Zooxanthellae diversity in the coral genus *Madracis*

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Abstract

The coral genus *Madracis* is probably the second most abundant Caribbean coral. Like all reef-building corals it lives in symbiosis with dinoflagellates. These unicellular algae named zooxanthellae, live as endosymbionts in a wide variety of marine invertebrates. Zooxanthellae were originally believed to be a single pandemic species *Symbiodinium microadriaticum*. Recent work utilizing genetic data, however, has shown that this is not the case. Zooxanthellae are not only highly diverse they are also found not obligately host-specific. Different zooxanthellae types have been found within a single host species and even within single coral colonies. Restriction Fragment Length Polymorphism (RFLP) analysis of SSrDNA zooxanthellae types in the coral genus *Montastrea* showed a strong correlation with depth. These results have led to the general view that corals adapt to different photic habitats by harbouring multiple zooxanthellae types. Here we present a similar RFLP analysis of zooxanthellae for another dominant reef building coral, *Madracis*. Surprisingly, no variation was found in zooxanthellae type either with respect to *Madracis* morphospecies or depth. This suggest that *Madracis* has other mechanisms that facilitate adaptation to light and the prevailing generalisation about symbiotic environmental adaptations needs to be re-evaluated.

Contents

Cover: photograph of the coral *Madracis mirabilis* on Curaçao (M. Vermeij) edited in combination with zooxanthellae (*Symbiodinium microadriaticum*) of unrealistic proportion (electron-microscopic image downloaded from internet). Actual size of zooxanthellae is approximately 10μm.

Introduction

The high biodiversity of the coral-reef ecosystem mainly depends on its structural complexity and high productivity. The calcareous reef framework harbours food, hiding place, substrate and brooding grounds for a variety of plants and animals. Stony (scleractinian) corals are important reef building organisms and therefore stand at the base of the tropical reef ecosystem. The high rate of calcification, responsible for the massive reef framework can only be accomplished through symbiosis with zooxanthellae. These phototrophic dinoflagellates occur as endosymbionts in many marine invertebrates and enable them to benefit from the high solar energy input (Muller-Parker & d'Elia 1997). Corals are vertically distributed throughout the entire euphotic zone (from 0m up to approximately 80 to 100m depth). The light intensity decreases with depth and therefore creates distinct photic habitats. In addition the three dimensional complexity of the coral reef also creates a variety of photic habitats within depths (Sheppard 1981, Chang *et al.* 1983).

Zooxanthellae Diversity

Zooxanthellae were originally believed to be a single pandemic species of endosymbiotic dinoflagellates *Symbiodinium microadriaticum*. This idea was based on the lack of phylogenetic data and morphological homogeneity. Research conducted by Blank & Trench (1985, 1986) and Blank (1986) on biochemical, behavioural and morphological observations of cultured zooxanthellae showed a more complex phylogeny and the existence of three additional *Symbiodinium* species (Trench & Blank 1987). Zooxanthellae have recently been described as belonging to at least 7 genera in 4 orders of dinoflagellates found in a wide range of marine invertebrates (Banaszak *et al* 1993, McNally *et al* 1994). However all zooxanthellae of the scleractinian corals so far identified belong to the genus *Symbiodinium* (Baker and Rowan 1997). Comparative DNA sequence analysis of the small subunit DNA revealed six genotypes of *Symbiodinium* (Rowan & Powers 1991a). Different zooxanthellae were found in different species of host, but taxonomically dissimilar hosts were also found to share closely related algae. Evolution between hosts and symbionts is not found related. This suggests that animal and algal lineages have maintained a flexible evolutionary relationship with each other (Rowan & Powers 1991a, 1991b). Furthermore different individuals of the same coral species harboured indistinguishable algae and some coral individuals harboured a mixture of zooxanthellae types while other corals exhibited a single type of symbiont (Rowan & Powers 1991a, Baker *et al.* 1997). Restriction Fragment Length Polymorphism (RFLP) analysis of two dominant Caribbean corals *Montastrea annularis and M.faveolata* (mountain corals) showed that each associated with three taxa of zooxanthellae that exhibited zonation with depth. Again some colonies apparently hosted multiple taxa of symbionts (Rowan & Knowlton 1995). Rowan named these three types of zooxanthellae A, B and C. Type A occurring at the highest light intensities, B at lower light intensities and C being most sensitive to light. These three zooxanthellae types can be regarded as three groups of related species of *Symbiodinium*. Sequence diversities within groups B and C imply that each group contains multiple species (Rowan & Powers 1991b).

Broad-scale distribution of zooxanthellae

Comparative studies on zooxanthellae diversity between scleractinian corals of the Caribbean and the Pacific Panama showed that corals of the Pacific Panama hosted only one of the three clades (type C) hosted by corals in the Caribbean (types A, B and C). Although the single clade found in the Pacific Panama is probably the most speciose of the three, it is surprising that symbiont diversity should be geographically restricted in this way. Particularly when non-scleractinian invertebrate hosts in the Pacific Panama can be found containing the A and B type of *Symbiodinium* (Baker & Rowan 1997).

The coral genus *Madracis*

Madracis is probably the second most abundant Caribbean coral next to *Montastrea*. *Madracis* is believed to be underestimated in it's importance because of the small size of the coral heads which make them easy to overlook. However the abundance of *Madracis* sp. is very high (observations Diekmann). *Madracis* is the current subject of two PhD projects. There are eight morphological *Madracis* species identified from which five can be found on Curaçao. These five morphospecies are: *Madracis mirabilis, M. pharensis, M. decactis, M. senaria* and *M. formosa* (descriptions by Wells 1973). Diekmann is using ribosomal DNA Internal Transcribed Spacer (ITS) sequences and morphometric trees in order to find out whether the five morphospecies of the Caribbean coral genus *Madracis* are genetically distinct. An investigation of the diversity of zooxanthellae in *Madracis* is a natural extension of Diekmann's project with the aim to find more about the life-history of *Madracis* and the symbiosis with its zooxanthellae. So far preliminary results show differences between coral sequences from *M. mirabilis, M. senaria, M.formosa* and *M.pharensis*. *M. decactis* is placed in the same clade as *M. pharensis* (Diekmann unpublished).

Given the similarity of abundance and distribution between *Montastrea* and *Madracis* we expect to see a similar distribution of zooxanthellae types related to depth as was found by Rowan and Knowlton (1995). In order to test this we investigated all five morphospecies over their range of depth at Buoy 1 Curaçao. The expected outcome is that more than one zooxanthellae type will be found in the coral genus *Madracis* and variation will be correlated with depth and perhaps with the different morphospecies of *Madracis*. RFLP analysis on the small subunit rDNA (ssRNA) (Rowan & Knowlton 1995: Rowan & Powers 1991a, 1991b) was used to distinguish different taxa of zooxanthellae within the coral genus *Madracis*. If the expected outcome is not found the prevailing generalisation of hosting multiple zooxanthellae in order to adapt to distinct photic habitats needs to be reviewed as that would imply other mechanisms facilitate adaptation.

Material & Methods

Sample collection and preservation

Samples of five different morphospecies of *Madracis* were collected over a depth range of 3-40m at one site (Buoy 1) on the southwestern coast of Curaçao, Dutch Antilles (Fig. 1). The morphospecies did not inhabit the whole depth range. *M. mirabilis* was found at 3-15m and *M. formosa* only below 30m. *M. decactis, M. senaria* and *M. pharensis* were collected along the complete depth range (Table 1). The coral samples were chopped off with a chisel and a stone or snapped off by hand. Several pieces of each colony of approx. 2-5 cm were collected. Samples were transported to the surface in labelled ziplock bags and preserved in 75% alcohol at 4°C.

