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Molecular identification of symbiotic dinoflagellates in Pacific corals in the genus *Pocillopora*

H. Magalon · J.-F. Flot · E. Baudry

Abstract This study focused on the association between corals of the genus *Pocillopora*, a major constituent of Pacific reefs, and their zooxanthellae. Samples of *P. meandrina*, *P. verrucosa*, *P. damicornis*, *P. eydouxi*, *P. ligulata* and *P. molokensis* were collected from French Polynesia, Tonga, Okinawa and Hawaii. *Symbiodinium* diversity was explored by looking at the 28S and ITS1 regions of the ribosomal DNA. Most zooxanthellae were found to belong to clade C, sub-clade C1, with little differentiation between populations. Interestingly, individuals of *P. damicornis* harbored sub-clade C1, clade D and clade A, depending on location. The symbiotic association of *P. damicornis* with its zooxanthellae may be somewhat more flexible than those of other *Pocillopora* species.

Introduction

Most tropical corals live in symbiosis with photosynthetic algae, the zooxanthellae (reviewed in Trench 1993). Most zooxanthellae belong to the genus *Symbiodinium* and 11 species have now been defined based on morphological, physiological and molecular criteria (reviewed in Baker 2003). Diversity of zooxanthellae from a wide range of hosts has been explored using molecular techniques with different levels of resolution. The first studies used allozymes and Restriction Fragment Length Polymorphism (RFLP) to differentiate between zooxanthella lineages (Rowan and Powers 1991). Later, *Symbiodinium* molecular phylogenetics became dominated by the study of nuclear DNA coding for ribosomal RNAs (nrDNA): 18S nrDNA encoding the small ribosomal subunit (SSU), 28S nrDNA encoding the large ribosomal subunit (LSU) and the internal transcribed spacers (ITS1 and ITS2) located in between (reviewed in Baker 2003). Later studies turned to chloroplast DNA (23S rDNA) and mitochondrial DNA sequences (Santos et al. 2002a, b, 2003; Takabayashi et al. 2004). Recently, microsatellite markers have also been used to explore *Symbiodinium* diversity, particularly at the population level (e.g., Santos and Coffroth 2003; Magalon et al. 2006). All these studies have defined multiple clades (reviewed in Coffroth and Santos 2005) that can further be subdivided into “sub-clades” or “types” (based on ITS1; e.g., van Oppen et al. 2001, and based on ITS2 DGGE profiles; e.g., LaJeunesse 2001). Clades were first thought to correspond to ecological types with different photoacclimatory abilities (Rowan and Knowlton 1995). Studies on zooxanthellae depth distribution and on their responses to stress conditions have attempted to determine the characteristics of each clade. However, these categories are highly simplistic and artificial since differential sensitivity to

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thermal stress among the various species of *Symbiodinium* seems to be distributed across all clades (Tchernov et al. 2004), as is sensitivity to irradiance (Savage et al. 2002). Researchers became interested in examining zooxanthellae diversity over a wide range of hosts and geographic locations. For instance, van Oppen et al. (2001) examined symbiont association across a large number of coral species belonging to the genus *Acropora* along the Great Barrier Reef and LaJeunesse (2005) published a complete survey of clade C diversity, encompassing nearly 100 host genera across the Atlantic and the Indo-Pacific.

In this study, zooxanthellae diversity was investigated in six *Pocillopora* species (Veron 2000): *P. meandrina*, *P. verrucosa*, *P. damicornis*, *P. eydouxi*, *P. ligulata* and *P. molokensis*, collected from French Polynesia, Tonga, Hawaii and Japan. *Pocillopora* is a major constituent of Pacific reefs where it may play an important role in coral reef ecosystems. Many studies have reported that *P. damicornis*, *P. verrucosa* and *P. eydouxi* from different regions [Japan (Hirose et al. 2000, 2001; Harii et al. 2002), Eastern Pacific (Glynn et al. 1991), Maldives (Sier and Olive 1994) and South Africa (Kruger and Schleyer 1998)] contained zooxanthellae in their oocytes prior to spawning, indicating maternal symbiont transmission. Even if *P. meandrina*, *P. ligulata* and *P. molokensis* are not mentioned specifically in these studies, it is probable that these latter species also transmit their zooxanthellae vertically since they are closely related to the ones cited above. Clade-specific 28S nrDNA primers were used and the ITS1 region was sequenced to investigate (1) whether the different species of the *Pocillopora* genus harbor different zooxanthellae, and (2) whether the association between the host and its symbiont is specific.

