

Diversity of zooxanthellae from corals and sea anemones after long-term aquarium culture

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*Aquarium systems allow technically sophisticated experiments that promise new opportunities to answer urgent questions about reef coral biology, for instance assessing the responses to decreasing environmental pH and/or increased temperatures. Over recent years, long-term culture and (predominantly asexual) propagation of corals has become possible in such systems. At present however, only limited data are available that clarify whether or not responses of the coral holobiont are dominated by the acclimatization to life in captivity or continue to reflect, for example, taxonomic differences seen in nature. We studied the diversity of the symbiotic algae in corals and sea anemones after long-term aquaculture by analysis of their small subunit (SSU) ribosomal DNA gene. A field sample of *Acropora clathrata* from the Arabian Gulf which was used as a control contained clade C zooxanthellae. The aquarium corals also harboured clade C symbionts, but sequencing of the SSU DNA suggested that the analysed animals host different subclades. A prevalence of clade C is also found among corals from the Indo-Pacific region, the origin of most of the aquarium samples. An individual of the temperate sea anemone *Anemonia sulcata* (viridis) contained clade A symbionts, similar to those found in nature, even after nearly 10 years of co-culture with sea anemones (*Entacmaea quadricolor*) and corals hosting clade C symbionts. The results indicate that the specific host–symbiont association occurring in nature appears to persist over > 2 years timescales in captivity, with no mixing of symbionts between hosts maintained in the same aquarium or apparent selection of stress-tolerant symbiont strains such as clade D.*

Keywords: corals, sea anemones, aquarium culture, mesocosm, fluorescent proteins, Zooxanthellae, symbiosis, *Symbiodinium*

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INTRODUCTION

Anthropogenic impacts and climate change are threatening the survival of coral reefs worldwide (Hughes *et al.*, 2003; Hoegh-Guldberg *et al.*, 2007). The rapid decline of reefs requires urgent reform of scientific approaches in order to understand the processes underlying the degradation (Hughes *et al.*, 2010). Studies of corals in mesocosms or tank systems under controlled conditions allow sophisticated experiments that hold a great promise to yield new insights in coral biology and are ideally suited to complement traditional field studies. The impact of *ex situ* experiments is strongly dependent on how close to natural corals behave in captivity. Tremendous progress has been made over the last 20 years in improving techniques for coral culturing that allow a sustainable propagation of corals not only by natural or induced fragmentation (many species), but also by sexual reproduction (e.g. *Pocillopora* and *Tubastrea*) (Petersen *et al.*, 2007). Long term culture of corals offers the opportunity to establish distinct model strains for which information can be accumulated over time. Despite the obvious benefits of working with

established models (Weis *et al.*, 2008), questions are often raised over the extent to which aquarium corals still show natural physiological responses in experimental treatments or if their responses are distorted by the acclimatization to life in captivity. The fact that corals can thrive for decades in experimental systems (Smith *et al.*, 2009) already suggests that basic physiological processes must function well. However, the relationship between corals and their symbiotic algae (zooxanthellae) shows a degree of flexibility and corals have been shown to change their dominant symbiont type at both the population and individual levels in response to changes in their specific environment (Baker, 2003; Van Oppen *et al.*, 2009). These changes might result from 'symbiont shuffling', where there is a shift in dominance of existing algal types within the host, or by 'symbiont switching', where the host acquires new algal strains from the environment (Baker, 2003; Van Oppen *et al.*, 2009). The degree to which corals are capable of changing their algal partners in nature is still controversially discussed (Goulet, 2006). Evaluating the diversity of zooxanthellae strains in aquarium corals promises insights into the general stability of the coral–algal relationship (Smith *et al.*, 2009). Moreover, the precise knowledge of the two partners of the symbiotic association facilitates the interpretation of the responses exhibited by experimental models to imposed stress conditions. The present study surveyed the zooxanthellae diversity in the experimental

