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Identity and diversity of coral endosymbionts (zooxanthellae) from three Palauan reefs with contrasting bleaching, temperature and shading histories

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Abstract

The potential of corals to associate with more temperature-tolerant strains of algae (zooxanthellae, *Symbiodinium*) can have important implications for the future of coral reefs in an era of global climate change. In this study, the genetic identity and diversity of zooxanthellae was investigated at three reefs with contrasting histories of bleaching mortality, water temperature and shading, in the Republic of Palau (Micronesia). Single-stranded conformation polymorphism and sequence analysis of the ribosomal DNA internal transcribed spacer (ITS)1 region was used for genotyping. A chronically warm but partly shaded coral reef in a marine lake that is hydrographically well connected to the surrounding waters harboured only two single-stranded conformation polymorphism profiles (i.e. zooxanthella communities). It consisted only of *Symbiodinium* D in all 13 nonporitid species and two *Porites* species investigated, with the remaining five *Porites* harbouring C•. Despite the high temperature in this lake (> 0.5° above ambient), this reef did not suffer coral mortality during the (1998) bleaching event, however, no bleaching-sensitive coral families and genera occur in the coral community. This setting contrasts strongly with two other reefs with generally lower temperatures, in which 10 and 12 zooxanthella communities with moderate to low proportions of clade D zooxanthellae were found. The data indicate that whole coral assemblages, when growing in elevated seawater temperatures and at reduced irradiance, can be composed of colonies associated with the more thermo-tolerant clade D zooxanthellae. Future increases in seawater temperature might, therefore, result in an increasing prevalence of *Symbiodinium* phylotype D in scleractinian corals, possibly associated with a loss of diversity in both zooxanthellae and corals.

Keywords: adaptation, coral bleaching, Scleractinia, *Symbiodinium* clades, thermo-tolerance, zooxanthella

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Introduction

Mass coral-bleaching events have greatly increased in frequency and intensity over the past 30 years (Wellington *et al*. 2001). In 1998, bleaching affected coral reefs in over 50 countries (Wilkinson 2000), with 70–99% mortality recorded at some sites in the Seychelles, Maldives and Arabian Gulf (Goreau *et al*. 2000), and 30–50% of corals lost in the Micronesian Republic of Palau (Bruno *et al*. 2001). The bleaching of corals and other tropical invertebrates containing symbiotic algae (zooxanthellae) is often observed when sea surface temperatures rise 1–2 °C above the long-term summer maximum and when sea conditions are unusually calm. Temperatures exceeding the upper tolerance limits of the zooxanthella–host complex induce physiological stress, damage photosystem II (Lesser 1996; Jones *et al*. 1998), and disrupt the fine-tuned balance between coral and algae (Brown 1997). As a consequence, the pigmented zooxanthellae are expelled from the host coral and the corals pale or whiten, the phenomenon of 'bleaching'. In bleached corals, zooxanthella densities are reduced by 60–90% and chlorophyll concentrations in the remaining zooxanthellae may be 50–80% lower (Porter *et al*. 1989; Glynn 1996) resulting

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in reduced photosynthesis, the arrest of skeletal growth (Tudhope *et al*. 1992) and loss of tissue energy reserves (Szmant & Gassman 1990; Fabricius 1999). When stress-inducing conditions are severe (e.g. temperatures > 2 °C above the local long-term summer maximum) or prolonged (e.g. 1 °C temperature elevation for several weeks) many colonies of bleaching-sensitive species will die (Glynn 1996; Loya *et al*. 2001). Surviving colonies of more resilient species are eventually repopulated by zooxanthellae and skeletogenesis recommences within a year, however, reproduction may continue to fail for up to three consecutive years until energy storages are replenished (Michalek-Wagner & Willis 2000).

The link between coral bleaching and seawater temperature is well established (Glynn 1993; Goreau & Hayes 1994; Brown 1997; Hoegh-Guldberg 1999); however, high irradiance and low currents further enhance thermal stress (Lesser *et al*. 1990; Fitt & Warner 1995; Glynn 1996; Brown 1997; Nakamura & van Woesik 2001). Coral species differ widely in their bleaching susceptibility (Marshall & Baird 2000; Loya *et al*. 2001) and thermal tolerances can vary even within colonies as a function of prior exposure to temperature and irradiance (Dunne & Brown 2001; Brown *et al*. 2002). Furthermore, an increasing amount of evidence exists that the genetic identity of zooxanthellae also plays a key role in the corals' resistance and resilience to bleaching (Rowan *et al*. 1997; Glynn *et al*. 2001; Toller *et al*. 2001; Bhagooli & Hidaka 2003; Baker *et al*. 2004).

We used unique geophysical settings in coral reefs of Palau to investigate the genetic identity and diversity of coral-associated zooxanthellae on a coral reef that is thriving at temperatures that are chronically elevated by > 0.5 °C, commonly exceeding bleaching thresholds in the surrounding waters. Despite the warm waters, no substantial bleaching mortality is recorded from corals on this reef that are shaded for parts of the day by steep reef walls. We compared the coral–zooxanthellae associations and coral communities of this warm and partly shaded reef with those on two nearby reefs: both have generally cooler temperatures, but one is also shaded and did not experience bleaching in 1998, whereas the other is exposed to high irradiance and suffered ~90% coral mortality in the (1998) mass bleaching event. These *in situ* data complement previous data on environmentally induced changes in the zooxanthella–coral associations that were predominantly based on experimental short-term exposures of corals or anemones to bleaching-inducing levels of irradiance or temperature (Baker 2001; Kinzie *et al*. 2001; Toller *et al*. 2001; but see also Hoegh-Guldberg *et al*. 2002). They may contribute to understanding the potential of coral reef systems to cope with warming conditions — knowledge that is critical for predicting the medium-term future of coral reefs while sea surface temperatures continue to rise (Hoegh-Guldberg 1999; West & Salm 2003).

