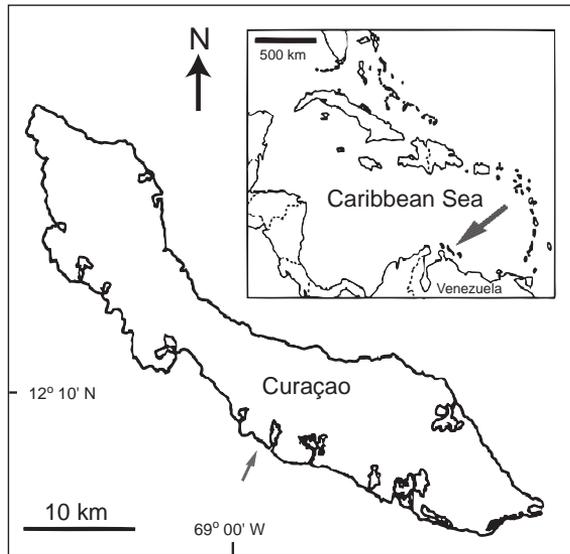


Figure 2.1 The Caribbean Sea (inset), Curaçao and the study site CARMABI Buoy One (arrows).

and morphological plasticity within species (Wells 1973a; Fenner 1993). The species have distinct depth distributions (Vermeij and Bak 2003, P.R. Frade, unpublished). At the study site, the encrusting or submassive *Madracis pharensis* (Heller 1868) and *Madracis senaria* (Wells 1973a) are found across all depths (5 to > 60 m) and the nodular *Madracis decactis* (Lyman 1859) occurs from 5 to 40 m; the branching species *Madracis mirabilis* Duchassaing and Michelotti 1861, *Madracis carmabi* (Vermeij *et al.* 2003a) and *Madracis formosa* (Wells 1973a) are restricted to, respectively, shallow- (2-25 m), mid- (20-40 m) and deep-water (> 30 m) habitats (Figure 2.2). All species are easily distinguished based on colony morphology. Diekmann *et al.* (2001) showed that the morphological species distinction only corresponds to monophyletic genetic-based groups for *M. mirabilis* and *M. senaria*, while the other morphospecies form a paraphyletic “species” complex. *Madracis pharensis* and *M. decactis* show morphological overlap and their separation as different species is debated (Fenner 1993; Diekmann *et al.* 2001; Vermeij *et al.* 2007a). In our study, encrusting and nodular colonies were classified as *M. pharensis* and *M. decactis*, respectively.

All studied *Madracis* species are brooders and release larvae throughout the year (Vermeij *et al.* 2003b). Both vertical and horizontal symbiont transmission modes have been reported within the genus. *Madracis senaria* and *M. mirabilis* release zooxanthellate planulae with a distinct symbiont ring at the oral end. *Madracis pharensis* and *M. decactis* are the only species for which only aposymbiotic planulae were observed (Vermeij *et al.* 2003b), suggesting horizontal symbiont transmission. Observations on *M. carmabi* suggest vertical transmission (P.R. Frade, unpublished). No information is available for *M. formosa*.

Previous ITS-rDNA sequence analysis survey (Diekmann *et al.* 2003) showed that *M. mirabilis* harbours type B13 *Symbiodinium*, while all other species harbour type B7 (nomenclature LaJeunesse 2002). Type B15 *Symbiodinium* was only described for a single *M. formosa* colony (Diekmann *et al.* 2003).

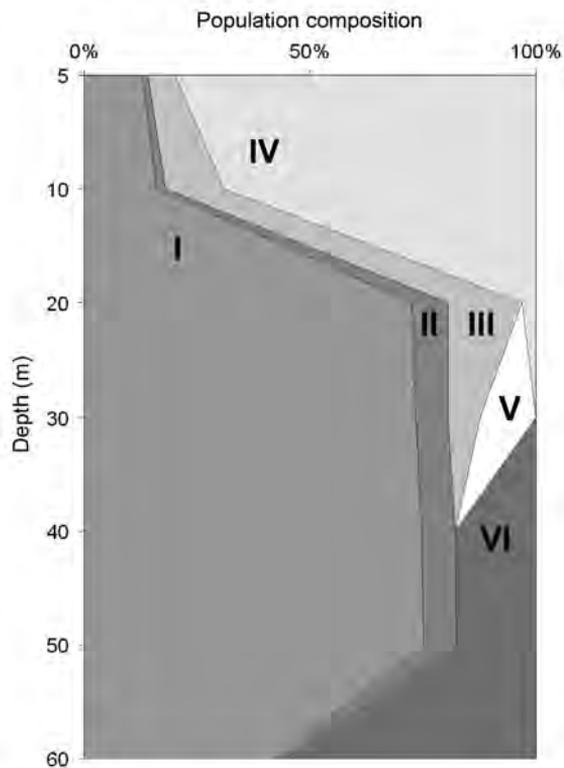


Figure 2.2 *Madracis* spp. distribution over the reef slope at study site Buoy One. I – *M. pharensis*, II – *M. senaria*, III – *M. decactis*, IV – *M. mirabilis*, V – *M. carmabi*, VI – *M. formosa*. Data Vermeij and Bak (2003), P.R. Frade (unpublished).