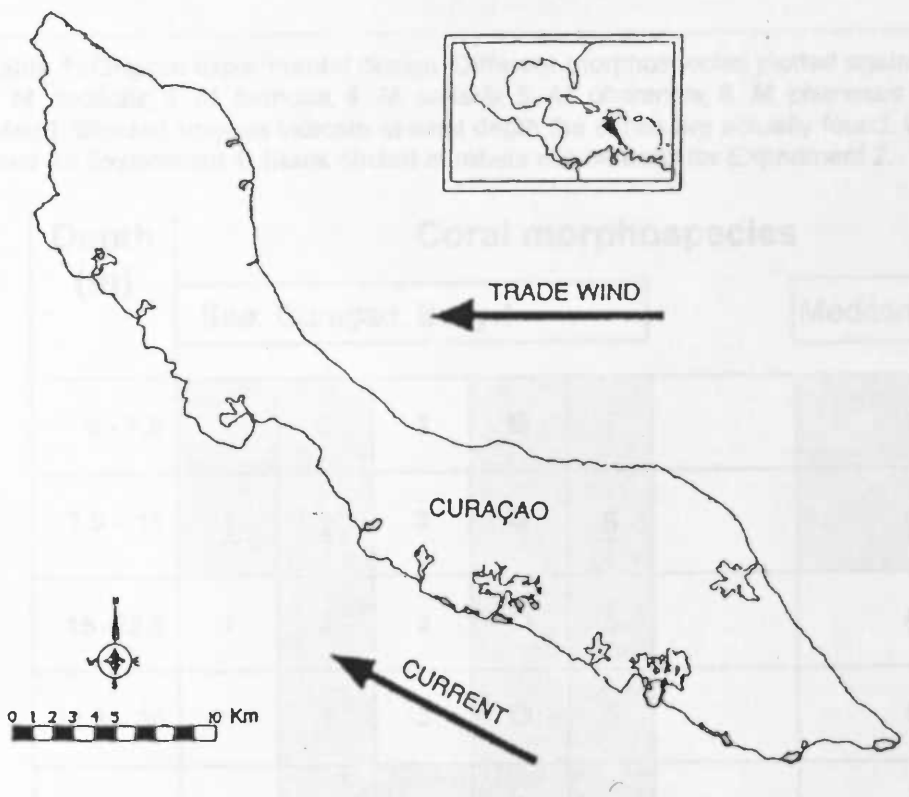


Figure 1: Map of Curaçao, sample site indicated with arrow.

Sampling design

Experiment 1: Samples of five *Madracis* morphospecies; *Madracis senaria, M. decactis, M. mirabilis, M. pharensis, M. formosa* were taken from one site (Buoy I) at Curaçao, Netherlands Antilles. The samples were taken as close to the same depth as possible in order to compare the occurrence of the zooxanthellae between the different morphospecies. This will address questions 1 and 2.
The Mediterranean *M. pharensis* (with zooxanthellae) has been used as an out-group in the *Madracis* phylogeny. Since we want to compare the zooxanthellae data with the *Madracis* phylogeny data, we will also include the Mediterranean species in the zooxanthellae investigation. Additionally the coral species *Stephanocoenia michilini* has been used as an outgroup.

Experiment 2: One morphospecies, *M. senaria* (Nr.4), is sampled at depths varying from 7-33m in order to examine variation in zooxanthellae within one morphospecies. This addresses question 3. If results show different other morphospecies will be examined at a depth range.

Table 1: Original experimental design. Different morphospecies plotted against depth: 1. *M. mirabilis*, 2. *M. decactis*, 3. *M. formosa*, 4. *M. senaria*, 5. *M. pharensis*, 6. *M. pharensis* from the Mediterranean (Med.). Shaded squares indicate at what depth the corals are actually found. Underlined numbers are used for Experiment 1. Black circled numbers will be used for Experiment 2.

Depth (m)	Coral morphospecies						
	Site: Curaçao, Buoy 1						Mediterranean
0 - 7.5	1	2	3	❹	5		6
7.5 – 15	1	2	3	❹	5		6
15 -22.5	1	2	3	❹	5		6
22.5 – 30	1	2	3	❹	5		6
30 ↓	1	2	3	❹	5		6

DNA Extraction

DNA extraction of the zooxanthellae was performed on the preserved coral-samples.

Method 1: The upper layer of the coral sample was scraped of and pulverised in 1 ml CTAB (consisted of 1.4M NaCl; 20mM EDTA; 100mM Tris-HCl pH=8; 2% CetylTrimethylAmmoniumBromide) and 18 µl Proteinase K (1%, 10 mg/ml) and mixed. The CTAB extraction buffer was heated to 50°C. Samples were incubated for 2-3 hours in a water bath at 50°C and shaken frequently. Followed by 30 minutes at 60°C. Extraction was done twice with one volume phenol (100 µl CIA (chloroform/isoamylalcohol 24:1) was added to prevent inversion of the water layer) and twice with one volume CIA. In order to precipitate, one volume isopropanol and 5% 4M NaAc were added. Precipitation time was at least 15 minutes. Samples were centrifuged in an eppendorf centrifuge at full speed (14.000 rpm) for 30 minutes. Subsequently the samples were washed two times with 1 ml 80 % ethanol and were vacuum dried in an execator for 20 minutes. In order to acquire the main stock the DNA was dissolved in 50 µl 0.1xTE and stored at -20°C.

Method 2: The upper layer of the coral samples was scraped off in liquid nitrogen. Small pieces were pulverised to powder with liquid nitrogen as a whole. The 1% SDS extraction buffer (sodium dodecyl sulfate, H_2O 1/100 pH = 7,2) was heated to 50°C and 900 µl DNA extraction buffer was added to the powder together with 3 µl β-mercapto. After mixing the samples were incubated for 2-3 hours in a water bath at 55°C and shaken frequently. Samples were incubated for 30 minutes at 60°C. Following steps according to method 1 from; "Extraction was done twice... and stored at -20°C." were followed accept for precipitation 10% 4M NaAc was added.

Method 3: Same as 1 only with SDS 1% as extraction buffer.

Gene Amplification

Sequence analysis of the ssRNA has proven to be a very useful method to distinguish different types of zooxanthellae (Rowan & Powers 1991a, b; Rowan & Knowlton 1995). The ribosomal cistron is a tandemly repeated unit that consists of conservative subunits and fast evolving Internal Transcribed Spacers (ITS) (Fig. 2). We are able to look at such a conservative part of the genome because dinoflagellates and corals are phylogenetically far apart.

Polymerase Chain Reaction (PCR) was used to amplify small subunit ribosomal RNA (ssRNA) from total nucleid acid samples. Zooxanthellae specific primers ss3Z and ss5Z (an equimolar mixture of two oligonucleotides) were used in combination with universal PCR-primers ss5 and ss3 according to Rowan and Knowlton (1995) and have been described in Rowan & Powers (1991a, 1992) (Fig. 3). Generally 1 µl DNA-template of the 10x diluted sample was used for gene amplification (no data was available on the quantity of DNA in the samples). Some samples acquired a different amount of template in order to optimise the amplification product. Initially 25 µl reactions (1 µl total nucleid acid) were done for experimenting and 100 µl reactions (4 µl total nucleid acid) were used to obtain enough PCR-product. Water and mix were added in a 1:1 proportion. Mix consisted of Mg-free Buffer (Promega) (2,5 µl Mg free buffer (after dilution) consists of: 10 mM Tris-HCl, 50 mM KCl and 0,1% Triton® x-100. Final concentration 1mM Tris-HCl, 5mM KCl), dNTP, $MgCl_2$, bidest, Taq DNA polymerase (Promega) and a combination of zooxanthellae-specific primers ss3Z and ss5Z and universal primers ss3 and ss5 (Table 3). The PCR-file (apparatus: Perkin Elmer Cetus) consisted of 34 cycles of the following thermal profile (file 36): time delay 4 min at 94°C; step cycle 1 min 94°C; 2 min 56°C; 3 min 30 at 72°C. Link to 1 cycle file 95: 1 min 94°C; 2 min 56°C; 8 min 72°C. Link to soakfile 11: 10°C.
PCR-products were purified with the GeneAmp PCR-kit according to the manufacturers' instructions.

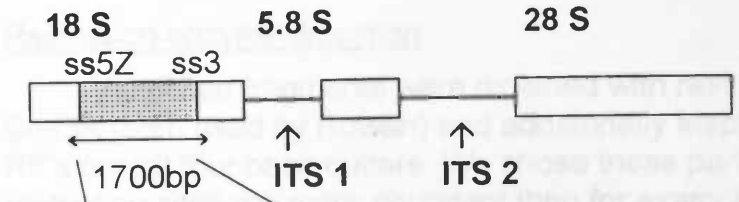
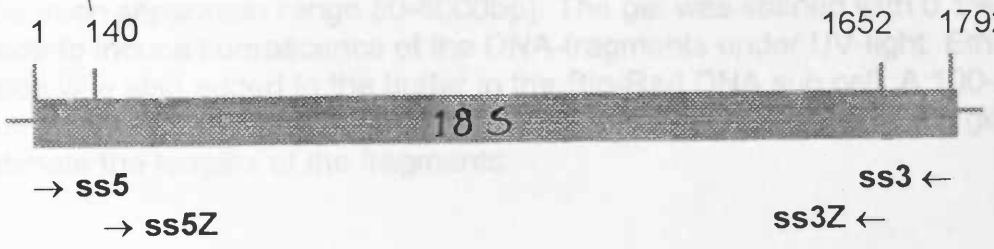


Figure 2: Schematic presentation of the nuclear ribosomal DNA cistron with small subunit (18S), the 5.8S, large subunit (28S) and ITS (internal transcribed spacer). The target fragment of interest marked by an universal (ss3) and a specific primer (ss5Z) is ca. 1700bp.



ss3: 5'-GATCCTTCCGCAGGTTCACCTACGG-AAACC-3'
ss5: 5'-GGTTGATCCT-GCCAGTAGTCATATGCTTG-3'

ss3Z: 5'-AGCACTGCGTCAGTCCGAATAATTCAC-CGG-3'
ss5Z: 5'-GCAGTTATAATTTATTTGATGGTCACTGCTAC-3' and
5'-GCAGTTATAGTTTATTT-GATGGTTGCTGCTAC-3'

Figure 3: Schematic diagram of the 18S RNA. Locations of the universal and zooxanthellae specific primers and design. Nucleotide positions are numbered according to *Symbiodinium* ssRNA sequences (Rowan & Powers 1992).