Materials and methods

Research location and field methods

The reefs of the main Palau Island group (Republic of Palau) experienced sea surface temperatures of 30–31 °C, 1.0–1.25° above the long-term summer maximum for about 16–18 weeks in the summer of 1998 (Bruno *et al*. 2001). Although strongly habitat- and species-specific, an overall total 30–50% of coral colonies in Palau bleached, and due to the length of the bleaching event the mortality of bleached corals was near total. Some habitats, such as outer reef slopes with a preponderance of bleaching-prone species, had mortalities of over 90% for all corals (PL Colin, personal observation). In contrast, many of the fringing reefs of the Rock Islands, which are partially shaded by their steep reef slopes and overhanging island vegetation, experienced less bleaching and much lower mortality. We investigated the genetic identity and diversity of zooxanthellae in corals from three reefs of the Rock Islands, which are characterized by contrasting temperature and irradiance environments, and contrasting bleaching histories during the (1998) bleaching event (Fig. 1). The reefs are hydrographically well connected, and characterized as follows:

1 Oikull Reef, a platform reef at the eastern edge of the Rock Islands (7°19.65′ N, 134°33.34′ E). No data from pre-1998 were available from this particular site, however, observational data exist from similar sites in the proximity where few corals survived the severe 1998 mass bleaching event. We therefore assumed that the low live coral cover at Oikull (4%) and the high number of dead standing coral colonies was due to bleaching mortality. The reef is surrounded by open water, at > 500 m distance from the next island, and slope angles are generally < 20°. Corals experience high irradiance and sea-surface temperatures that reflect those of the large oceanic water body.
2 Taoch Bay Fringing Reef, along the western side of one of the larger karst islands (7°16.59′ N, 134°25.63′ E). The reef has steep walls (> 60° slopes) and is located at the inner end of a semi-enclosed bay surrounded by steep karst island walls that shade the corals until late morning. Although temperature stratification may occur locally on calm days, there are no major physical barriers that restrict water exchange with the larger oceanic water body. Hence, this reef generally experiences a lower total diurnal photon flux, and a sea-surface temperature that traces closely that of the oceanic water body. There are no records or signs of damage (i.e. low abundance of dead corals) from the (1998) bleaching event.
3 A previously unnamed karst lake (7°15.09′ N, 134°22.54′ E, hereafter referred to as '*Heliofungia* Lake' because of the predominance of the coral *Heliofungia actiniformis* below 10 m depth in the lake) that contains a coral reef with

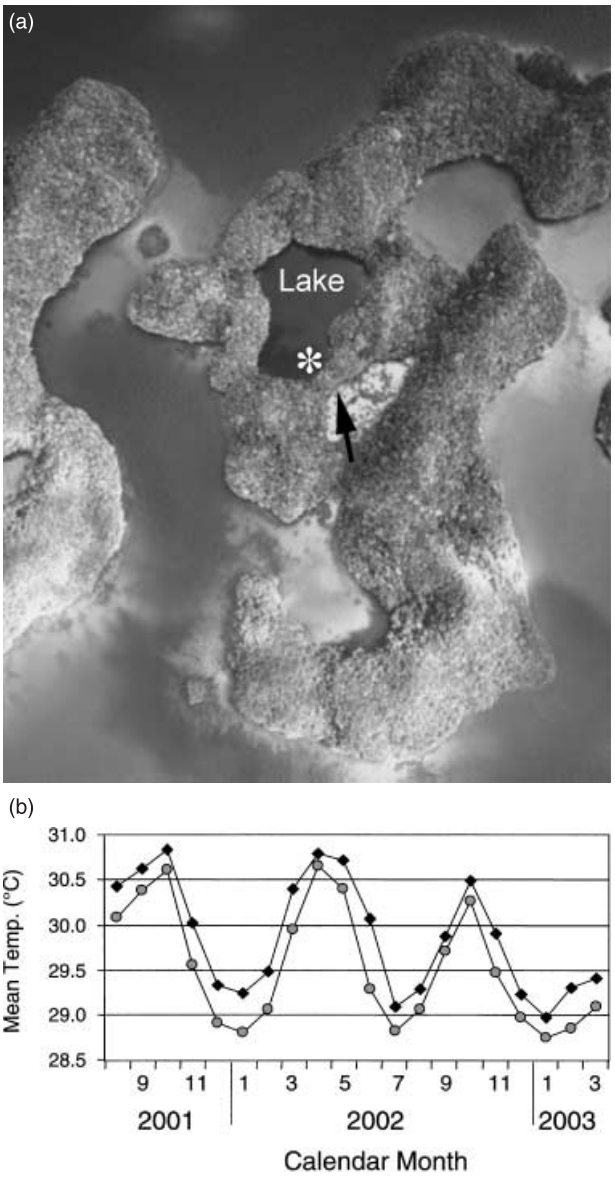

Fig. 1 Aerial photo of *Heliofungia* Lake in the Palauan Rock Islands, and mean monthly water temperatures in the lake (black) and in a well-flushed channel outside the lake (grey). The arrow marks the location of the subsurface tunnel entrance to the lake, and the asterisk marks the location of the tunnel exit, and of the temperature loggers in the lake (at 2 and 12 m depth).

> 50° steep walls (Fig. 1). The lake measures ~200 × 100–150 m in surface dimensions with an approximate area of 2.14×10^4 m^2 (irregular shape), averages ~18 m in depth (volume: ~3.85×105 m^3) and is holomictic. It is connected to a shallow outside basin (< 5 m deep and enclosed by rock islands except for a small opening to the more general rock island channel area) by two > 1-m diameter tunnels (20 m long) situated ~1.5 m below spring high-water level, plus other smaller cracks through the donut-shaped island. Tides are semidiurnal and average ~1.0 m in amplitude. The lake is well connected (little damping) to the shallow outside basin, so its tidal range would be virtually the same, except for very low tides. The tunnels are above sea level on a spring low tide, hence air-filled, so at some point in the tidal cycle these stop transporting water between the lake and the basin. This would determine the lowest level the lake could reach, although the basin might have a water level somewhat lower (perhaps 0.2–0.3 m) on spring low tides. Given these constraints, the average water exchange in the lake per tidal cycle through the tunnels and cracks (typical 1.0 m tide) is ~21 000 m^3 twice a day, thus theoretically the water in the lake could be completely exchanged every 8–9 days. Although there is no strong vertical stratification of the lake (mean temperature difference between 2 and 13 m depth: +0.05 °C), the exchange sources are all shallow and located in only one area of the margin, so the lower depths of the lake are certainly exchanged less often.

Two temperature loggers were deployed in the lake at 2 and 13 m depth down slope and laterally from the exchange tunnels for 20 months (Fig. 1). Although not in the direct path of the exchanging water, these loggers were within ~20 m of an exchange tunnel, so regularly saw water coming into or out of the tunnels. Fifteen other temperature-logging stations are deployed throughout Palau, including both lagoon and offshore waters. The reefs at Oikull and Taoch are part of these generalized areas. During 2000–01, stations in lagoons areas were 29.45 °C (mean temperature, range 28.2–30.6 °C). Outer reef stations were 29.1 °C (mean temperature, range 27.4–30.0 °C) during the same period. Because of the sheltered conditions and water intake from a shallow outside basin that warms rapidly, the lake water is on average 0.5 °C warmer (maximum deviation: +2.2 °C) than the larger oceanic water body around Palau. Monthly mean temperatures in the Lake exceeded 30 °C in 9 of 20 months during the observation period in the Lake, with a mean monthly temperature record of 29.91 °C (28.51–31.78 °C) (Fig. 1). The reef walls and 10–30 m island walls are steep and shade parts of the reef for several hours per day. Again, there are no records or signs of damage (i.e. low abundance of dead corals) by the (1998) bleaching event, however, ~10% of corals of most families (in particular Heliofungia, Poritidae and Faviidae) were blanched (pale brown) in October 2001, when the temperature loggers recorded 31.3 °C in the upper 2 m and 30.8 °C at 12 m depth during that week.