Materials and methods

Sample collection

Samples of *Pocillopora* spp. were collected between December 2000 and July 2004, from locations and depths shown in Table 1. All individuals were identified based on their morphology following Veron (2000). Total DNA extraction was performed as described in Magalon et al. (2004).

PCR amplifications of zooxanthellae nrDNA

Zooxanthella diversity was first explored by amplifying the 28S nrDNA region. Following preliminary sequencing, primers specific to clades A, B, C and D were designed manually by aligning the LSU region from the different clades available in GenBank (Table 2); primers were subsequently tested with the software package Primer3 (Rozen

and Skaletsky 2000). Specificity was checked by amplifying and sequencing the 28S nrDNA region from pure cultures of *Symbiodinium* clades A, B, C, D (kindly provided by T.C. LaJeunesse). PCR amplifications were carried out as described in Magalon et al. (2004) with an annealing temperature of 54°C. PCR products were run on 1% TBE-agarose gels. Amplifications were repeated using 30 and 40 cycles to detect zooxanthella types present in a lesser proportion.

To investigate zooxanthella diversity in more detail, the Internal Transcribed Spacer 1 region (ITS1), which is a non-coding region that evolves faster than the 28S nrDNA (Santos et al. 2002a), was sequenced. Two primers were used: one designed in the 3'-end of the 18S DNA [ITS-for: 5'-CGG TGA ATT ATT CGG ACT GAC-3'; reverse of SYM3, modified from Hunter et al. (1997)] and the other in the 5.8S region [ITSint-rev: 5'-GAA TTG CAG AAC TCC GTG-3' (reverse of ITSintfor2 from LaJeunesse and Trench (2000))]. The PCR protocol was identical to that of 28S nrDNA amplification, with the exception of the reaction volume which was 30 µl instead of 10 µl. Amplified products were purified using the QIAquick PCR purification kit (Qiagen) following the manufacturer's instructions and the ITS1 region was directly sequenced in both directions using 3.2 pmol of primer. Reagents and cycling conditions were as specified in the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems, Foster City, California).

DNA sequence analysis

Chromatograms were checked using Chromas2 software (<http://www.technelysium.com.au>), and sequences aligned using BioEdit5 (Hall 1999). As double peaks were present in some sequences, the different rDNA types for such individuals were inferred by considering their sequence as the superposition of different sequences with no ambiguities and by comparing with those of other individuals of the population that did not exhibit ambiguities (Clark 1990; Stephens et al. 2001). ITS1 sequences were aligned to other related sequences of symbionts from different hosts and geographic regions obtained from GenBank (through Blast search, <http://www.ncbi.nlm.nih.gov/BLAST/>). Phylogenetic analyses using Neighbor-Joining (based on a Kimura two-parameter distance matrix) and Maximum Parsimony were conducted on aligned data sets using Mega2 (Kumar et al. 2001). Maximum Likelihood analysis was performed using PAUP* version 4.0b10 (Swofford 2001). A bootstrap re-sampling was conducted for 1,000 replicates to assess relative branch support (Felsenstein 1985). As ITS sequences from the major *Symbiodinium* clades (i.e., A, B, C and D) are highly divergent and difficult to align, sequences were analyzed separately for each clade.