Collection of corals

Fragments of the six *Madracis* morphospecies (Vermeij and Bak 2003), *M. mirabilis*, *M. decactis*, *M. pharensis*, *M. senaria*, *M. formosa* and *M. carmabi*, were collected with hammer and chisel at four depths (5, 10, 25 and 40 m). All colonies were sampled for two different positions: the horizontal top ($\alpha = 0^\circ$) and the seaward-facing vertical side ($\alpha = 90^\circ$), except for 40 m (only top position was sampled). We collected 276 samples from 158 colonies. For details on sample size for species, depths and colony position, *see* Results. All samples were taken from July to September 2005 with the exception of *M. carmabi* (same period 2004). Care was taken to sample adult, healthy colonies exposed to the depth-mediated light gradient (Vermeij and Bak 2002). Samples were placed in individual seawater-filled plastic bags and immediately transported at constant temperature to the laboratory, where coral tissue was removed with a sterile scalpel and preserved in 95% ethanol at -20°C until further DNA extraction. Ethanol was washed out before DNA extraction.

Genetic analyses

Total nucleic acids were extracted using the UltraClean Soil DNA kit by MoBio. Polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE) fingerprinting of the *Symbiodinium* ITS2-rDNA region were coupled for each DNA extract (LaJeunesse 2001,2002). This distinguishes between DNA fragments with as little as 1 base-pair (bp)

change (Schafer and Muyzer 2001). The ITS2 region was amplified using primers designed by LaJeunesse and Trench (2000): the 5.8S internal primer “ITSintfor2” and the ITS flanking primer “ITS-Reverse” (Coleman *et al.* 1994), now modified with a 39-bp guanine cytosine-rich clamp extension and named “ITS2clamp” (LaJeunesse and Trench 2000). Amplification took place on a TGradient thermal cycler (Biometra) using the “touchdown” protocol (LaJeunesse 2002) to ensure PCR specificity, but with a final annealing temperature of 50°C (15 cycles) after a 10°C total decrease over 20 cycles (0.5°C after each). ITS2 amplified products (100 ng) were electrophoresed for 14 h at 100 V at a constant temperature of 60°C on DGGEs (30-70%) using a Bio-Rad DCode system. Gels were stained with SYBR Gold (Molecular Probes) for 30 min and photographed over a Dark Reader transilluminator (Clare Chemical Research).

Discrete bands from the denaturing gels were excised, eluted at 4°C in 15 μ L of 1x PCR buffer (GenScript) for 48 h and later re-amplified using the same protocol and primer set but now without the GC-clamp addition (LaJeunesse 2002). The re-amplified ITS2 PCR products were purified using a QIAquick kit (QIAGEN) and cycle sequencing was achieved separately in both directions as specified in the ABI PRISM BigDye Terminator Cycle Sequencing ready reaction kit (Applied Biosystems). Reaction products were analysed in an ABI PRISM 310 genetic analyser (Applied Biosystems) and the obtained sequence chromatograms were checked manually using SEQUENCING ANALYSIS version 3.0. Consensus sequences were obtained in AUTOASSEMBLER version 2.0 (Applied Biosystems). These sequences were uploaded in GenBank BLAST searches (Altschul *et al.* 1997) and imported with their closest relatives into the ARB software (Ludwig *et al.* 2004), to identify *Symbiodinium* ITS2 diagnostic bands. To limit sequencing effort, all digital DGGE band profiles were compared using the software FINGERPRINTING II (Bio-Rad) and the presence of diagnostic bands was analysed.

Environmental parameters

Ambient seawater temperatures ($\pm 0.1^\circ\text{C}$) were recorded hourly with StowAway TidbiT temperature loggers (Onset) at the depths of 2.5, 5, 10, 25 and 40 m for a whole year leading towards and including the sampling period; daily averages were calculated.

The depth-mediated light gradient was measured around solar noon (± 30 min) on the 19th of September 2005, under a cloudless sky and no wind, using a photosynthetic active radiation (PAR: 400-700 nm) cosine-corrected LI-192SA flathead light sensor connected to a LI-1000 data logger (Li-Cor). Irradiance ($\mu\text{mol photons m}^{-2} \text{ s}^{-1}$) was recorded with increasing depth resolution from 44 m of depth up to sea (sub) surface, for each of two directions: light coming in straight from above ($\alpha = 0^\circ$) and light coming from aside into the reef ($\alpha = 90^\circ$). This included a minimum of 10 measurements for each depth to minimize the effect of light fluctuations due to sea surface refraction.