Ninety small coral fragments (generally 1–2 cm in size) belonging to 38 scleractinian species (21 genera, 9 families, Table 1) were collected from upper colony surfaces or branch tips at 5–6 m depth at the three reefs in October 2001. Samples were taken only from old colonies that were large enough

Table 1 List of families, genera and species of corals, and total number of colonies sampled from three Palauan reefs. Also tabled are the colonies dominated (= 90% concentration) by zooxanthellae of strain C•, other mixed strains of clade C (labelled with C*), or clade D. Numbers in brackets denote the number of colonies found to harbour a certain set of *Symbiodinium* strains. 'M' demarcates the single colony with a 50–50% mix of clades C and D. Note the dominance of clade D in *Heliofungia* Lake, and of strain C• in *Porites*

Family	Genus	Species	Total	Oikull	Taoch	*Heliofungia* Lake
Acroporidae	*Acropora*	*brueggemanni*	2		C*, D	
	Acropora	*horrida*	1		D	
	Acropora	*subglabra*	1		D	
	Astreopora	*myriophthalmata*	4	D	D (3)	
Agariciidae	*Hydnophora*	*rigida*	2			D (2)
Faviidae	*Cyphastrea*	*chalcidicum*	1	D		
	Cyphastrea	*serailia*	3		D (3)	
	Echinophyllia	*echinoporides*	1			D
	Echinopora	*lamellose*	5	C*		D (4)
	Favia	*stelligera*	1	C*		
	Favites	*helicora*	1			D
	Goniastrea	*aspera*	8			D (8)
	Goniastrea	*australensis*	1			D
	Goniastrea	*edwardsii*	2	D	D	
	Goniastrea	*pectinata*	3	C*	C*, D	
	Goniastrea	*retiformis*	1	D		
	Leptastrea	*transversa*	1	D		
	Leptastrea	*pruinosa*	1		D	
	Leptastrea	*purpurea*	2		C*, D	
	Montastrea	*magnistellata*	1			D
Fungiidae	*Ctenactis*	*crassa*	4	C*, D, M	D	
	Cycloseris	*hexagonalis*	1		D	
	Fungia	*concinna*	1			D
	Fungia	*danae*	1	C*		
	Heliofungia	*actiniformis*	4			D (4)
Merulinidae	*Merulina*	*scabricula*	5	C*	D	D (3)
	Pavona	*varians*	1	C*		
Mussidae	*Lobophyllia*	*corymbosa*	4		C* (4)	
	Lobophyllia	*pachysepta*	4			D (4)
Pectinidae	*Pectinia*	*alcycornis*	2			D (2)
Pocilloporidae	*Pocillopora*	*damicornis*	2		D (2)	
Poritidae	*Porites*	*attenuata*	1	C•		
	Porites	*australensis*	1	C•		
	Porites	*cylindrical*	4	C•	C•	C•, D
	Porites	*lichen*	3		C•	C• (2)
	Porites	*monticulosa*	1			D
	Porites	*rus*	7	C• (4)	C• (2), C*	
	Porites	sp.	2			C• (2)
Total: 9 families	21 genera	38 species	90	21	27	42

to have clearly settled before 1998 and hence had survived the bleaching event. Corals were identified to species level (Veron 2000), and preserved in 90% chemically pure ethanol. The aim was to characterize reefs rather than to compare and characterize individual coral species, thus the collection included all major coral families and many of the genera that were representative for each location. Forty-two colonies were sampled in the *Heliofungia* Lake Reef, 21 at Oikull Reef and 27 samples at Taoch Reef. The families Faviidae, Fungiidae, Merulinidae and Poritidae were represented in samples from all three reefs, whereas representatives of the families Acroporidae, Agariciidae, Mussiidae and Pocilloporidae were only found at one or two of the reefs.

To characterize the coral communities, four to five transects were video recorded each at 3 and 10 m depth at the three reefs. The transect length was determined by 5-min timed swimming on scuba, and corals were video recorded from 0.5 m above the substrate. The taxonomic composition of hard coral communities and benthic cover were later analysed by determining coral genera and substrate types on equally spaced stop-frames along the videotapes underneath 40 fixed points (English *et al*. 1997).

Laboratory methods

DNA extraction, polymerase chain reaction, cloning and sequencing. DNA was extracted from a tiny piece of ground-up coral using the DNeasy™ Tissue Kit (Qiagen) following the manufacturer's protocol for animal tissues. DNA was eluted from the Qiagen column twice with 150 μL elution buffer. To distinguish between zooxanthella strains, the nuclear ribosomal DNA internal transcribed spacer (ITS)1 region was amplified as described in van Oppen *et al*. (2001). The forward primer was fluorescently labelled with HEX or TET for detection on the GelScan (2000) system (Corbett Research) using single-stranded conformation polymorphism (SSCP) analysis.

SSCP analysis. One microlitre of polymerase chain reaction (PCR) product was mixed with (1–25 μL, depending on the yield) formamide gel-loading dye (Sambrook *et al*. 1989). Samples were denatured for 3 min at 95 °C and snap-cooled on ice. One microlitre of each sample was loaded onto a 4% nondenaturing TBE-polyacrylamide gel (20 cm) and pulsed for 10–15 s, after which the remaining sample was flushed from the well. Approximately 15 mL solution was used for each gel [1.5 mL 40% acrylamide (37 : 1 bisacrylamide/acrylamide (Sigma), 12.3 mL ddH_2O, 0.9 mL 10× TBE (Amresco), 0.38 mL 80% glycerol]. Thirty microlitres of TEMED and 75 μL 10% ammonium persulfate were added to start polymerization prior to pouring the gel. Gels were run on the Gelscan2000 (Corbett research) for ~40 min (1200 V, 22 °C) with 0.6× TBE buffer. Reference samples of known ITS1 sequence were run alongside the samples on each gel. SSCP profiles were scored manually from the gel-picture produced by the Gelscan2000.