Table 3: Master mix for one 25 µl PCR-reaction.

Components	Concentration	Final concentration
5,2 µl bidest		
2,5 µl dNTP	8 mM	0,8 mM
2 µl $MgCl_2$	25 µM	2 mM
0,12 µl Taq	5 u/µl	0,6 units
0,25 µl primer 1 (ss3Z or ss3)	50 µM	0,5 µM
0,25 µl primer 2 (ss5ZA, s5ZB or ss5)	50 µM	0,5 µM

Restriction enzyme digestion

Amplified fragments were digested with restriction endonucleases (RE) Taq1, Sau3A (both used by Rowan) and additionally MspI, HAEIII, HinFI and CfoI. These RE's are all four base cutters. We chose these particular RE because four base restriction sites are more abundant then for example 6-base cutters. Amount of PCR-product used for digestion was 100ng.

The RFLP method (Restriction Fragment Length Polymorphism) was used for analysing the zooxanthellae sequence variation. The resulting fragments from the digestion were separated on length by electrophoresis in a Bio-Rad DNA sub-cell. Approximately 3 µl of orange G (loading dye) was added to the total volume of digest (20 µl) and pipetted into the wells. The agarose gel consisted of 2% RESponse Regular PCR-agarose (Biozym bv) and 1% RESult LE agarose (Biozym bv). RESponse is the replacement for NuSieve used by Rowan (former Biozym product and no longer available) and has the same qualities. The mix of RESponse and normal agarose was chosen, based on the high resolution capacities of RESponse (like Nusieve) which enables smaller fragments to be detected (RESponse/RESult combination separation range 50-8000bp). The gel was stained with 0.1% ethidium bromide to induce fluorescence of the DNA-fragments under UV-light. Ethidium bromide was also added to the buffer in the Bio-Rad DNA sub cell. A 100-basepair marker (marker 3, Pharmacia) was run along with the products on each gel in order to estimate the lengths of the fragments.

Table 4: Information on restriction enzymes

RE	Base-cutter	Buffer	Incubation temp.	Brand
TaqI	T↓CGA	One-Phor-All buffer	65°C	Pharmacia
MspI	C↓CGG	One-Phor-All buffer	37°C	Pharmacia
CfoI	GCG↓C	Sure/cutbuffer L	37°C	Boehringer-Mannheim
Sau3A	↓GATC	Incubation buffer A	37°C	Boehringer-Mannheim
HaeIII	GG↓CC	NEbuffer 2	37°C	Biolabs
HinFI	G↓ANTC	NEbuffer 2	37°C	Biolabs

DNA sequencing

Five PCR-products were cloned and sequenced (Table 5) in order to be certain that it was the zooxanthellae fragment we had amplified. These cloned products were also digested with restriction enzymes and analysed (Fig. 8). Exact fragment lengths were obtained from sequenced clones using the program DNA * MapDraw.

Table 5: Overview of cloned PCR-products and primer combinations used. The primers ss5 and ss3 are universal. The primers ss5Z, ss5Zb and ss3Z are zooxanthellae specific primers.

Sample	Primer 1	Primer 2
A55 (*M. mirabilis* from 2,2m)	ss5	ss3
A55	ss5Z	ss3
A55	ss5Z	ss3Z
B3 (*M. decactis* from 4,7m)	ss5ZB	ss3
B13 (*M. decactis* from 34,7m)	ss5	ss3

Results

No variation was detected among morphospecies, within morphospecies or with depth in all our digested fragments of the zooxanthellae of the *Madracis* morphospecies on Curaçao. No variation was found between deep and shallow coral samples and no variation between the five morphospecies (Fig. 4, 5 and 6). The *M. pharensis* of the Mediterranean (outgroup) and the *Stephonacoenia michilini* indicate different digestion patterns. The uncut fragment of sample B13 (*M. decactis* from 34,7m) was used as a control.

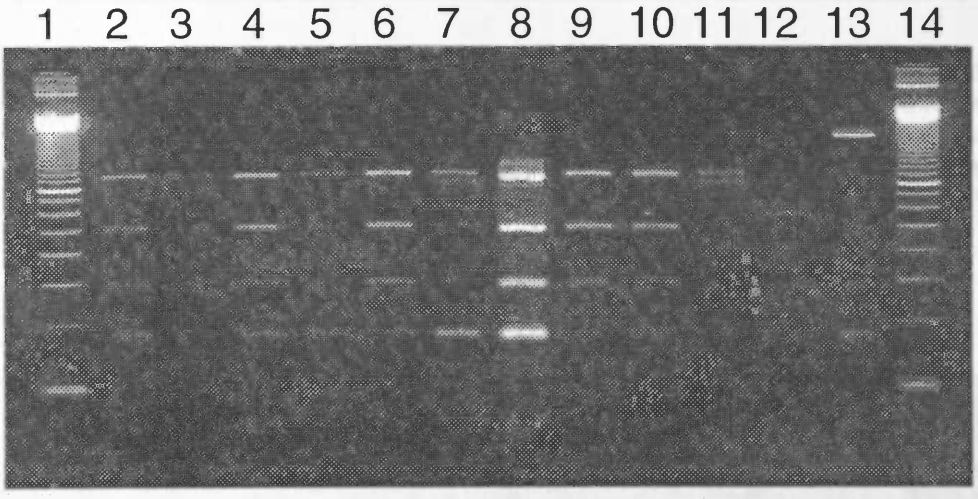


Figure 4: Restriction digests of 9 zooxanthellae samples of 5 *Madracis* morphospecies cut by restriction enzyme TaqI. Lane 1 and 14 represent markers (100 bp). Lane 13 represents the control (uncut fragment B13). Lane 11 and 12 are the outgroups respectively *M pharensis* from the Mediterranean and *S. michilini*. Lane 2 to 10 represent zooxanthellae RFLP genotypes of the 5 *Madracis* morphospecies of deep and shallow sites. Respectively: A55, A9, B3, B13, C25, C35, D3, D13 and E4 (Table 7).

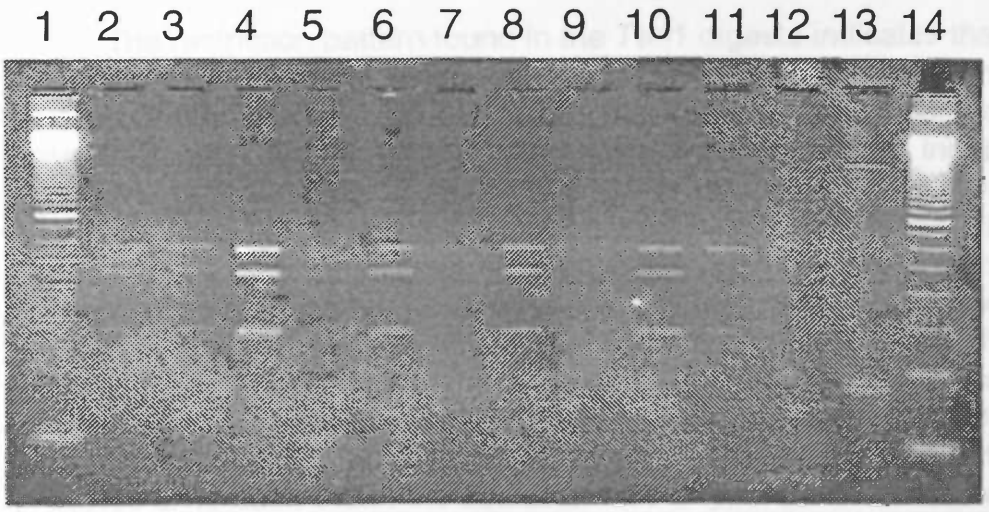


Figure 5: Restriction digests of 9 zooxanthellae samples of 5 *Madracis* morphospecies cut by restriction enzyme MspI. Lane 1 and 14 represent markers (100 bp). Lane 13 represents the control (uncut fragment B13). Lane 11 and 12 are the outgroups respectively *M pharensis* from the Mediterranean and *S. michilini*. Lane 2 to 10 represent zooxanthellae RFLP genotypes of the 5 *Madracis* morphospecies of deep and shallow sites. Respectively: A55, A9, B3, B13, C25, C35, D3, D13 and E4 (Table 7).