The spectral distribution of light along the water column was measured around solar noon (± 30 min) on the 8th of October 2004, using the approach described above for total PAR irradiance. Spectral irradiance ($\text{mW m}^{-2} \text{ nm}^{-1}$) was recorded with 3 nm resolution for the 350-900 nm range using a Ramses-ACC hyperspectral radiometer (TriOS) with a cosine response sensor.

Statistical analyses

Multiple linear regression was used to analyse all environmental data. Depth and month

(included to accommodate season-related variance) were tested as factors in temperature variation. Besides depth, light direction ($\alpha = 0^\circ$ and $\alpha = 90^\circ$) was used to calculate the vertical attenuation coefficient K_d of the water for total PAR irradiance. For spectral irradiance, K_d s were calculated for each wavelength interval using the same approach (statistics not shown) and the effect of wavelength and light direction on K_d was further tested. All irradiance data was ln-transformed before analyses.

Logistic regression was used to separately model the distribution (presence/absence) of each symbiont ITS2-type found, assuming no interference between types. We tested the null hypothesis that the presence of a symbiont type was not related to any of the predictor variables (host species, depth and colony surface position), including only data representing a complete cross-factorial sampling design: species *M. pharensis*, *M. senaria*, *M. decactis* and *M. mirabilis* originated from depths of 5, 10 and 25 m ($n = 235$). We used a binomial distribution for the error terms and a logit link function. A backward and forward model selection method was applied to identify the variables that accounted for a significant amount of the variation in the response variable (Zuur *et al.* 2007). The adequate model was reached when no variable could be either deleted or added to the model without causing a statistically significant change in deviance (χ^2 distributed).

All statistical tests were performed at a significance level of 0.05. Interactions between main factors were tested when applicable. Modelling was carried out using the software BRODGAR (version 2.5.2), linked to the statistics package R (version 2.4.0).

RESULTS

Environmental parameters

Figure 2.3 shows the measured environmental parameters for the Buoy One reef location. Mean seawater temperature (Figure 2.3a and b) decreases with depth (0.02°C per meter, in average) and shows seasonal variation, through which the depth effect is irregular (interaction between depth and month; $F_{11,1702} = 23.822$, $P < 0.001$). Lowest temperatures occur at 40 m. This is caused by frequent (daily to weekly, data not shown) thermocline movement over the deep reef slope, leading to increased temperature oscillations at this depth (absolute measured minimums rarely below 25°C). Yearly averages (including season effect) are $27.8 \pm 0.8^\circ\text{C}$ for 2.5 m, $27.7 \pm 0.9^\circ\text{C}$ for 5 m, $27.5 \pm 0.9^\circ\text{C}$ for 10 m, $27.4 \pm 1.0^\circ\text{C}$ for 25 m and $26.9 \pm 0.8^\circ\text{C}$ for 40 m.

Total PAR irradiance (Figure 2.3b) decreases exponentially with depth for both $\alpha = 0^\circ$ and $\alpha = 90^\circ$, with K_d s of 0.07 m^{-1} and 0.05 m^{-1} , respectively, and subsurface values of about 1500 and $240 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$, respectively (interaction between depth and light direction; $F_{1,586} = 85.765$, $P < 0.001$). With depth, there is increasing similarity between downwelling ($\alpha = 0^\circ$) and side coming light ($\alpha = 90^\circ$) that consequently reduces the variation of light over the colony surface.

The spectral distribution of the light field (Figure 2.3d), similar for $\alpha = 0^\circ$ and $\alpha = 90^\circ$ (K_d unrelated to light direction; $P = 0.18$), is modified with depth, changing towards the blue and green part of the spectrum, as K_d reaches minimum values at this spectral region (significant effect of wavelength on K_d ; $F_{1,332} = 1029.0$, $P < 0.001$).