Cloning and sequencing. For several coral samples showing multiple SSCP bands, the zooxanthella ITS1 PCR product was cloned to isolate all SSCP electromorphs present on the gel. The aim of this undertaking was to identify the bands but also to examine whether each SSCP band represents a different sequence or whether some are additional conformations, an artefact known to sometimes occur in SSCP analysis (Sunnucks *et al*. 2000). Inserts of positive clones were PCR amplified with the original primers and run on an SSCP gel along with the original PCR products to verify that all SSCP bands had been obtained and also to exclude any new bands, which may be the result of PCR errors. For cloning, nonfluorescently labelled PCR products were mixed with 6 μL Orange G loading dye and loaded on a 1% agarose-TAE gel, along with a 1 kb size marker. PCR products were excised from the gel and spun through a no. 1 Whatman paper filter for 2 min at 2300 *g* in a bench top Eppendorf centrifuge. These purified products were cloned into a pGEM-T and pGEM-T Easy Vector Systems (Promega). For sequencing, inserts of white colonies were amplified using vector primers, run on an agarose gel and spun through Whatman paper as described previously. Products were quantified and sequenced on an ABI 310 Genetic Analyser (Perkin–Elmer).

Determination of relative abundance of different zooxanthellae strains. A set of experiments was performed to determine the sensitivity of SSCP analysis, and to assess whether relative abundances of specific strains can be estimated from relative band intensity on the SSCP gels. The assumption we make here is that no significant differences exist in ribosomal DNA (rDNA) copy number between clade C and D zooxanthellae. ITS1 PCR products of a clade C and a clade D strain were cloned into the pGEM-T vector using standard procedures. A positive clone from each transformation was analysed using SSCP to verify its identity and each plasmid was subsequently purified on Qiagen spin columns following the manufacturer's protocol. Both plasmids were initially diluted ~10^4 times to equal concentrations (0.01 ng/μL), after which a 1–100-fold dilution series was prepared. Next, a sample of each step of the dilution series of strain C was mixed with an equal volume of the 1× dilution of strain D, and vice versa, to obtain samples with the two mixed DNA templates of a range of known ratios. Nine microlitres of 7 ng/μL genomic DNA of *Acropora millipora* isolated from sperm (no zooxanthellae) was added to 1 μL of each mixture to introduce genomic complexity. These samples were then PCR amplified and run on a SSCP gel as described above. For each sample, the relative intensity of the two (C and D) bands was visually estimated. This experiment was carried out in duplicate, as well as with an initial 10^3 instead of a 10^4 dilution of the plasmids, to assess the results over a wide sample DNA concentration.

Data analysis

Sequences were proofread and aligned in SEQUENCHER 4.1 (Gene Codes Corp.). A Bayesian inference of phylogeny was followed using the software package MR BAYES version 3.0b4 (Huelsenbeck & Ronquist 2001) under the HKY85 model of sequence evolution, as the likelihood ratio test (as implemented in the program MODELTEST; Posada & Crandall (1998)) revealed that the this model has the best fit to both data sets. No prior probabilities were defined. The analysis was run with four independent chains and for 5 million generations, of which 1.25 million were discarded (burn-in).

A principal components analysis (PCA) on square-root transformed video transect data was used to determine patterns in coral composition and community structure among the three reefs and two depths zones (S-PLUS, Statistical Sciences 1999). Vectors representing highest representation of coral genera were superimposed on

the PCA biplot, and the relative generic richness of individual transects was marked by the 'thermometer' symbols, with a complete symbol fill representing highest and empty symbols representing lowest richness in the data set.

Results

Phylogenetic patterns in zooxanthella strains

Zooxanthellae of clades C and D were represented in the corals from Palau. The ITS1 sequences from this study have been submitted to GenBank (Accession nos AY456926–AY456956 for the clade C sequences and AY457947–AY457967 for the clade D sequences). Figure 2A and B shows phylogenetic trees of the rDNA ITS1 sequences from clade C and D zooxanthellae obtained in this study, combined with those from the Indo-Pacific region previously published in our laboratory (van Oppen *et al*. 2001; van Oppen 2004). Several distinct *Symbiodinium* C and D ITS1 subclades and types can be distinguished, but only the ones published previously are named (van Oppen *et al*. 2001; van Oppen 2004). The unnamed C strains are pooled and called C_n throughout the text, the unnamed D strains are pooled and referred to simply as D. We are confident that C1–3, C• and C+ represent distinct strains based on ecological and taxonomic distributions (van Oppen *et al*. 2001; Ulstrup & van Oppen 2003; van Oppen 2004). For any of the other ITS1 types we are uncertain (these may represent intragenomic variants) and have therefore grouped them into C_n. With regard to *Symbiodinium* clade D, it is possible that we have only characterized two strains, the normal D and D• but further studies are required to clarify this. Because of this uncertainty of new ITS1 sequence representing new strains or intragenomic variants, we have not named them. Moreover, the current nomenclature of *Symbiodinium* strains identified using DNA sequences is problematic, as different researchers use different regions of the rDNA cistron to distinguish between strains (e.g. ITS1 in the van Oppen laboratory and ITS2 by LaJeunesse 2002), and we have refrained from adding to this nomenclatorial confusion here by not introducing more names.

Relative abundances of zooxanthella strains

Band intensity on SSCP gels is a good indication of relative abundance of a particular zooxanthella strain (Fig. 3). However, strains present at a relative abundance of < 5–10% cannot be detected with this method. The experiment was repeated at a 10-fold higher plasmid concentration, which yielded the same results (not shown), indicating that the estimation of relative abundances of zooxanthella strains from SSCP gels is independent of the DNA concentration in the PCR reaction.

Coral–zooxanthellae associations on the three reefs

The identity and relative abundance of the *Symbiodinium* strains sampled in corals from the three reefs are listed in Table 1 and shown graphically in Fig. 4. SSCP band intensity was taken into account when calculating the relative abundances of zooxanthellae. Many colonies contained zooxanthellae of both clades, as well as a number of different ITS types from each clade, however, in all but one colony either clade C or D were represented at = 90% concentration.

At Oikull Reef 21 colonies (16 species) were sampled, which all had survived the bleaching event 3 years earlier on this reef. In total 12 zooxanthella communities (defined here as SSCP banding patterns) were distinguished within these 21 colonies (Fig. 5). Most of the colonies (71%) harboured 100% clade C zooxanthellae. Only one colony (*Astreopora myriophthalmata*) contained 100% clade D zooxanthellae, but four colonies (*Goniastrea retiformis*, *G. edwardsii*, *Ctenactis crassa* and *Leptastrea transversa*) harboured 90% clade D zooxanthellae, complemented by traces of C2 or C_n. One colony (*Ctenactis crassa*) contained a 50–50% mix of clade C and D zooxanthellae.

At Taoch Reef 27 colonies (16 species) were sampled, and 10 zooxanthella communities were distinguished within these colonies. About half of the colonies harboured a diverse range of combinations of C types and no clade D zooxanthellae. Two colonies contained 100% clade D zooxanthellae, and 48% of colonies contained 90% clade D zooxanthellae when all samples were included (62% after excluding *Porites*).