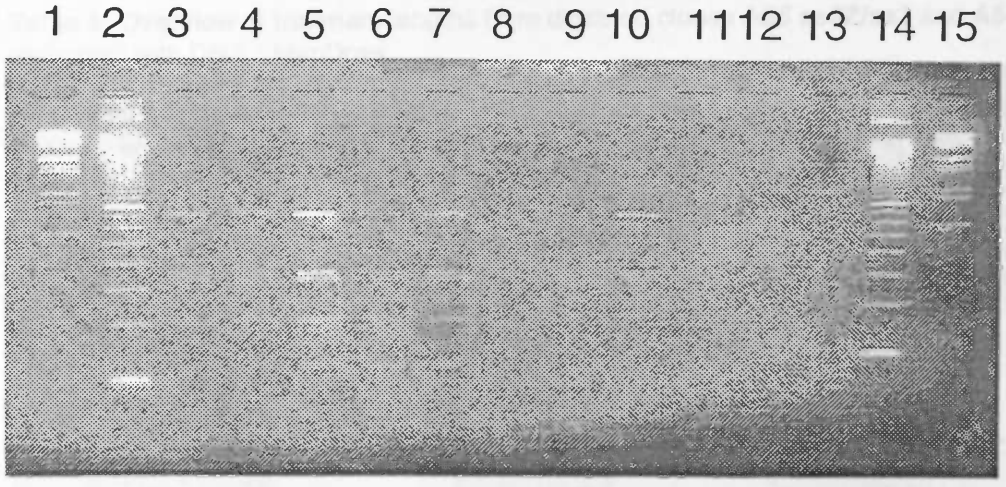


Figure 6: Restriction digests of 8 zooxanthellae samples of 5 *Madracis* morphospecies cut by restriction enzyme CfoI. Lane 2 and 14 represent markers (100 bp). Lane 1 and 15 Marker 3. Lane 13 represents the control (uncut fragment B13). Lane 11 and 12 are the outgroups respectively *M pharensis* from the Mediterranean and *S. michilini*. Lane 3 to 10 represent zooxanthellae RFLP genotypes of the 5 *Madracis* morphospecies of deep and shallow sites. Respectively: A55, A9, B3, B13, C35, D3, D13 and E4 (Table 7).

The restriction pattern found in the Taq1 digests indicates that the zooxanthellae type found in the coral genus *Madracis* coincides with the B-type found in *Montastrea annularis*. Exact fragment lengths are presented in Table 8. In order to compare the restriction pattern found in zooxanthellae inhabiting the coral genus *Madracis* with zooxanthellae types of *M. annularis* a phylogenetic tree (Fig. 7) was made based on zooxanthellae 18S partial sequences. This phylogenetic tree shows that the zooxanthellae type found in *Madracis* falls in the same group as the zooxanthellae type B from *Montastrea* but isn't exactly the same type B.

The phylogenetic tree shows that within one clade genetic differences occur but on a much smaller scale. These small variations within one zooxanthellae type were also found in one case of the digested *Madracis* clones (Fig. 8). Digested clone from PCR-product B3 (*M. decactis*) shows a divergent restriction pattern. This indicates a variation within the zooxanthellae B type. Baker and Rowan (1997) also found variations within zooxanthellae types A, B and C.

Table 8: Overview of fragment lengths from digested clones **A55 ss5Z/ss3** and **A55 ss5Z/ss3Z** estimated with DNA * MapDraw.

Fragment	Enzymes					
	TaqI	CfoI	MspI	Sau3A	HaeIII	HinFI
A55 ss5Z/ss3	892	685	568	760	945	689
	504	350	452	504	520	347
	302	332	282	128	176	204
		204	271	106	64	150
		117	128	102		124
		11		53		113
		8		23		71
				22		19
				11		
				4		
A55 ss5Z/ss3Z	859	688	568	759	942	690
	508	346	459	501	419	341
	167	329	272	110	174	124
	37	216	249	76	62	115
		13	27	55		113
		2		32		101
		2		20		69
				11		19
				8		

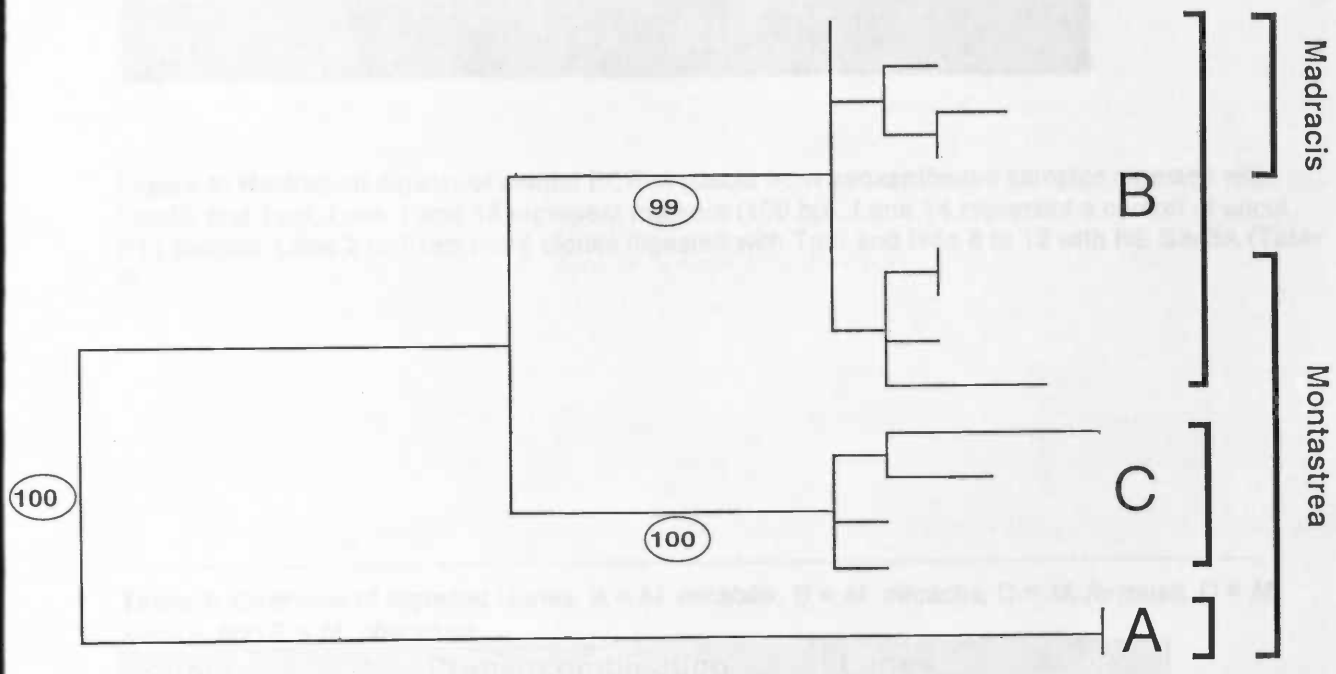


Figure 7: Unrooted phylogenetic tree of zooxanthellae based on partial 18S sequences (PAUP analysis). Bootstrap values are circled. A, B and C represent the three zooxanthellae types. The corals im which the zooxanthellae types are found (*Montastrea* sp. and *Madracis* sp.) are superimposed

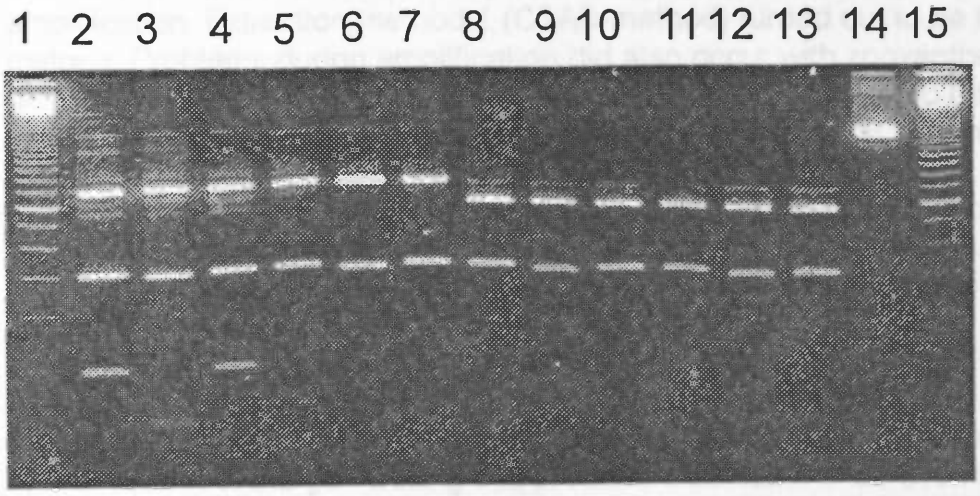


Figure 8: Restriction digests of cloned PCR-products from zooxanthellae samples digested with Sau3A and TaqI. Lane 1 and 15 represent markers (100 bp). Lane 14 represent a control of uncut B13 sample. Lane 2 to 7 represent clones digested with TaqI and lane 8 to 12 with RE Sau3A (Table 9).