Table 1 Host species (*P. Pocillopora*), collection site, sample code, collection depth (m) and symbiont types

Host species and collection site	Sample code	Depth (m)	Symbiont type
Society Archipelago, French Polynesia			
Moorea Island			
Site Mo1			
<i>P. meandrina</i> (3)	H2	13	C1.4
Site Mo2			
<i>P. meandrina</i> (3)	T4	13	C1.4
Site Mo3			
<i>P. meandrina</i> (4)	V8, MmP1	13	C1.4
<i>P. meandrina</i> (2)	MmL1, MmL2	5–6	C1.1, C1.4
<i>P. verrucosa</i> (5)	CR40, MvP1	13	C1.3, C1.4
<i>P. verrucosa</i> (1)	MvL2	5–6	C1.3
<i>P. damicornis</i> (2)	MdL1	5–6	D1 ^a ; C1 ^b
<i>Acropora nasuta</i> (2)	Acro1	5–10	A1 ^a , C2 ^b
Tahiti Island			
<i>P. meandrina</i> (4)	Ta30, TmP1	13	C1.4
<i>P. meandrina</i> (2)	TmL1	5–6	C1.4
<i>P. verrucosa</i> (2)	TvP1, TvP2	13	C1.3, C1.4
<i>P. verrucosa</i> (2)	TvL1	5–6	C1.3
<i>P. damicornis</i> (2)	TdL1	5–6	D1 ^a , C1 ^b
Bora-Bora Island			
<i>P. meandrina</i> (3)	Bo10	13	C1.4
Tongatapu Island, Tonga			
<i>P. meandrina</i> (2)	To8, To22	2–5	C1.3, C1.4
<i>P. meandrina</i> (3)	To30	12–15	C1.3, C1.4
Hawaii			
<i>P. meandrina</i> (3)	02Oahu18	3–4	C1.1, C1.4
<i>P. damicornis</i> (3)	02Oahu04	1–2	C1.4
<i>P. eydouxi</i> (1)	02Oahu30	5–6	C1.1
<i>P. ligulata</i> (3)	02Oahu24	3–4	C1.2
<i>P. molokensis</i> (2)	02Oahu33	20–40	C1.2
Okinawa, Japan			
Maejima			
<i>P. meandrina</i> (4)	04Oki001, 04Oki003, 04Oki007, 04Oki008	5–10	C1.1, C1.4
<i>P. eydouxi</i> (2)	04Oki005	5–10	C1.1, C1.4
Oku			
<i>P. damicornis</i> (3)	04Oki040	5–6	C1.4
Amuro			
<i>P. damicornis</i> (2)	04Oki127	2–5	C1.1
Bise			
<i>P. damicornis</i> (3)	04Oki099	2–3	A1 ^a

Numerals in parentheses next to host names indicate the number of colonies independently sampled. Symbiont type annotated C1.n are designated from this study and belong to sub-clade C1 (van Oppen et al. 2001)

^a sensu LaJeunesse

^b sensu van Oppen

Results and discussion

Amplifications using the clade-specific primers showed that all corals sampled contained *Symbiodinium* clade C, except *P. damicornis* colonies from Bise (Okinawa, Japan) that associated exclusively with clade A. It is noteworthy that

P. damicornis from Moorea and Tahiti lagoons (French Polynesia) harbored clades C and D simultaneously. Fifty-eight clade C, 4 clade D and 3 clade A ITS1 sequences were obtained (GenBank accession numbers EF455494–EF455528). Among the 58 clade C ITS1 sequences, there were 15 variable and 13 parsimony informative positions

Table 2 Specific primers amplifying the different *Symbiodinium* clades 28S DNA

Amplified regions	Primer sequences	Product length (bp)
Clade A 28S DNA	28A-for 5'-ATT GTG GCC TTT AGA CAT ACT ACC-3' 28A-rev 5'-CAA TCC ACA CAC ACC TGA ATC C-3'	504
Clade B 28S DNA	28B-for 5'-GCT GGA AAC CGA TAC ATC TGT G-3' 28B-rev 5'-GTA AAT GCT CAC ACA TAA GCA GTC-3'	408
Clade C 28S DNA	28CD-for 5'-AGT CAC GCT CCT CGG AAT TG-3' 28C-rev 5'-TCA CAG CAG CCT ACA GCA ATC-3'	206
Clade D 28S DNA	28CD-for 5'-AGT CAC GCT CCT CGG AAT TG-3' 28D-rev 5'-TGC CAA CTC ACT CCT AGA CAA G-3'	255