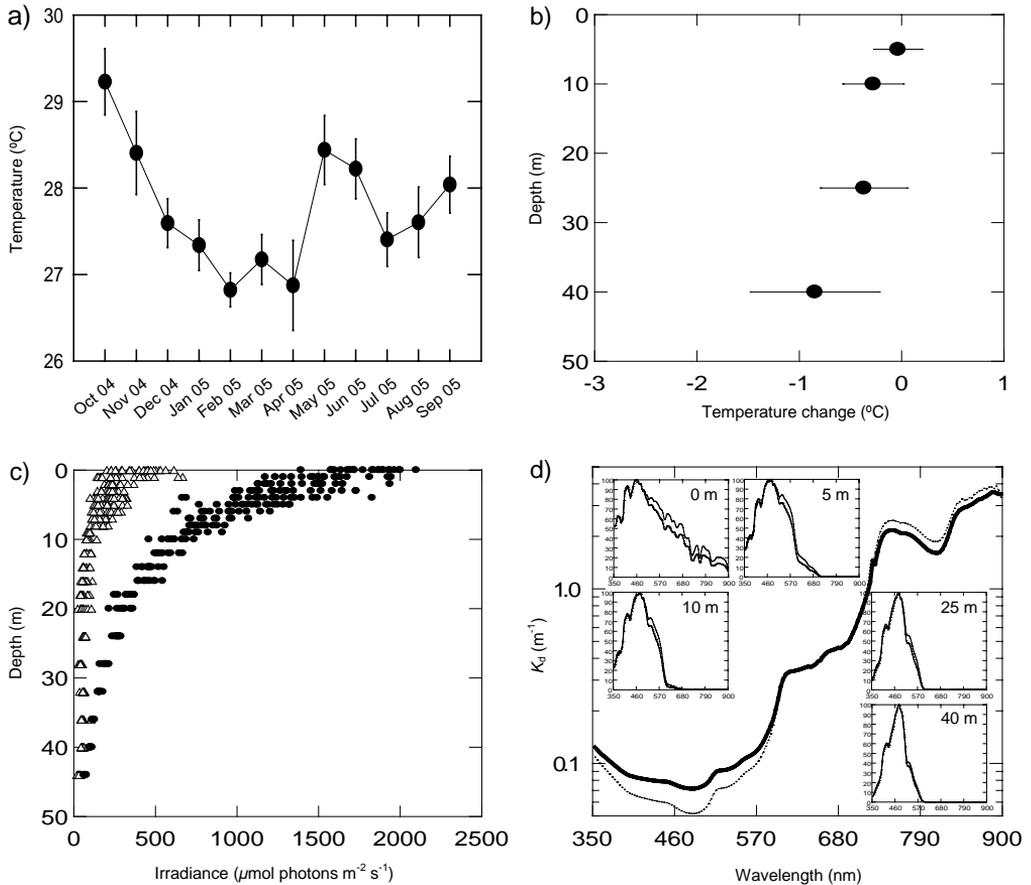


Figure 2.3 Environmental parameters over the reef slope at study site Buoy One. **(a)** Monthly average temperature ($^{\circ}\text{C} \pm \text{SD}$) at 2.5 m depth from October 2004 to September 2005. **(b)** Temperature ($^{\circ}\text{C} \pm \text{SD}$) expressed as the mean difference between temperature at 2.5 m and at sampled depths. **(c)** Total PAR irradiance ($\mu\text{mol photons m}^{-2} \text{s}^{-1}$) for two directions: (\bullet) straight from above ($\alpha = 0^{\circ}$) and (Δ) from aside into the reef ($\alpha = 90^{\circ}$). **(d)** Spectral distribution (nm) of the vertical attenuation coefficient K_d (m^{-1}) for light from two directions: (—) straight from above ($\alpha = 0^{\circ}$), (---) from aside into the reef ($\alpha = 90^{\circ}$); inner graphs show light spectral distribution for subsurface (0m), 5 m, 10 m, 25 m and 40 m (irradiance normalized to maximum value for each respective depth).

DGGE profiles and ITS2 phylogeny

The ITS2 DGGE band profiles were highly repeated and each distinct signature characterised by a bright diagnostic band accompanied by faint bands. A total of 81 bands were successfully sequenced to validate the fingerprinting. Obtained sequences have been deposited in the National Center for Biotechnology Information (NCBI, USA) GenBank (accession numbers EF450546-EF450626). Not all faint bands were amenable to sequencing, but when they were, the resulting sequence was identical to a diagnostic band. Here, the differential migration of bright diagnostic and vague bands is most likely the result of anomalous melting behaviour caused by secondary structural conformations of identical DNA fragments (Buckler *et al.* 1997; Michikawa *et al.* 1997).

Some samples showed more complex profiles designating a mix of two distinct *Symbiodinium* ITS signatures. In this case, “quartet” band profiles were often present because of heteroduplex

formation (also confirmed by sequencing), characteristic of re-annealing of nonextended heterogeneous DNA single strands during PCR extension saturation effect (Buckler *et al.* 1997). Because the sequencing of very vague diagnostic bands was usually unsuccessful, the FINGERPRINTING II (Bio-Rad) band assignment properties were set to consequently neglect such vague bands (representing less than 10% of the lane total band pixel density). Rarely, vague band sequences were derived from the coral host ITS2 region, as recognised by GenBank BLAST searches (Altschul *et al.* 1997).