Table 9: Overview of digested clones. A = *M. mirabilis*, B = *M. decactis*, C = *M. formosa*, D = *M. senaria* and E = *M. pharensis*.

Sample	Primer combination	Lanes
A55	ss5Z/ss3	2 and 8
B3	ss5Z/ss3	3 and 9
B13	ss5Z/ss3	4 and 10
A55	ss5/ss3	5 and 11
B3	ss5/ss3	6 and 12
B13	ss5/ss3	7 and 13

Technical problems associated to DNA extraction, PCR amplification and digestion

Problems arose both during the extraction of the DNA and during the amplification. Extraction method 1 (CTAB-method) turned out to be the most suitable method. Problems during amplification did also occur with zooxanthellae DNA extracted according to method 1. The electrophoresis showed no bands at all or the bands were to weak and not sufficient for RE-digestion. A list with modifications to the amplification method is presented in Table 6.

Primer-dimers of unusual brightness occurred at the bottom of the gel. All combinations of universal and zooxanthellae specific primers were used but only the ss3/ss5Z combination seemed to work. We also tried reamplifying but the quality of the PCR-product declined.

Additional bands with the length of 170bp appeared in most PCR-products and subsequently in restriction enzyme digests. These extra bands did not intervene with the restriction patterns. The additional fragments were lost after cutting the band of interest out of the gel. During this procedure zooxanthellae DNA was lost and the remainings were not sufficient for cutting with restriction-enzymes. These fragments remain unexplained and were also found by Rowan (Rowan & Powers 1991a)

Restriction enzymes Sau3A, HaeIII and HinFI did not completely digested the PCR-products and are therefore not included in the results. Restriction patterns of different morphospecies and over range of depth seemed to be alike (see appendices).

Table 6: Modifications applied in order to obtain better PCR-products

Modifications:
→ Different $MgCl_2$ concentrations in the master mix varying from 0,08 to 2 mM, best result 2,0 mM. → Variations in annealing temperature 50°C, 53°C, 55°C and 56°C were made. Samples showed varying preferences. → Different amounts of template (2, 4 an 6µl) and different dilution's (stock template, 10x and 100x diluted) were used for amplification. Samples showed varying preferences since concentration of extracted zoox. DNA varied between samples and was unknown. For example; sample D4 worked best at 2 µl template DNA and sample D14 with 2 µl. → Combinations of ss3, ss5, ss3Z and ss5Z were used → Adding Taq right before placing the samples in the PCR machine instead of adding Taq to the mix.

The original plan of comparing morphospecies over the entire depth range (40m) was not feasible due to no or insufficient PCR-yields. Samples that provided a sufficient PCR-product for RFLP analysis are listed in Table 7.

Table 7: Overview of samples used in the restriction analysis.

Morphospecies	Shallow samples (m)	Deep samples (m)
A = *M. mirabilis*	**A55** (2,2)	**A9** (19,8)
B = *M. decactis*	**B3** (4,7)	**B13** (34,7)
C = *M. formosa*		**C25/C35** (39,6)
D = *M. senaria*	**D3** (10,8)	**D13** (32,3)
E = *M. pharensis*	**E4** (7,0)	

Discussion

We did not find any variation between the RFLP patterns of the zooxanthellae inhabiting the coral genus *Madracis*. No genetic variation on the level of the three zooxanthellae types A, B and C distinguished by Rowan and Powers (1991a) was found over a range of depth from 3 to 40m and no variation was found between the five morphospecies of *Madracis* on Curaçao. The zooxanthellae restriction pattern found is comparable with that of type B observed by Rowan in *M.annularis* and *M. faveolata* but not exactly similar. Variations within the three clades A, B and C were also described by Rowan (1998).

Our hypothesis "finding zooxanthellae variation over a depth range and perhaps between the different morphospecies" was based on the research conducted on *Montastrea annularis* and *M. faveolata* by Rowan & Knowlton (1995). Rowan showed variation in zooxanthellae types within one species over a depth range and also found some colonies hosting multiple types of symbionts. He suggested that this common occurrence of polymorphic, habitat-specific symbioses challenged conventional understanding of the unit of biodiversity (Rowan & Knowlton 1995). Since we did not find any variation the prevailing generalisation about symbiotic environmental adaptations between corals and zooxanthellae needs to be re-evaluated as that would imply that other mechanisms facilitate adaptation. Important questions arise with our results. Why does *Madracis* have only one zooxanthellae type; did this genus lose the ability to contain multiple zooxanthellae types or did *Madracis* never had multiple zooxanthellae types? If *Madracis* lost the ability is it then a question of not needing multiple zooxanthellae and having other mechanisms to facilitate adaptations? What are the differences on an ecological and morphological level? Seeing that these different zooxanthellae types enable *Montastrea* to grow in ecological distinct habitats why is it that *Madracis* inhabits an even larger depth-range and thus with more extreme light conditions? Can we give a physiological explanation? The different aspects; evolution, ecology, morphology, physiology and geography will be discussed.

Zooxanthellae diversity in scleractinian corals of the Caribbean versus the Panama Pacific

In order to put the results on zooxanthellae diversity within the coral genus *Madracis* into a broad context we compared our results with research conducted on zooxanthellae diversity in general. Baker and Rowan (1997) surveyed 28 species of scleractinian corals from the Caribbean (Panama, Bahamas, US Virgin Islands) and 11 species from the Pacific Panama (Gulf of Panama, Gulf of Chiriqui, Panama) for their zooxanthellae diversity. From the total of 28 Caribbean coral species only 21% hosted multiple zooxanthellae types in contrary to 79% that hosted a single symbiont type. Clearly corals hosting one zooxanthellae type are more common then presumed and hosting multiple types is the exception of the rule. Rowans prevailing generalisation "corals harbouring multiple zooxanthellae types in order to survive different photic habitats" needs to be reviewed. Attention needs to be focused on corals harbouring one zooxanthellae type since little is known about the alternative mechanism that these corals have in order to facilitate adaptation.

The same survey conducted by Baker showed major differences in genetic

diversity between the symbionts of the scleractinian corals of the Pacific Panama and the Caribbean. Scleractinian corals of the Pacific Panama all show the same symbiont belonging to clade C in contrary to Caribbean corals who host *Symbiodinium* belonging to three clades A, B and C (Baker & Rowan 1997). This is extra surprising because other invertebrates inhabiting the Pacific Panama do host zooxanthellae from clade A and B (Rowan & Powers 1991,1992).

The closure of the Central American Seaway (the formation of the Isthmus of Panama) in the Pliocene (3,5 mya) may have influenced the contemporary distribution of scleractinian zooxanthellae (Johnson & Budd 1995). We know that late Triassic scleractinian corals inhabiting shallow-water carbonate complexes of the Tethys were predominantly zooxanthellate (Stanley & Swart 1994). We have no information of the identity of the types of zooxanthellae that inhabited those corals. Consequently we cannot draw conclusions about whether the Pacific Panama corals lost their capacity for containing multiple zooxanthellae types or that the Caribbean scleractinian corals developed this characteristic separately.

The formation of the Isthmus of Panama gave rise to distinct ecological differences between the Eastern Pacific and the Caribbean, what once was one continuous area. Perhaps it were these ecological differences that induced a different evolutionary scenario for the corals and their zooxanthellae in the Caribbean and thereby zooxanthellae type A and B evolved after the closure of the Isthmus. An ecological difference is the higher turbidity and lower light irradiance on the eastern Pacific reefs in relation to the Caribbean reefs (Dana 1975). This could explain the differences in zooxanthellae evolution for they are closely correlated to available light in order to photosynthesise. However the occurrence of one symbiont type C in the scleractinian corals is not consistent in the other invertebrate hosts of the Eastern Pacific and this makes it even more complicated and interesting. Perhaps the scleractinian corals of the Eastern Pacific have no need for other zooxanthellae types then C in the same way the coral genus *Madracis* is specific for type B. Maybe the answer to this loyalty to one single symbiont lies in the complex physiology of the corals and the interaction processes with the zooxanthellae.