(gaps included) for a total of 367 positions. There was no difference between the four clade D sequences, nor between the three clade A sequences. Phylogenies inferred using three different methods (NJ, MP and ML) were similar in topology and branch supports (data not shown). *Pocillopora* clade C sequences fell in the subclade C1 (sensu van Oppen et al. 2001) and were classified into four groups (named C1.1 to C1.4, see Fig. 1a). The C1.1 group comprised symbionts from *P. damicornis*, *P. meandrina* and *P. eydouxi* sampled in Hawaii, Japan and French Polynesia. Group C1.2 contained all the symbionts of *P. ligulata* and *P. molokensis*, with the symbionts from the latter clustering into a separate group. Group C1.3 was only found in *P. meandrina* and *P. verrucosa* from French Polynesia and from Tonga. The C1.4 group contained most of the symbionts found in the different *Pocillopora* species considered in this study (except *P. ligulata* and *P. molokensis*). *P. damicornis* colonies from Moorea and Tahiti lagoons harbored subclade C1 zooxanthellae and type D1 zooxanthellae (sensu LaJeunesse 2002) at the same time (Fig. 1b). Interestingly, *P. damicornis* colonies collected in Bise (Okinawa, Japan) were found to associate with type A1 (sensu LaJeunesse 2001) (Fig. 1c).

Only 13 colonies were found to harbor more than one ITS sequence simultaneously. The fact that a single host colony can harbor several different sequences may be explained in two ways. First, nrDNA is organized in tandem repeats and may be not homogenized by concerted evolution (Marquez et al. 2003). Thus, an individual cell may contain intragenomic variation (LaJeunesse 2002). Alternatively, each sequence could correspond to a unique zooxanthella type, which implies that several different zooxanthella lineages are present in the same host. The fact that the different sequences found in the same colony can also be found individually in other hosts makes the second explanation more likely.

P. meandrina, *P. verrucosa*, *P. damicornis*, *P. eydouxi*, *P. molokensis* and *P. ligulata*, which originated from geographically distant regions (French Polynesia, Tonga, Japan, Hawaii), harbored closely related symbionts belonging to the clade C, particularly subclade C1 ($\theta_{ITS1} = 0.0073$; $\pi_{ITS1} = 0.05315$, with θ and π two estimators of nucleotide

diversity). This may be related to the mode of symbiont transmission in *Pocillopora*. It is frequently thought that maternal transmission results in lower symbiont diversity than horizontal transmission, which permits the acquisition of exogenous (and potentially novel) zooxanthellae every generation. In the case of horizontal transmission, the initial uptake of zooxanthellae by juvenile corals during natural infection may be specific or non-specific, depending on the particular host (e.g., Coffroth et al. 2001; Little et al. 2004). Exclusively vertical transmission, where a correlation between host and symbiont is expected, should result in a coevolution of the host and its symbiont (Diekmann et al. 2003). However, van Oppen (2004) found that zooxanthella diversity was similar for two coral genera differing in their mode of symbiont transmission.

Samples of *Pocillopora* collected from distant geographic regions and different habitats were found to associate with subclade C1. However, *P. molokensis* and *P. ligulata* from Hawaii associate specifically with C1.2 symbionts. *P. meandrina*, *P. verrucosa*, *P. damicornis* and *P. eydouxi* associate with closely related zooxanthella types belonging to the groups C1.1, C1.3 and C1.4. Members of subclade C1 were found in other studies to associate with very different corals such as *Acropora*, *Fungia*, *Porites* (Fabricius et al. 2004) and *Plesiastrea* (Rodriguez et al. 2003). They are thus considered host-generalists and may be ancestral to symbionts belonging to specialist subclades (LaJeunesse 2005).