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aquarium of the Coral Reef Laboratory at the National Oceanography Centre, Southampton. Where available, we selected different colour morphs of the species for the study in order to evaluate the potential influence of the host pigments on the zooxanthellae complement. The striking differences in the visual appearance of the colour morphs result from the differential expression of green fluorescent protein (GFP)-like proteins (Wiedenmann, 1997; Wiedenmann *et al.*, 2002; Leutenegger *et al.*, 2007a, b; Oswald *et al.*, 2007; D'Angelo *et al.*, 2008). Green and red tones are caused by green and red fluorescent proteins, whereas blue morphs derive their colour from a dominant expression of purple-blue non-fluorescent chromoproteins (Alieva *et al.*, 2008; D'Angelo *et al.*, 2008). In many corals, the expression of the GFP-like proteins is regulated by the intensity of the incident blue light (D'Angelo *et al.*, 2008). In contrast, the expression of these pigments in sea anemones and at least in some corals is largely independent of the light climate (Leutenegger *et al.*, 2007a, b).

Zooxanthellae clades were determined by diagnostic restriction digest. For this purpose, the DNA region encoding the small subunit (SSU) ribosomal RNA of zooxanthellae was amplified and digested with *TaqI* and *DpnII* (Rowan & Powers, 1991; Bythell *et al.*, 1997; Savage *et al.*, 2002). The analyses were supported by cloning and sequencing of the amplified SSU rDNA fragments.

MATERIALS AND METHODS

DNA isolation

DNA was isolated from zooxanthellae using a modification of a previously described method (Doyle & Doyle, 1990). Tissue was homogenized in sterile filtered seawater. The homogenate was filtered through a 0.1 mm mesh to remove large cellular or skeletal debris. Zooxanthellae were precipitated from the solution by centrifugation (5 minutes/500 × g/4°C). The zooxanthellae pellet was re-suspended in 1 ml lysis buffer containing: 2% cetyl-trimethylammonium-bromide (CTAB); 1.4 M NaCl; 0.5% 2-mercaptoethanol; 2% polyvinylpyrrolidone (PVP); 20 mM EDTA; 10 mM Tris-HCl, (pH 8.0); and incubated at 60°C for 30 minutes in a water bath. Subsequently, the DNA solution was thoroughly mixed with 1 ml chloroform: isoamyl alcohol (24:1) followed by centrifugation (5 minutes/20,800 × g/4°C). The aqueous phase was removed and the extraction was repeated twice using 1 ml phenol:chloroform:IAA (25:24:1) and 1 ml chloroform. The DNA was then precipitated from the solution by adding an equal volume of isopropanol and centrifugation (5 minutes/20,800 × g/4°C). The supernatant was removed and the DNA pellet was washed with 1 ml 70% ethanol followed by a further centrifugation step (5 minutes/20,800 × g/4°C). After removal of the supernatant, the DNA pellet was dried, subsequently dissolved in 50 µl TE and stored at -20°C for further analyses. DNA concentration and purity were determined with a Nanodrop-ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, USA).

Amplification of zooxanthellae SSU rDNA

The SSU rDNA gene was amplified using zooxanthellae-specific polymerase chain reaction (PCR) primers ss5z and

ss3z (Rowan & Powers, 1991). Reaction mixtures with a final volume of 50 µl contained 1mM dNTPs, 0.5 µM of each oligonucleotide, 2.5 U *Taq* Polymerase (Promega, Madison, WI, USA) and 3 mM MgCl₂. Fragments were amplified in a MyCycler Thermal Cycler (Biorad, Hertfordshire, UK) using the following cycling steps: initial denaturation: 2 minutes/94°C, 40 cycles (denaturation 30 seconds/94°C; annealing 30 seconds/59°C; elongation 2 minutes/72°C) and final elongation: 5 minutes/72°C. The PCR products were separated on ethidium bromide stained agarose gels. Bands of the expected size of ~1.6 kB were purified from the gel using the JETQuick PCR Product Purification kit (Genomed, Löhne, Germany).