At the *Heliofungia* Lake Reef 42 colonies were sampled, and the total number of zooxanthella communities in these 42 colonies was only 2 (Fig. 5). Only 5 of the 42 colonies (17 species) sampled contained 100% clade C zooxanthellae, and all five belonged to the genus *Porites* in which zooxanthellae are transmitted maternally to the gametes. These colonies were associated with a single *Symbiodinium* C strain (C•). No traces of other C strains were found in *Heliofungia* Lake, hence C1, C2, C3 and C_n that all occurred outside *Heliofungia* Lake were absent in the lake. The large majority of colonies from this reef (all nonporitid species plus two colonies of *Porites*) harboured 100% clade D zooxanthellae, with multiple sequences of phylotype D represented.

At all three reefs, most *Porites* colonies were associated with the zooxanthella strain C• that was not recorded in any of the remaining coral genera: most *Porites* colonies were dominated by this strain, however, additional traces of C1 and C2 were found in a small number of Oikull and Taoch colonies. Two *Porites* colonies in *Heliofungia* Lake (one of the four colonies of *P. cylindrica* investigated, and the only colony of *P. monticulosa* investigated) harboured clade D only, and one *P. rus* colony from Taoch Reef harboured C1 only.

Fig. 2 Bayesian inference of the phylogeny of zooxanthellae. (A) Clade C zooxanthellae, (B) clade D zooxanthellae. Sequences obtained in this study have a 'P' code and are given in bold, regular font. Values above the branches are posterior probabilities.

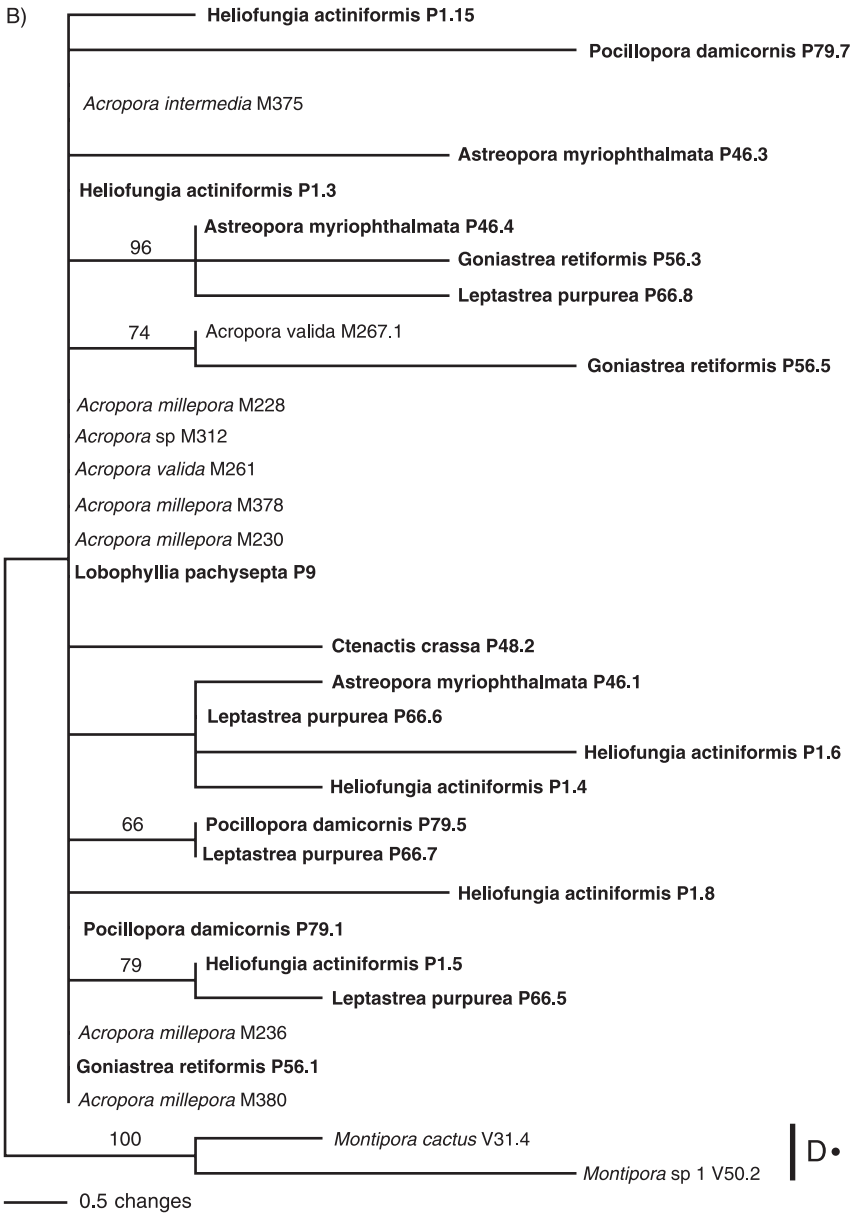

Fig. 2 *Continued*

The level of replication in coral species both within and between reefs was low, rendering it difficult to determine whether the host–zooxanthella association in any one colony was mostly determined by location or by species affiliation. Partly due to the low level of replication, a majority of the 39 coral species appeared to be exclusively associated with either clade C or D. However, a total of 30% of species from which more than one colony was tested (6 of 20) were found to vary between clades C and D. In five cases, colonies of the same species contained different clades within the same reef (*Acropora brueggemanni*, *Goniastrea pectinata* and *Leptastrea purpurea* at Taoch Reef, *Ctenactis crassa* at Oikull and *Porites cylindrica* at *Heliofungia* Lake Reef; Table 1). Two of seven species sampled at two reefs had different clades at different reefs. Only two coral species (*Merulina scabricula* and *Porites cylindrica*) were sampled at all three reefs, and in both cases colonies harboured different clades at different reefs.

In summary, *Heliofungia* Lake Reef harboured a very uniform zooxanthella community. Zooxanthella community types were more diverse at Taoch and Oikull Reefs, with Oikull Reef corals commonly dominated by C strains, and Taoch having relatively even proportions of corals dominated by C and D clades. The difference in combinations of zooxanthella strains between *Porites* and all other taxa was more pronounced than the differences between Oikull and Taoch reefs, but otherwise our data revealed little species specificity in the coral–zooxanthellae associations. Thus, the associations between coral and zooxanthellae were characterized by (i) a high level of symbiont specialization

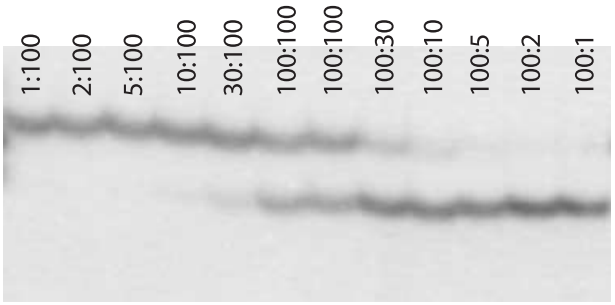

Fig. 3 SSCP gel image, based on dilution and mixing of two plasmid batches. The image displays the link between band intensity and relative abundances of strains of zooxanthellae, and the limits of sensitivity of the SSCP technique for determining zooxanthellae that occur in relatively low concentrations.