Unexpectedly, *P. damicornis* collected from Moorea and Tahiti lagoons harbored clades C and D simultaneously, as observed in the Great Barrier Reef (Ulstrup et al. 2006), whereas *P. verrucosa* and *P. meandrina* collected at the same location were found to associate only with clade C. Conversely, *P. damicornis* collected at Bise (Okinawa, Japan) harbored only type A1 zooxanthellae, suggesting that this species might have co-evolved with this zooxanthella type at this specific (and remote) location (Barneah et al. 2004), or that other symbiont types were not detected due to the small sample size used (3 specimens). Thus, *P. damicornis* seems more capable of associating with several different clades compared to *P. verrucosa* and

Fig. 1 Neighbor-Joining trees of *Pocillopora* zooxanthellae based on the ITS1 sequences. Bootstrap values are given above the branches. Samples from this study come from French Polynesia (*in red*), from Tonga (*in violet*), from Japan (*in green*) and from Hawaii (*in blue*). Numbers in parentheses are the number of colonies showing identical sequences. Samples of which the sample code is followed by a dot and a number (e.g., *P. verrucosa* TvP2.1) have been reported to exhibit two ITS sequences. **a** Clade C zooxanthellae. Group names which are followed by a dot and a number (e.g., C1.1) are originated from this study, the other from van Oppen et al. (2001) and from Fabricius et al. (2004). **b** Clade D zooxanthellae. Types in *bold* are from LaJeunesse (2001). The notation D• comes from Fabricius et al. (2004). **c** Clade A zooxanthellae. Types in *bold* (e.g., A1) are from LaJeunesse studies

P. meandrina. Furthermore, *Acropora nasuta* collected on the outer reef slope of Moorea harbored zooxanthellae belonging to subclades C2 and A1. This shows that these types of symbionts were present and locally available for the hosts inhabiting Moorea, although these types were not

found in the *Pocillopora* species of this region. So these particular associations between coral colonies and zooxanthella symbionts can be the result of recognition mechanisms, either by the symbiont or by the host, the influence of environmental conditions, competition between symbio-

Fig. 1 continued

nts within the host tissues, or a combination of these different mechanisms. The environment does not seem to play a major role, as species sharing the same environmental conditions do not harbor the same zooxanthellae and inversely, as species from different habitats and from distant regions harbor the same zooxanthellae (with exception of *P. damicornis*, which shows a particular geographic association pattern). It appears that host identity (i.e., the internal host environment and/or host-symbiont specificity) might be one of the main factors governing the *Pocillopora*–*Symbiodinium* symbiosis. But these conclusions have to be taken with caution because of the small sample size and the lack of resampling through time of the sampling scheme.

Clades of zooxanthellae have been hypothesized to differ in their thermotolerance (Kinzie et al. 2001), which may contribute to holobiont thermotolerance. Here most *Pocillopora* colonies harbored symbionts belonging to subclade C1 (groups C1.1, C1.3 and C1.4), regardless of their habitat.

Samples were obtained from the outer reef slope (Polynesia and Tonga at 12–15 m depth) as well as lagoon (*P. damicornis* collected in Hawaii, 1–2 m depth). Symbionts belonging to subclade C1 are not structured by depth and can be considered as generalists, since they are found in different habitats and at different depths. Type A1 was found in *A. nasuta* on Moorea outer reef slopes (13 m) and also in *P. damicornis* from Bise (2–3 m), in a pool exposed to high irradiance and high sea-surface temperature during low tide periods. Hence, this clade appears to be adapted to stressful environmental conditions, as previously reported (Kinzie et al. 2001). Clade D was only found in *P. damicornis* from Moorea and Tahiti in shallow lagoons. Clade D symbionts are often found in association with hosts inhabiting turbid waters with variable temperature (van Oppen et al. 2001), chronically warm waters (Fabricius et al. 2004) or in cases where hosts are recovering from a bleaching event (e.g., Baker et al. 2004; van Oppen et al. 2005).

A possible explanation for the association of *P. damicornis* with clades C and D simultaneously, is that *P. damicornis* colonies subjected to bleaching (Gleason 1993) might have reassociated with better adapted zooxanthellae acquired from the environment. However, it is not clearly demonstrated that corals which vertically transmit their symbionts can acquire zooxanthellae from the environment.

In conclusion, this study demonstrated that (1) most corals of the genus *Pocillopora* associate with zooxanthellae that are weakly differentiated from each other, all belonging to subclade C1, (2) no particular pattern of zooxanthellae depth zonation is observed in subclade C1, (3) *P. damicornis* is more flexible than other corals of the genus *Pocillopora* by harboring symbionts belonging to clades A, C and D.

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