Another possible explanation could be the long-distance immigration of coral species from the Indo-Western Pacific. During the Pleistocene (1.5 my) all Eastern Pacific hermatypic corals were lost. The shallow -water hermatypic corals found in the Eastern Pacific today have their closest affinities with Indo-Pacific corals (Dana 1975). Since the Indo-Pacific is considered the centre of evolutionary radiation (Briggs 1966) it could be that these ancient corals with their long evolutionary history lost zooxanthellae type A and B and exhibit a much stronger relationship between host and zooxanthellae type.

Evolutionary aspects

Scleractinians first became prominent in the shallow epicontinental seas of the Mesozoic Tethys. These late Triassic (± 210 mya) scleractinian corals were predominantly zooxanthellate, like their living counterparts from present day reefs (Stanley & Swart 1994). It was in these shallow-water carbonate complexes of the Tethys where they rapidly evolved into an essentially pantropical fauna (distributed throughout the tropics) in which most modern families were present by the mid-Tertiary (30 mya). In the later Tertiary, the original fauna differentiated into the two

isolated faunas of the tropical Atlantic and Indo-Pacific regions that are now completely distinct at the species level (Potts 1983).

The genus *Madracis* is a member of the scleractinian family Pocilloporidae. *Madracis* is restricted to the Caribbean, the western Pacific and the Indian ocean. The exceptions are small populations of *Madracis* in the Galapagos (azooxanthellate) and the Mediterranean (with zooxanthellae). Specimens of *Madracis* from the Miocene and the Pliocene bear little resemblance to the fauna present in the Caribbean during the Quaternary (Swedberg 1994). This change is probably related to large-scale faunal turnover due to acceleration in rates of extinction and origination during the late Pliocene (between 1 and 4 mya). The turnover episode appears to be related to long-term changes in climatic and oceanic circulation patterns across the Caribbean resulting from the closure of the Isthmus of Panama at about 3.5 mya (Johnson & Budd 1995). Colony size has been the most important ecological characteristic determining extinction rate. Over the past 22 m.y. species with large colonies were less likely to become extinct than species with small or intermediate sized colonies. Corals with small massive colonies were most vulnerable to extinction (Johnson & Budd 1995).

Montastrea annularis is a classic example of a large colonial species with low extinction rates (Johnson & Budd, 1995). According to Johnson and Budd's reasoning the small colonies formed by *Madracis* species deal with high extinction rates. Perhaps the *Madracis* species complex originated more recently and has undergone a shorter period of evolution than *Montastrea*, which is a large long-lived species (Johnson & Budd, 1995). Maybe the difference in life-history strategy between *Montastrea* and *Madracis* reflects the different role of zooxanthellae in their life-history strategies through time. One should be careful to generalise the beneficial effects of hosting multiple zooxanthellae types for all corals. Ecological characteristics often show trade-offs and hosting multiple zooxanthellae might be just one of the possibilities for a species to adapt to different photic habitats. It must be taken into account that *Madracis* specimens before the Quaternary could have been morphological distinct (Swedberg 1994) and are therefore not comparable to small colonies today.

Fossil data on Neogene and recent occurrences imply that *Madracis decactis* and *M. formosa* are older than *Montastrea annularis* (Budd *et al.* 1992, 1994). Having a longer evolutionary history, the relationship between host and zooxanthellae type might be stronger. *Madracis* could have chosen the best zooxanthellae type out of three. This theory is supported by *Montastrea cavernosa* that is also older than *M. annularis* and harbours only one zooxanthellae type (Billinghurst *et al.* 1997). In order to seriously consider this theory the phylogeny of *Madracis* and *Montastrea* species needs to be researched more extensively.

Ecological aspects

Rowan suggests symbiont zonation by depth strongly supports the theory that hosting different types of zooxanthellae permits corals to acclimate or adapt to different photic habitats (Rowan & Knowlton 1994). The depth range examined for *Montastrea* is 1 to 14 meters. We did not find any variation in zooxanthellae for *Madracis* over a range of 1 to 40 meters. Meaning *Madracis* adapted to an even

larger depth range of 40 meters with more extreme photic habitats without the assistance of multiple zooxanthellae. What mechanism does *Madracis* facilitates in order to survive these extreme habitats? In order to come up with alternative mechanisms we need to look into the physiological, morphological and biochemical characteristics of both the corals and the zooxanthellae.

Rowan also suggests that bleaching could promote adaptive changes in coral-zooxanthellae associations (Rowan & Knowlton 1994). Bleaching involves either the mass expulsion of zooxanthellae or the loss of photosynthetic pigments within individual zooxanthellae. Bleaching often occurs following periods of elevated seawater temperatures (Lesser 1997). Having more than one zooxanthellae type could diminish the severity of bleaching. During a bleaching event only type C was seen expelled from a coral colony hosting a mixture of zooxanthellae types while the other type was able to withstand the stressful conditions (Rowan *et al.* 1997). In relation to *Madracis* not having multiple zooxanthellae *Madracis* was also never reported bleached. Even during the peak of mass bleaching events in 1995 on Bonaire a *Madracis* colony was found unbleached surrounded by several bleached colonies of different coral species (observations Diekmann). A possible explanation for the occurrence of only one zooxanthellae type in *Madracis* could be the lack of bleaching which may provide the potential for establishment of new zooxanthellae populations in corals (Buddemeier & Fautin 1993). Very speculative but interesting is the idea: If *Madracis* isn't subjective to bleaching in the extent other (bigger) corals are what mechanisms does *Madracis* then have to make it so successful? Has *Madracis* indeed chosen the best zooxanthellae type out of three? Or is it the coral physiology that allows *Madracis* to persist during stressful conditions? Seeing they live in symbiosis it could very well be a combination of host and symbiont characteristics.
-Note: the lack of reported bleached *Madracis* colonies may also be explained by the preference for the more obvious larger corals that are bleached.

Morphological aspects

Comparing morphological conditions of *Madracis* and *Montastrea* coral colonies shows enormous differences in growth form. *Montastrea* colonies being massive mountain-shaped in contrary to sampled *Madracis* colonies that are small and "cauliflower" shaped, encrusted or branching irregular forms. The massive *Montastrea* form exhibits distinct shade and sun-sides allowing different photic habitats within one coral colony. *Montastrea* seemed to have adapted to these varying light intensities by harbouring multiple zooxanthellae types. Within one *Madracis* colony no distinct sun or shade sides comparable with *M. annularis* are present due to the coral form and the occurrence of only one zooxanthellae type is thus logical. It probably doesn't need more than one type because of the heterogeneous exposure of light. Large *Madracis* colonies with comparable sizes to *Montastrea* have been sighted on the northern coast of Curaçao (observations Diekmann). Environmental conditions vary between the northern and southern coast. The northern coast being highly exposed to wave action and strong currents. Smaller *Madracis* colonies are probably too fragile to survive these extreme conditions.

Physiological aspects

Different corals exhibit a wide range of life-history strategies. New colonies can arise through both sexual and asexual reproduction. Reproduction strategies are also variable: oviparous species broadcast sperm and eggs directly into the water column, while viviparous species brood their larvae internally (Johnson, Budd & Stemann 1994). The life history of *Madracis* remains obscure and is presently under investigation. Preliminary observations suggest that *Madracis* releases its fertilised eggs in the environment which take approximately two days to develop into larvae which settle, a variation on a classic brooding species (Diekmann personal comment).

Zooxanthellae can be acquired directly from the parent or from the surrounding seawater (Muller-Parker & D'Elia 1997). Observations on *Madracis* morphospecies on Curaçao showed white larvae with a dark band possibly consistent of zooxanthellae. This band subsequently spread through the larvae (Vermeij personal comment). This could imply that the symbionts are inherited from the parents. Finding only one zooxanthellae type could strengthen this supposition since recruitment of symbionts from the environment induces the possibility of ending up with different zooxanthellae types. No published data exists dealing with amounts or diversity of free living symbionts in the ambient seawater. Corals might have a recognition mechanism to select only one zooxanthellae type as their own. Other types would subsequently be denied. Comparable with the immunological concept of self-nonself recognition (Trench 1997). If *Madracis* developed such a mechanism it would strengthen the supposition that *Madracis* shows specificity for one algal type.

Iglesias-Prieto and Trench (1994) analysed three species of *Symbiodinium* and found that the symbionts effectively modified their photosynthetic machinery in respond to changes in photon flux density. Subsequently the different species of *Symbiodinium* respond differently to equal variations in light. This suggests the role of photo-adaptation as an important part of niche diversification (Iglesias-Prieto & Trench 1994). This mechanism is very likely to be the driving force behind the evolution of the A, B and C zooxanthellae types (Rowan & Knowlton 1994). If the algae exhibit different adaptations to the photic environment this may also limit their distribution and that of their hosts. An alternative mechanism of the corals other than hosting multiple zooxanthellae types is harbouring one type of zooxanthellae that is able to survive under different light intensities (generalist).