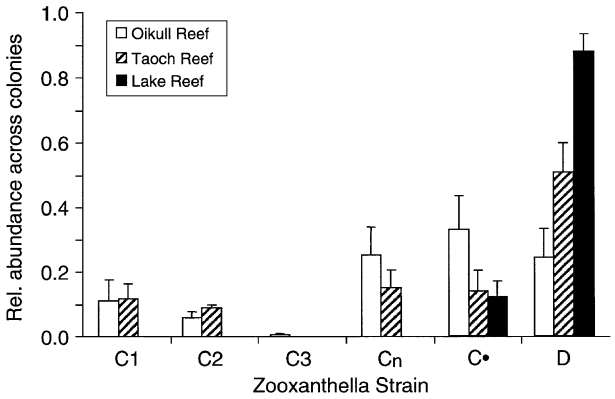

Fig. 4 Relative abundance of the different zooxanthella strains on the three study reefs, averaged over all scleractinian coral samples (including species of *Porites*; note that the presence and frequency of host species vary between reefs). Error bars are 1 SE. The geophysical settings and bleaching history of the three reefs are as follows: Oikull Reef — generally cool, relatively high diurnal photon flux, high bleaching-related mortality in 1998; Taoch Reef — generally cool, reduced diurnal photon flux, no bleaching-related mortality in 1998; Lake Reef (*Heliofungia* Lake) — relatively warm, reduced diurnal photon flux, no bleaching-related mortality in 1998.

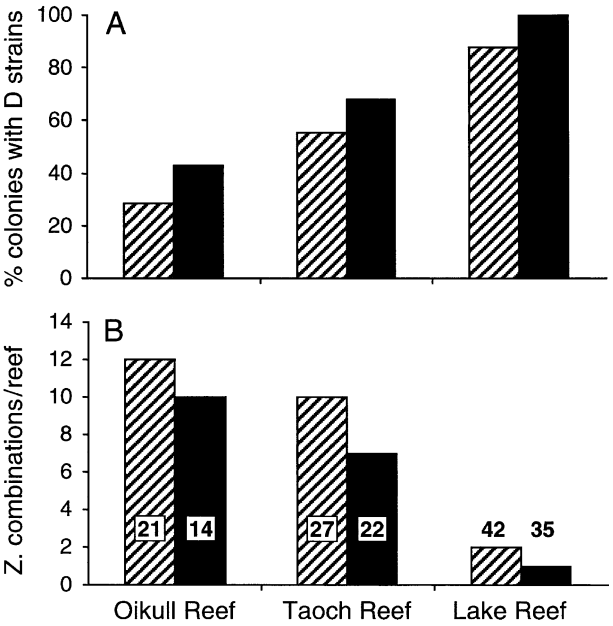

Fig. 5 Comparison of zooxanthella strains across reefs, with and without inclusion of the maternally transmitting *Porites* (hatched and black bars, respectively). (A) Proportion of coral colonies that contain clade D zooxanthellae at > 5–10% of their total zooxanthella numbers. (B) Plot showing the total number of zooxanthella communities per reef (based on the SSCP profile of the zooxanthella rDNA ITS1 region). Bold numbers indicate the number of colonies sampled.

on one reef (independent of coral species) contrasting with a high level of zooxanthella diversity elsewhere, and (ii) a high level of symbiont specificity in one coral genus (*Porites*) and one zooxanthella strain (independent of reef) contrasting with an apparently low level of symbiont–host specificity in many of the other corals and zooxanthellae.

Patterns in the coral communities

Coral cover and community composition differed strongly among the three reefs. Hard coral cover at Oikull Reef averaged 5.4% at 3 m and 2.3% at 10 m depth three years after the (1998) mass bleaching event. In contrast, coral cover at Taoch Reef was 66 and 27% at 3 and 10 m depth, respectively, whereas at *Heliofungia* Lake Reef it was 41 and 37%, respectively.

Hard coral richness (defined as the number of genera distinguished in 40 sampling points per transect) averaged 4.3 ± 0.8 per transect at *Heliofungia* Lake Reef. At Oikull Reef, it was 2.1 ± 0.6 SE, and four times higher at Taoch Reef (8.0 ± 0.9). The bleaching-sensitive coral families Acroporidae and Pocilloporidae were missing in the lake community and were rare at Oikull Reef. The principal components analysis indicates that most species were associated with the cool Taoch Reef where no bleaching damage had occurred (Fig. 6). The *Heliofungia* Lake Reef community was characterized by high abundances of *Heliofungia*, faviids (especially *Echinopora*), Poritidae and *Merulina*. Taoch Reef was distinguished from the other reefs by the presence of a number of mussid and faviid genera, *Astreopora*, and the bleaching-sensitive *Acropora*. Oikull Reef had few characteristic genera except for *Diploastrea*, *Pocillopora*, *Montipora* and *Goniopora* that all occurred in low abundances. There were no clear differences between the two depth zones at Oikull and the *Heliofungia* Lake communities, whereas the two depths at Taoch reef differed slightly in relative abundances of a number of species.

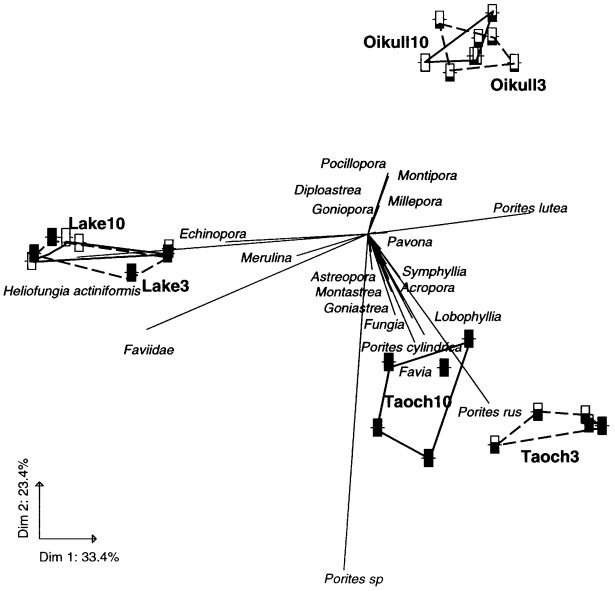

Fig. 6 Scleractinian coral communities at three Palauan Reefs (two depths) with varying bleaching, temperature and irradiance histories (principal components biplot of double-square root transformed video transect data). Dashed lines connect sites at 3 m depth, solid lines connect sites at 10 m depth. The fill of the symbols indicates generic richness (relative number of genera distinguished per transect). Only vectors of the more common coral genera are displayed for clarity.