Figure 2.4 shows the different band profiles obtained, labelled with their ITS type affiliation (LaJeunesse 2002; Diekmann *et al.* 2003). Three clade B *Symbiodinium* ITS types previously described (types B7, B13 and B15) were found to be present (Figure 2.5, also includes reference sequences retrieved from GenBank). The *Symbiodinium* ITS2 region was 198, 197 or 194 bp in length, for B7, B13 or B15, respectively. Among these *Madracis* symbionts, the alignment of the whole amplified fragment shows 11 phylogenetically informative sites, all located within the ITS2 region, including two indels (one 4 bp, one 1 bp) and several single base substitutions. ITS2 types B13 and B7 differ only by minor sequence variation (one indel and one substitution), while ITS2 type B15 shows broader differences. Besides the informative sites, there was inconsequential, random variation that could not be attributed to band level or any of the explanatory ecological variables considered. Consensus sequences were identical to previously published sequences for the same types, with the exception of type B15, for which the single sequence published to date (AF458603) includes sequencing ambiguities. Because AF458603 was still the closest match and because its original sequence was obtained from a *Madracis formosa* (Diekmann *et al.* 2003), we attribute the same nomenclature to our sequences. No other type was found to be present, even if eventually all different band levels were sequenced. Whenever a mixed occurrence of B7 and B13 diagnostic bands was registered, two faster melting heteroduplex bands were often visible (Figure 2.4).

Symbiont population composition

Readable PCR-DGGE band profiles were achieved for a total of 276 samples. For sample size of species, depth and colony surface position, *see* Figure 2.6. To each sample, one of

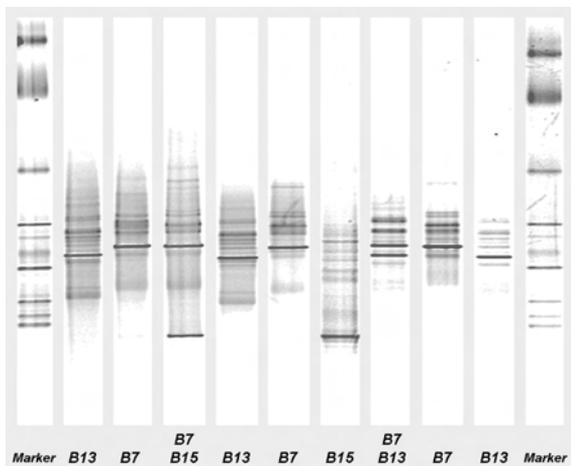


Figure 2.4 *Symbiodinium* spp. Diagnostic PCR-DGGE ITS2 band profiles (negative image) representative of the different symbiont type combinations found. Marker contains no phylogenetic information.

the following symbiont population keys was attributed: B7, B13, B15, B7 + B13 or B7 + B15 (types B13 and B15 never co-occur). Type B7 *Symbiodinium* dominates the symbiont population within the *Madracis* genus, being present in all different host species, at all depths and in all colony positions (Figure 2.6). B13 type is typically restricted to *Madracis mirabilis*, the shallow-water branching species, and is present in more than half of this species' colonies at each depth. The other ITS2 type detected, B15, is mostly found in *Madracis pharensis* at and below 25 m, present in about half of the colonies. B15 also occurs in about one-fourth of *M. formosa* colonies. Unlike *M. mirabilis*, *M. pharensis* and *M. formosa*, the other three species, *M. decactis*, *M. senaria* and *M. carmabi*, exclusively form associations with type B7 *Symbiodinium* throughout the whole sampled depth range and regardless of colony position. The single exception was the top position of a *M. senaria* colony harbouring type B13 at 5 m. Symbiont identity did not vary within the colony in most cases (Figure 2.6), with top and side harbouring the same symbiont types.

Symbiont distribution, as modelled by logistic regression for each ITS2 type, was found to be host species related in all three cases: B7 ($\chi^2 = 100.167$, d.f. = 3, $P < 0.001$), B13 ($\chi^2 = 191.999$, d.f. = 3, $P < 0.001$) and B15 ($\chi^2 = 33.045$, d.f. = 3, $P < 0.001$). Depth had a significant effect on the presence of symbiont types B7 ($\chi^2 = 5.591$, d.f. = 1, $P < 0.05$) and B15 ($\chi^2 = 24.889$, d.f. = 1, $P < 0.001$) but not of type B13 ($P = 0.79$). Presence of type B7 was found to be further explained by an interaction between host species and depth ($\chi^2 = 22.201$, d.f. = 3, $P < 0.001$). Surface position was never found to have a significant effect on symbiont presence ($P > 0.70$ in all cases).

The statistical significance of depth-related symbiont variation is a consequence of symbiont distribution in *M. pharensis*, where type B15 gradually replaces B7 from shallow to deep environments. Running a new logistic regression model, describing the distribution of type B15 for *M. pharensis* along the whole sampling depth gradient, allowed us to predict that the *M. pharensis* population will be fully dominated (> 95% colonies) by this symbiont type at depths ≥ 54 m. Interestingly, six colonies (four *M. pharensis* and two *M. formosa*) sampled at a depth of 60 m (same season 2006) harboured exclusively B15 *Symbiodinium* (results not shown).