Individual zooxanthellae of the same type change their photosynthetic systems, including the light harvesting ability of photosynthetic units (amounts of pigment) and the rate of carbon fixation (enzymatic adaptations) in order to acclimate to different light intensities. Zooxanthellae in corals from shaded habitats usually contain more chlorophyll and thus are more efficient at light capture; the size of their light-harvesting units is large. Zooxanthellae in corals in high light environments contain less photosynthetic pigments, in smaller light-harvesting units, but have high rates of carbon fixation by containing more photosynthetic units. Light intensity also varies on a daily and seasonal basis, and zooxanthellae are likely to acclimate to these changes (Muller-Parker & D'Elia 1997). Perhaps the acclimatisation's by individual zooxanthellae are sufficient for the coral species of *Madracis* to enable them to survive the depth range of 70m.

No information is currently available about the position of the zooxanthellae in

the coral hosts of the genus *Madracis*. The general idea is that zooxanthellae are situated in the gastrodermis (sometimes referred to as endodermis). The position of the symbiont in the host could very well be of importance since zooxanthellae that are situated near the surface will be more exposed to irradiance. Symbionts situated deeper in the cellular layers of the coral are protected from harmful ultraviolet light by UV-absorbing pigments and bacteria in the host cells and in the mucus layer (Muller-Parker & D'Elia 1997). Hosts that harbour their symbiont close to the surface might therefore need additional mechanisms like multiple zooxanthellae types.

Conclusions

Only one zooxanthellae type was found within the coral genus *Madracis*. No variation was found between the different morphospecies or over a range of depth. The zooxanthellae restriction pattern found is comparable with that of type B found by Rowan in *M.annularis* and *M. faveolata* but not exactly similar. In the discussion evidence is provided to substantiate that harbouring multiple zooxanthellae types as an environmental adaptive mechanism used by corals unjustly became the general view. The more widespread mechanism used by scleractinian corals is hosting a single symbiont type. Our results suggest that the "*Montastrea* model" in which multiple zooxanthellae types are distributed with depth is not general but probably the exception. Until a broader survey of corals is undertaken the photoadaptive advantage conferred should not be generalised.

Suggestions for future research

Sample size

Due to the amplification problems sample size remained low. Finding no variation between the 10 coral samples requires extensive sampling since it is easily discarded by the idea that finding no variation doesn't mean that there is no variation. It is therefore advisable to extend our sample size in order to strengthen the results.

Different DNA region

The variation we seek is on the level of the clades A, B and C distinguished by Rowan and Powers (1991a). RFLP analysis of zooxanthellae rDNA will not always differentiate closely related taxa within the A, B and C clades because it distinguishes between taxa based on their similarity at only a limited number of restriction sites (Rowan & Powers 1991a). No variation within the coral genus *Madracis* was found in the small subunit rDNA. The more variable large subunit should allow the identification of more closely related *Symbiodinium* species within the B clade. It would be very interesting to see if smaller variations can be found within the B type and if these differences possibly correlate with depth.

Amplification problems

Problems that occurred during amplification could have been caused by our preservation method. We preserved the coral samples on 75% alcohol whereas Rowan used fresh material preserved on dry ice at -70°C (Rowan & Knowlton 1995). Perhaps zooxanthellae DNA concentration in relation to coral DNA concentration obtained from non-fresh material by scraping of the coral tissue is too low and disturbs amplification. For further research corals could be rinsed off and zooxanthellae could be collected from fresh material directly out of the sea in order to obtain higher zooxanthellae concentrations.

Evolutionary aspects

In order to seriously consider the theory that *Madracis decactis* and *M. formosa* are older than *Montastrea annularis* (Budd *et al.* 1992, 1994) and therefore have a stronger host-zooxanthellae relationship the phylogeny of *Madracis* and *Montastrea* using fossil data, needs to be researched more extensively.

Ecological aspects

In vitro experiments with *Madracis* in combination with other coral species kept under equal regulated stress factors could be done in order to test whether *Madracis* has a higher resistance to environmental stress than other coral species.

Morphological aspects

No investigations on the genetic variation of zooxanthellae within the large *Madracis* colonies situated on the northern coast of Curaçao were done. If these giant coral colonies show no variation it would strengthen the supposition that *Madracis* contains other mechanisms then the arrangement of different zooxanthellae types to facilitate environmental adaptation.

Physiological aspects

Experiments in which *Madracis* larvae and older colonies are only exposed to zooxanthellae types A and C, might give some information about the acquirement of zooxanthellae by the species *Madracis*. This could also be done in combination with stressful conditions inducing the coral to bleach and subsequently provide only symbionts other than B.

To gain insight on the relation between the positioning and the diversity of the zooxanthellae comparative histology analysis of *Madracis* and *Montastrea* might be a useful method.

Geographical aspects

Since samples were only taken at one site (Buoy 1 on the south-western coast of Curaçao) a possible explanation for finding only one zooxanthellae type within the coral genus *Madracis* is a location specific phenomenon. To establish whether these findings obtained for Curaçao are general further RFLP studies of *Madracis* sp. are needed from other sites. For instance from Bonaire because coral samples are already available from this site.

Acknowledgements

I would like to thank my supervisors Onno Diekmann, Jeanine Olsen and Wytze Stam for their guidance. Especially Onno for the enormous amount of time and energy spent and Jeanine for all the suggestions in order to improve my seminar and report, which were very educational and helpful. Furthermore I would like to thank Stella Boele-Bos for her help with the practical work and Mark Vermeij for his field information about the *Madracis* larvae.

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Appendix

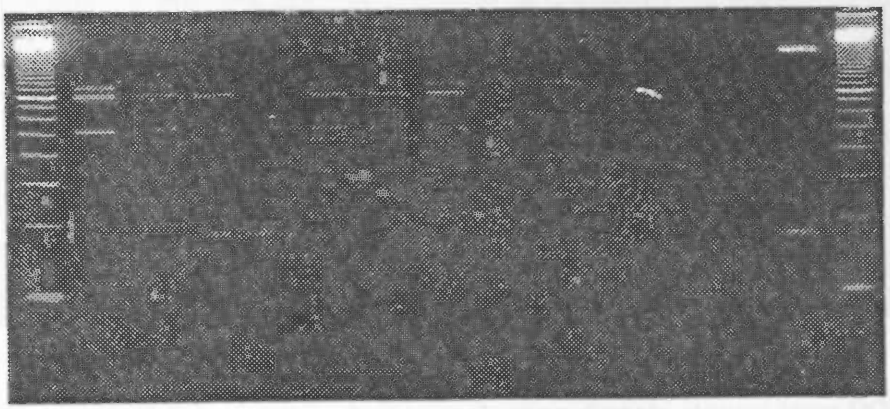

Figure 9: Ten zooxanthellae restriction digests obtained from the 5 *Madracis* morphospecies (same samples used in figure 4, 5 and 6) digested with RE Sau3A.

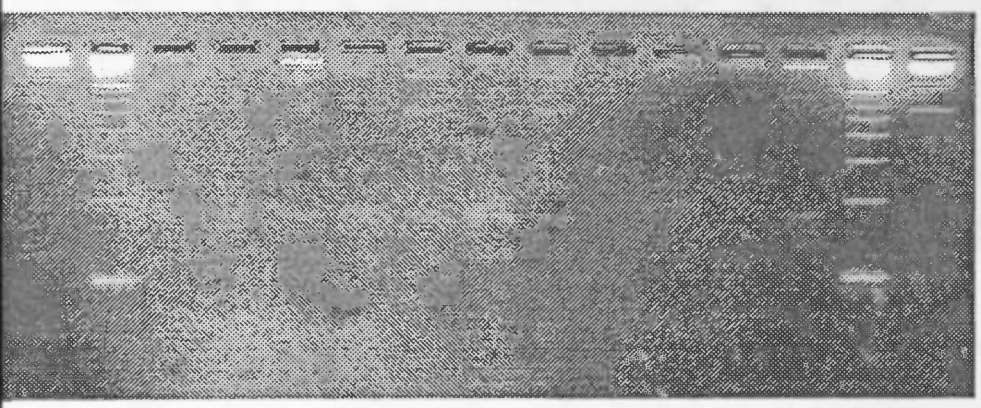

Figure 10: Ten zooxanthellae restriction digests obtained from the 5 *Madracis* morphospecies (same samples used in figure 4, 5 and 6) digested with RE HaeIII.