Discussion

The dominant types and relative abundances of zooxanthellae associated with reef corals differ greatly among three Palauan reefs with contrasting temperature and shading exposure and bleaching history. Differences are particularly noticeable in the proportion of colonies dominated by zooxanthellae of phylotype D. All coral colonies sampled from the warm and shaded *Heliofungia* Lake Reef, except for some *Porites* colonies, harbour 100% *Symbiodinium* phylotype D, without any measurable traces of additional clades of zooxanthellae. Outside the *Heliofungia* Lake, fewer than half of the colonies harbour D zooxanthellae, and of these most colonies also contain traces of C strains rather than housing 100% D clade zooxanthellae.

Different clades and strains of zooxanthellae appear to differ in their thermo-tolerance (Kinzie *et al*. 2001; Bhagooli & Hidaka 2003), which is likely to contribute to the thermo-tolerance of the holobiont (the whole animal–dinoflagellate association). The fact that corals at *Heliofungia* Lake and Taoch Reef did not experience extensive bleaching mortality in 1998 may be related not only to the shading, but also to the higher thermo-tolerance of colonies housing clade D rather than clade C zooxanthellae. Baker *et al*. (2004) found a significantly higher proportion of clade D zooxanthellae in corals that had survived the severe bleaching in the Arabian Gulf than in corals from the Red Sea, a pattern that coincides with greater temperature fluctuations and higher salinity in the former compared with the latter region. Glynn *et al*. (2001) also showed that far-eastern Pacific corals harbouring clade D symbionts had suffered less from the (1998) coral-bleaching event than those that associated with symbionts of a different genotype. Similarly, Toller *et al*. (2001) found that clade D zooxanthellae ('clade E' *sensu* Toller *et al*. 2001 is called 'clade D' by most other authors; Baker 2003) are relatively stress-tolerant in two Caribbean *Montastraea* species experimentally exposed to changing light levels. On the Great Barrier Reef, the coral *Acropora millepora* associates with clade D zooxanthellae only on more turbid and sometimes warmer inshore reefs, whereas conspecific mid-shelf reef populations harbour clade C symbionts (van Oppen *et al*. 2001). A related species, *Acropora valida*, also harbours significantly more *Symbiodinium* D than C in low light and warm vs. high light and cooler environments (Ulstrup & van Oppen 2003). Hence, the evidence suggesting that type D zooxanthellae are relatively heat tolerant and light intolerant is compelling. The monopoly of holobionts associated with clade D zooxanthellae on *Heliofungia* Lake Reef across a wide range of coral species may therefore be explained by a chronic exposure of the algae to warmer water at shaded conditions. Seventy-five per cent of sampled coral species were found at least occasionally in association with clade D zooxanthellae. However, our sampling intensity was too small to establish whether those species that were missing in the lake are unable to associate with clade D. Additional research is needed to determine how flexible host–zooxanthellae associations are, i.e. whether a significant number of coral recruits can enter into an association with whatever zooxanthellae are well-suited at given local environmental conditions. Nevertheless, our data suggest an exclusive selection of clade D dominated holobionts in six of seven coral families (except *Porites*) in chronically warm water and shaded conditions, suggesting that clade D is the better-adapted endosymbiont at these environmental conditions. Further, at Toach Reef (where, like at *Heliofungia* Lake, the diurnal photon flux is reduced) corals associate twice as often with *Symbiodinium* clade D than at the high-irradiance Oikull Reef, possibly in response to differences in shading.

Coral species are known to differ in their thermo-tolerance and bleaching sensitivity. Temperature-sensitive coral genera, such as *Acropora* and *Pocillopora* (Hoegh-Guldberg & Salvat 1995; Marshall & Baird 2000) are absent from the Lake Reef, despite the availability of D endosymbionts and the fact that these corals are known to be able to associate with a range of symbionts from three phylogenetic clades (e.g. Pacific *Acropora* spp. can harbour *Symbiodinium* A, C and D; Loh *et al*. 2001; van Oppen *et al*. 2001). The complete absence of *Acropora* and other bleaching-sensitive taxa in the lake is probably not due to a lack of recruits from outside,

because populations of bleaching-sensitive species are located in the basin outside the *Heliofungia* Lake and other areas close by. *Acropora* is also present on the shaded Taoch Reef, indicating that the warm temperature rather than shading might be responsible for the failure of this and other bleaching-sensitive genera to thrive in the lake community.

The association of *Porites* colonies in the warm *Heliofungia* Lake with C• symbionts is remarkable. It matches the finding of a *Symbiodinium* strain, which is unique to *Porites* (LaJeunesse *et al*. 2003), named C15, and *Montipora* (van Oppen 2004). Both *Porites* and *Montipora* corals transmit their algal symbionts to the eggs. Thus, zooxanthella types are determined (to some extent) by those carried by the maternal colony rather than those in the water column available for uptake by recruits after settlement. The association of *Porites* with clade C in the warm lake can be explained in two ways. First, the thermo-tolerance of certain C-strains may be similarly high as that of *Symbiodinium* D, in particular in C-strains with strong host specificity such as *Symbiodinium* C•. Second, host factors may play an important role in determining the thermal tolerance of the holobiont. For example, *Acropora tenuis* colonies that harbour *Symbiodinium* C1 have a higher thermal tolerance than *A. millepora* colonies associated with *Symbiodinium* D from the same reef (van Oppen, unpublished data). The relative contribution of each of the symbiotic partners to thermal tolerance of the holobiont is, however, currently unknown. In our data, 30% of the coral species that were sampled more than once were found associated with more than one zooxanthella clade, despite the low number of replicate colonies sampled per species. Thus, with the exception of *Porites*, the proportion of coral species that showed flexibility in forming associations with different clades appeared to be relatively high.