DISCUSSION

The present study comprises an extensive ITS2-rDNA-based data set on the diversity of symbiont populations harboured by a single scleractinian genus for one location over a large depth range. It offers a detailed example of symbiont variation for closely related coral species that highlights the implications of ecological and evolutionary processes in coral-algal associations.

Symbiont variation and host specificity

The genus *Madracis* local endosymbiont population is dominated by clade B *Symbiodinium*, regardless of host species, depth or within-colony position (see also Diekmann *et al.* 2002; Diekmann *et al.* 2003). At a subcladal level, three ITS2 variants are present (types B7, B13 and B15 *Symbiodinium*), of which the distribution is clearly not random (Figure 2.6). Instead, the occurrence of these *Symbiodinium* types shows systematic patterns of distribution in different *Madracis* species over a depth- and colony landscape-mediated light gradient.

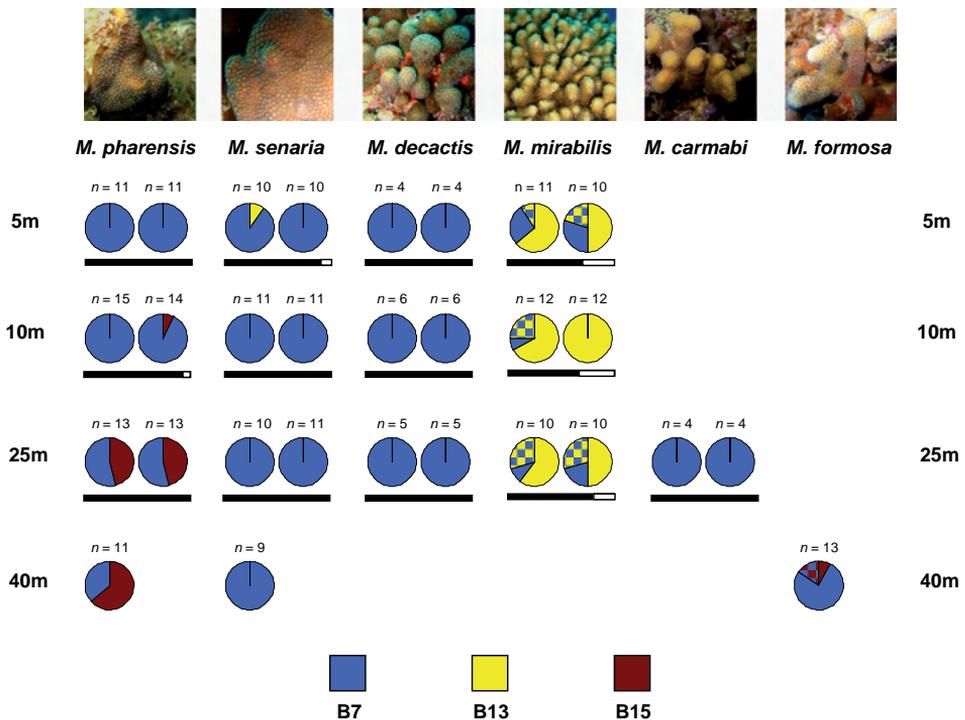


Figure 2.6 *Symbiodinium* spp. population ITS2-diversity for six *Madracis* morphospecies, four depths and two within-colony positions. For each species-depth combination, graphs represent: (left) horizontal top colony position ($\alpha = 0^\circ$) and (right) seaward-facing vertical side colony position ($\alpha = 90^\circ$). Note that at 40 m only the top positions ($\alpha = 0^\circ$) were sampled. Number of replicates (number of colonies) is shown over each data point. Black bars represent percentage of similarity between $\alpha = 0^\circ$ and $\alpha = 90^\circ$ for colonies sampled on both positions. Presence of two symbiont types within the same sample represented by mixed pattern of respective colours. For full colour version see Appendix (page 132).