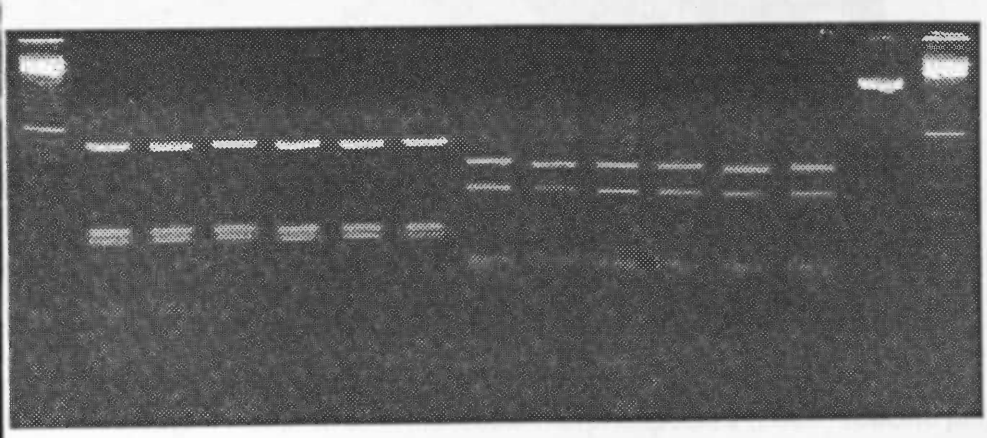

Figure 11: Twelve zooxanthellae restriction digests cloned from PCR-products from 5 *Madracis* morphospecies (same samples used in figure 8) digested with RE CfoI (lane 2 to 7) and MspI (lane 8 to 13).

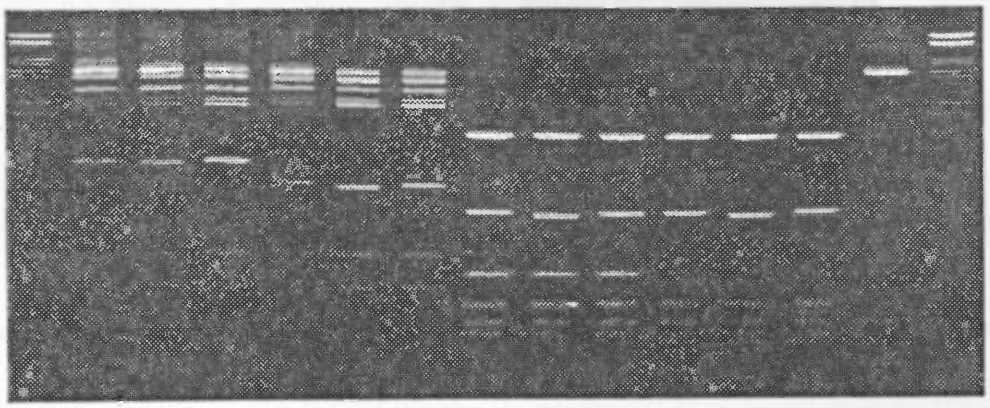

Figure 12: Twelve zooxanthellae restriction digests cloned from PCR-products from 5 *Madracis* morphospecies (same samples used in figure 8) digested with RE HaeIII (lane 2 to 7) and HinFI (lane 8 to 13).

Figure 13: Eight samples of 5 *Madracis* morphospecies (zoox. specific primers used) digested with Sau3A.

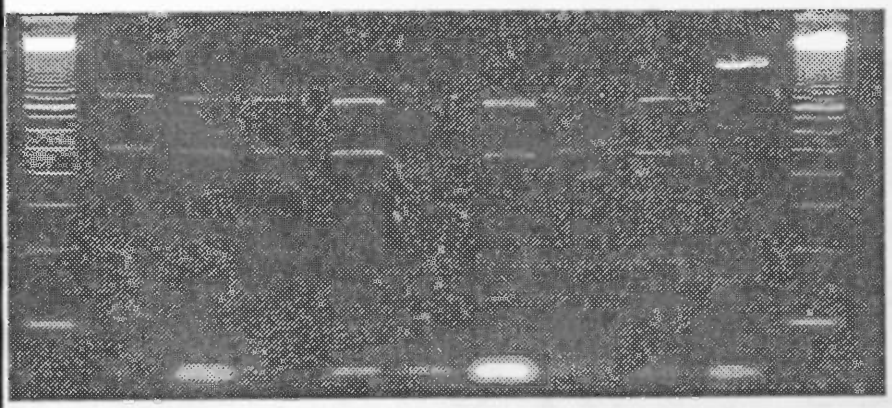

Figure 14: Eight samples of 5 *Madracis* morphospecies (zoox. specific primers used) digested with MspI.

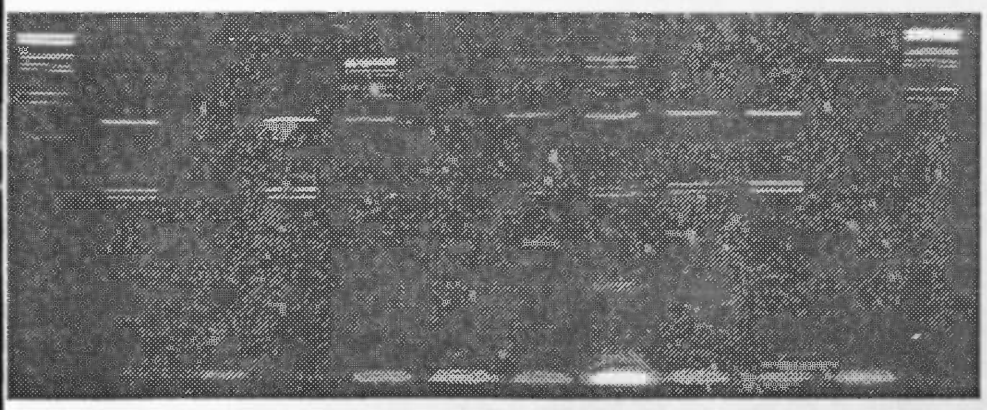

Figure 15: Eight samples of 5 *Madracis* morphospecies (zoox. specific primers used) digested with CfoI.

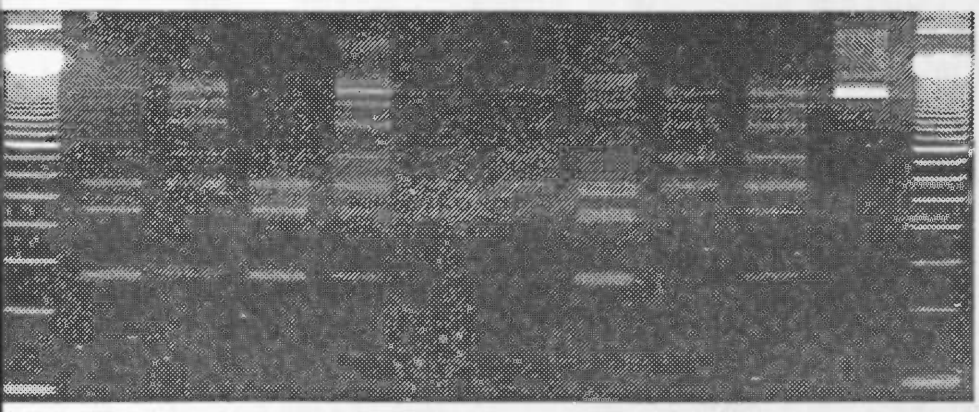

Figure 16: Eight samples of 5 *Madracis* morphospecies (zoox. specific primers used) digested with TaqI.

Figure 17: Eight samples of 5 *Madracis* morphospecies (zoox. specific primers used) digested with HaeIII.

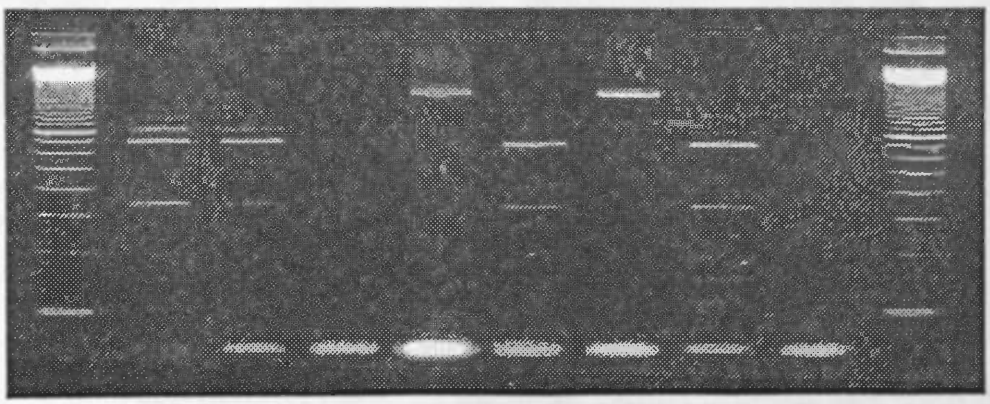

Figure 18: Eight samples of 5 *Madracis* morphospecies (zoox. specific primers used) digested with HinFI.