The zooxanthella community in *Heliofungia* Lake was remarkably uniform, with only *Symbiodinium* D monopolizing all disparate coral taxa except the maternally transmitting *Porites*. There are two potential explanations for this monopoly. First, an extreme (bottleneck) environmental event may have killed all other zooxanthella strains in *Heliofungia* Lake (corals, water column and sediments); thus only clade D zooxanthellae are available in high abundances in the water column, and this clade is likely to be taken up by newly settling coral recruits, and only coral recruits of those species can flourish that are able to associate with clade D. This argument of selection in the past is supported by the fact that other zooxanthella strains are only found in *Porites*. However, *Heliofungia* Lake is relatively well connected with the outside water body, thus if zooxanthellae disperse in the water column all strains should be imported on a regular basis from the outside and replenish the *Heliofungia* Lake Reef communities. A second hypothesis may be that all zooxanthella strains are imported into the *Heliofungia* Lake environment, however, clade D zooxanthellae are the most suitable symbionts for the local condition of warm water and periods of shading. Therefore, chronic rather than extreme environmental conditions give clade D zooxanthellae a competitive advantage over all non-D clade zooxanthellae, with selection either acting on the zooxanthellae in the free-living state or on the holobionts, by selection against those recruits that have associated with clade C zooxanthellae. The only counter-argument against this hypothesis of present-day selection against available clade C zooxanthellae is the presence of populations of *Porites* with clade C zooxanthellae types that thrive at the warm lake temperatures, but as discussed above, their thermal tolerance may be due to the unique nature of the host factors in *Porites*.

Biological diversity in communities that live at extreme environmental conditions is generally lower than in communities that live close to the environmental optimum of a group (Sheppard & Sheppard 1991). The *Heliofungia* Lake community supported only a subset of zooxanthella genotypes and holobionts found at Taoch Reef, which is located in a physically similar setting to that of the Lake Reef (at the inner end of a semi-enclosed bay surrounded by shading karst island walls, with low flushing rate, relatively low currents and a high level of protection from wave action). The most obvious difference between the two reefs is the seawater temperature, which in the *Heliofungia* Lake Reef is often at or beyond the upper temperature tolerance limit of most Palauan corals of 30–31 °C (Bruno *et al*. 2001; no temperature records from within the lake exist from the 1998 bleaching period, but the lake temperature may have continued to exceed outside seawater temperatures by some 0.5 °C).

Both adaptation and acclimatization have been widely discussed as potential mechanisms for corals to survive future globally warming conditions. Adaptation is defined as genetic change through natural selection in a population, whereas acclimatization is defined as phenotypic change in an organism, with both changes leading to characteristics that are superior in a given local environment. In relation to coral bleaching, both the terms 'adaptation' and 'acclimatization' have been used to describe the recombination of hosts with new genetic strains of zooxanthellae, depending whether the mechanism is believed to be the uptake of new external zooxanthellae containing new genotypes, or the upregulation of clades that previously existed as small traces inside the colony. It has been argued that the process of bleaching may provide the opportunity for the host to be repopulated with a different type of zooxanthellae that is better adapted to a changing environment and for removal of maladapted types (the 'adaptive bleaching hypothesis'; Buddemeier & Fautin 1993; Ware *et al*. 1996). Corals have been found to shift to dominance of a different zooxanthella type after experimental irradiance-induced bleaching (Toller *et al*. 2001). As corals

that appear totally white still have up to 1.0×104 cm^{-2} zooxanthellae in their tissues, remaining zooxanthellae are probably the main or only source of zooxanthellae to repopulate a surviving coral (Hoegh-Guldberg & Salvat 1995; Hoegh-Guldberg *et al*. 2002). We do not have historic data on the diversity of zooxanthellae and holobionts before the (1998) bleaching event killed large proportions of corals in Palau. However, our comparison of hydrographically well-connected coral reefs in contrasting light and temperature conditions indicates that some longer term 'adaptation' (i.e. a change in the genetic composition of its coral community towards holobionts associated with D-zooxanthellae) might have occurred in *Heliofungia* Lake. The outcome of this adaptation to chronically high temperatures is a low diversity of zooxanthellae, the absence of bleaching-sensitive coral taxa, and the absence of colonies that are unable to associate with zooxanthellae of clade D in the Lake.

If clade D is indeed more temperature tolerant, a shift from holobionts harbouring clade C *Symbiodinium* to those harbouring clade D might happen not only at *Heliofungia* Lake Reef, but also in other places in response to globally increasing sea-surface temperatures. A reduced diversity of zooxanthellae, dominated by clade D, may potentially represent a mode of adaptation on coral reefs at least in low-irradiance settings (e.g. deeper or turbid waters). If such adaptation took place, it could possibly reduce the frequency of coral damage through bleaching in corals that grow at low irradiance. Such scenario would indicate that low-light environments may host a pool of corals that are able to live at increasing temperatures and could re-seed the high-irradiance areas that are more prone to bleaching damage. The 29% of corals that contained clade D zooxanthellae at Oikull Reef at 5 m depth indicates that clade D can grow not only in shaded conditions, but also at high irradiance; however, more research is needed to determine whether clade D symbionts have helped these corals to survive the (1998) bleaching event on this reef.

The implications of such a potential shift to *Symbiodinium* D for the predicted widespread loss of coral reefs through globally increasing temperatures are unknown. Predicted temperature increases of 2–4.5 °C in the next 100 years could exceed the tolerance even of clade D zooxanthellae within less than 50 years (Cubash *et al*. 2001). Furthermore, diversity was substantially decreased in the warmer lake not only in terms of the number of genera represented in the coral community (compared with Taoch Reef), but also in terms of the number of zooxanthella communities per reef, and the mean relative number of putative strains found within individual colonies: the relative mean number of zooxanthella strains per colony that was distinguished by our method was 1.7 ± 0.2 (SE) in Oikull Reef and 2.2 ± 0.1 in Taoch Reef, whereas it was 1.0 ± 0.0 in *Heliofungia* Lake Reef. Any decrease in zooxanthella diversity would obviously compromise the capacity of corals to respond to other environmental variations; e.g. associations with *Symbiodinium* D may increase the thermo-tolerance of the holobiont, but can simultaneously have negative effects on other physiological characteristics (Little *et al*. 2004). Colonies containing more than one strain of zooxanthellae may be able to down- or upregulate individual strains in response to short- to medium-term environmental fluctuations; a capacity that is not given in the colonies growing in the warm lake.

The future of coral reefs crucially depends on the frequency of mass coral mortalities, and the speed of adaptation to the El Niño-related severe temperature peaks on top of the gradual increases in the baseline temperature. The few colonies that had survived the severe selection process at Oikull Reef in 1998 contained a high number of zooxanthella communities with potentially varying thermotolerances, and a large proportion of clade C zooxanthellae. Thus, despite the severity of the 1998 bleaching event, some holobionts associated with clade C did survive, and the apparently less temperature tolerant zooxanthellae were not fully removed from this reef. This indicates that, if adaptation should indeed occur, it could be a slow process: a better-adapted (and reduced) set of genotypes might gradually establish through series of extensive sublethal bleaching events that remove the maladapted zooxanthella genotypes and coral species. However, frequent mass bleaching mortalities, as experienced in 1998, would rapidly reduce genetic diversity of coral reefs and hence their potential to adapt to a presently changing environment.

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