The presence of clade B *Symbiodinium* has been extensively reported for the coral reef community of symbiotic invertebrates in the Caribbean region (LaJeunesse 2002; Goulet and Coffroth 2004; van Oppen *et al.* 2005), including scleractinian coral genera such as *Cladocora*, *Colpophyllia*, *Dendrogyra*, *Diploria*, *Dichocoenia*, *Eusmilia*, *Favia*, *Manicina*, *Meandrina*, *Porites*, *Siderastrea* and *Montastraea*. According to present knowledge, the three *Symbiodinium* types described here are exclusive to the coral genus *Madracis* with a single exception reported: the presence of type B7 (AY894812) in the Caribbean octocoral *Pseudopterogorgia bipinnata* (Boehnlein *et al.* 2005). LaJeunesse (2005) suggested these highly host-specific *Symbiodinium* types to be part of a diverse “species” assemblage, the result of diversification within Caribbean clade B in an eco-evolutionary radiation (diverging since 1.2-2.1 million years ago) from more ancestral lineages, such as type B1 *Symbiodinium*. This ITS B1 type (LaJeunesse 2001), analogous to (chloroplast large subunit ribosomal DNA defined) type B184 of Santos *et al.* (2003a) and considered the most ubiquitous lineage in the Caribbean (LaJeunesse 2002), still shows a considerable degree of fine-scale diversity and host-symbiont specificity as shown by microsatellite genotyping (Santos *et al.* 2004). By analogy, some within-type population structure not inferable by ITS2-rDNA sequence analyses may be present for the *Symbiodinium* B-types occurring in the coral genus *Madracis* (Pettay and LaJeunesse 2007).

The *Madracis* symbiont genotypes are highly specific for this coral genus but show different levels of specificity towards the different host morphospecies. Therefore, it is relevant to consider within-*Madracis* symbiont host specificity when describing ecological processes such as those related to depth zonation of ITS2-defined diversity. Type B7 *Symbiodinium* is a depth-generalist with rather low host specificity (dominant in all *Madracis* species) that occurs either as single symbiont type or in mixed populations (even at the colony level); in *Madracis pharensis*, this type is replaced by type B15 *Symbiodinium*, which occurs predominately at deep environments (≥ 25 m) and appears to have an intermediate host specificity, being restricted to *M. pharensis* and *M. formosa*; on the other hand, type B13 *Symbiodinium* is restricted to shallow water environments (≤ 25 m), although this may be masked by its high host specificity for *Madracis mirabilis*, a coral species that typically also does not occur deeper than 25 m (Vermeij and Bak 2003, P.R. Frade, unpublished).

Symbiont functional diversity

The reported symbiont type distribution suggests that, besides having different levels of host specificity, these three symbiont types also have different (but overlapping) depth zonation. This argues for the hypothesis that these three *Symbiodinium* ITS2-types correspond to genetic entities that are to a certain extent adapted to the heterogeneity created by host species and/or depth-related environmental conditions. This would relate to evolutionary pathways leading to niche or ecological diversification.

Symbiodinium sp. adaptation processes to environmental axes are only marginally known. Nevertheless, studies on isolates have demonstrated photoacclimation characteristic responses that are thought to represent adaptation and can therefore be expected to vary across symbiont types. Such responses include changes in cellular pigment contents, number and size of photosynthetic units, activities of CO₂-fixing enzymes and electron transport systems and growth rates (Chang *et al.* 1983; Iglesias-Prieto and Trench 1994, 1997b; Robison and Warner 2006).

Compared with corals such as the Caribbean *Montastraea* spp., we find a remarkable constancy in symbiont diversity within individual colonies of *Madracis*. The reported landscape-mediated light gradient over colony surface appears to have no effect on symbiont diversity. This, even if light changes an order of magnitude between top and side of colonies, variation comparable to major differences measured across depths. Intracolony constancy observed within species that have depth-correlated symbiont variation (i.e. *M. pharensis*) questions the role of light irradiance as the main driving factor. A case similar to *M. pharensis* is *Acropora cervicornis*, a species also showing no evidence for within-colony diversity despite clear patterns of symbiont depth zonation (Baker and Rowan 1997). Bathymetric symbiont variation such as in *M. pharensis* could be caused by environmental factor(s) other than light intensity. Possible alternatives, eventually acting synergistically, are light spectral distribution (Falkowski and Laroche 1991), temperature (Rowan 2004; Tchernov *et al.* 2004) or available nutrients (Fitt and Cook 2001). These are depth-variable parameters influencing *Symbiodinium* physiology that are not expected to vary significantly at colony microhabitat scale.

Although the analyses suggest temperature and light spectral distribution as being plausible explanations for the depth-related symbiont variation, the impact of total PAR irradiance, a major variable in algal distribution, cannot be excluded. If the B7 to B15 transition with depth

is related to photoadaptation, the dichotomy between depth- and colony landscape-mediated gradients indicates that *Madracis* colonies function, not as the sum of parts, but rather as a unit. Such a unit may be the result of competitive exclusion between symbionts (LaJeunesse 2002). Colony uniformity contrasts with symbiont distribution in the *Montastraea annularis* complex, where intracolony variation matches depth-based variation. Perhaps the difference is explained by colony size. *Madracis* adult colonies achieve only small sizes, surface areas < 100 cm² (or < 400 cm² for *Madracis senaria*), according to Vermeij and Bak (2003). This is much smaller than the adult size achieved by species for which intracolony variation is reported: *Montastraea* easily reaches surface areas of more than 4000 cm² (Vermeij *et al.* 2007b). Small colony size may not provide the necessary spatial range over which the patterns of symbiont competition or differential reproduction can develop. Possibly, the intracolony light scattering processes (Enriquez *et al.* 2005) provide the “sides” with more light than estimated based on incident light measurements. Functional colony uniformity finds support in studies that describe resource allocation among coral polyps at a linear scale of more than a dozen centimetres (Oren *et al.* 2001; Henry and Hart 2005).

An alternative explanation for the depth-related symbiont variation is that it has actually no functional significance, being instead the result of distinct rates of mutation propagation indirectly related to depth. Hypothetically, symbiont distribution could be driven by host distribution and related factors, such as coral species lineages and reproductive behaviour.

The intriguing presence of different symbiont types in different *M. pharensis* colonies living side-by-side under the same conditions (light field, temperature, etc.) can be explained by random events during the initiation of the symbiosis. This would occur in the absence of a clear competitive advantage of symbiont types under intermediate beneficial conditions (Warner *et al.* 2006).

Symbiont transmission mode

Symbiont distribution is often theoretically related to symbiont transmission mode. Several authors (e.g. LaJeunesse *et al.* 2004) propose that corals obtaining their symbionts by vertical inheritance are expected to have less diverse symbionts than corals acquiring their symbionts horizontally, from the surrounding environment. However, so far not much evidence has been found for this correlation between symbiont diversity and transmission mode. van Oppen (2004) showed that transmission mode does not affect symbiont diversity in acroporid corals. Within the genus *Madracis*, symbiosis is mostly very specific (e.g. *M. senaria* with B7) but there are examples of flexibility (e.g. *M. pharensis* with B7 and B15), suggesting the existence of distinct host symbiotic strategies coping with depth. *Madracis pharensis* is the only species within the genus for which bathymetric symbiont variation was found. Curiously, only aposymbiotic planulae have been reported for this species (Vermeij *et al.* 2003b), suggesting horizontal transmission. Hypothetically, an open transmission mode offers *M. pharensis* offspring the opportunity to associate with locally adapted symbiont types.

ITS2 region as a phylogenetic marker

There is ongoing debate on the usefulness of ITS region for coral phylogenetics (Vollmer and Palumbi 2004). For *Symbiodinium*, however, ITS phylogenies are widely used (e.g. Coffroth and Santos 2005; van Oppen *et al.* 2005; Thornhill *et al.* 2006; Warner *et al.* 2006). The

existence of some degree of intragenomic variation in the ribosomal array, with the presence of different ITS2 genotypes in the cistron repeats of the same organism (LaJeunesse 2002), invited comments (Gates *et al.* 2005); for the ITS1 region *see* van Oppen *et al.* (2005). Possible PCR-DGGE limitations in describing the diversity occurring within a mixture are debated by Appril and Gates (2007). In our study, we present DGGE fingerprinting profiles that are clear, highly repeatable and were validated by extensive sequencing. The disparities found in electrophoretic mobility strongly correlate to local host ecological patterns (e.g. replacement of B7 by B15 types in *M. pharensis* across depth). This supports the conviction that the resulting ITS2 diversity represents the natural biological variation and that fine-scale ITS2 variation may find a parallel in symbiont functionality differences. However, the absence of a clear niche diversification between the more similar B7 and B13 ITS2 types raises doubts on the distinction of these two types as ecologically distinctive forms. These may instead represent intragenomic variants or even diverging populations from an ancestral type (Romanski and Baker 2005). Further studies using other techniques (e.g. microsatellite loci, Pettay and Lajeunesse 2007) may show the degree of genetic isolation between these closely related ITS2 types.

Final considerations

There is only limited data on scleractinian symbiont diversity (Baker 2003; Coffroth and Santos 2005) and few studies have comprehensively explored intracladal *Symbiodinium* diversity in corals with large depth distributions (Warner *et al.* 2006). The deep reef, an important part of the coral reef ecosystem, has been largely ignored. The results presented in our study would have been substantially different if the sampling had been restricted to shallow depths (0-15m): the case of *M. pharensis*, with depth-related symbiont variation, would have been overlooked. Examining large depth distributions using large sample sizes increases the probability of finding scleractinian intraspecific symbiont diversity, as we are looking at an increased range of environmental interaction. On one hand, shallow water habitats are likely to be relatively more variable spatially and seasonally, therefore providing understanding of general environmental trends. On the other hand, including the deeper habitats provides the opportunity to address the whole range of natural variation that occurs along the environmental gradients represented over reef slopes. Unravelling the full picture of *Symbiodinium* diversity is essential in understanding the challenges posed to coral reefs by a rapidly changing environment.

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