Restriction fragment length polymorphisms

The purified PCR products were split in two aliquots which were digested with the restriction enzymes *TaqI* and *DpnII*, respectively, following the manufacturer's protocol (New England Biolabs, Ipswich, MA, USA). Digested fragments were analysed by electrophoresis on ethidium bromide-stained agarose gels. The length of the restriction fragments were determined relative to a size standard and used for the identification of zooxanthellae phylotypes as described previously (Rowan & Powers, 1991; Bythell *et al.*, 1997; Savage *et al.*, 2002).

Sequence analyses

For selected samples, the purified PCR fragments were cloned using the StrataClone kit (Agilent Technologies, Berkshire, UK). After purification of plasmids containing inserts of the expected size, two clones were sequenced for each species by a commercial provider (Macrogen, Seoul, Korea). Sequences were deposited at Genbank under the accession numbers (JN255733–JN255743). SSU sequences of *Symbiodinium* clades B, C, D and E were downloaded from GenBank and used for comparison. Sequences were aligned using MUSCLE (Edgar, 2004) and a phylogenetic tree was constructed applying the maximum likelihood method implemented in PHYML (Guindon *et al.*, 2004). Bootstrap analyses were performed using 100 replicates to evaluate the reliability of the tree topology (Felsenstein, 1985).

RESULTS AND DISCUSSION

Scleractinian corals and sea anemones are kept and propagated for experimental purposes in the Coral Reef Laboratory at the National Oceanography Centre of Southampton (NOCS) (<http://www.soton.ac.uk/soes/research/staff/jwlw07.page?>). Individuals have been co-cultured with each other in this and preceding aquarium systems together with numerous other hexacoral and octocoral taxa for periods between 2.5 and 10 years (Table 1). During this time, the organisms experienced comparable environmental conditions and were exposed to the same recirculated water body. This gave us the opportunity to evaluate if life in captivity affects the dominant zooxanthellae strains hosted by the experimental animals, i.e. via symbiont shuffling or exchange (Baker, 2003; Van Oppen *et al.*, 2009). DNA was isolated and parts of the *Symbiodinium* SSU rDNA gene were amplified using PCR primers ss5z and ss3z (Rowan & Powers, 1991).

Table 1. Origin of samples and their predominant zooxanthellae clades.

Species	Origin	Time in captivity ^a (years)	Zooxanthellae clade
Scleractinia			
<i>Acropora microphthalma</i>	–	>2.5	C
<i>Acropora millepora</i> (red morph)	Indo-Pacific	>2.5	C
<i>Acropora millepora</i> (green morph)	Indo-Pacific	>2.5	C
<i>Acropora millepora</i> (blue morph)	Indo-Pacific	>2.5	C
<i>Acropora pulchra</i>	Indo-Pacific	> 8	C
<i>Acropora clathrata</i>	Arabian Gulf	Field sample	C
<i>Montipora</i> sp. (plate-like) (purple-green morph)	Indo-Pacific	>2	C
<i>Montipora</i> sp. (plate-like) (brown morph)	Indo-Pacific	>2	C
<i>Montipora</i> sp. (plate-like) (red morph)	Indo-Pacific	>2	C
<i>Montipora</i> sp. (plate-like) (red morph)	Aquarium Wilhelma, Stuttgart, Germany	>2	C
<i>Montipora digitata</i> (red morph)	–	> 8	C
Actiniaria			
<i>Entacmaea quadricolor</i> (red morph)	Indian Ocean (1983), Aquarium Ulm (2000) Germany	27	C
<i>Entacmaea quadricolor</i> (green morph)	–	>1.5	C*
<i>Anemonia sulcata</i> (=viridis) var. <i>rufescens</i> (2000 collection)	Elba, Italy	10	A
<i>Anemonia sulcata</i> var. <i>rufescens</i> (2006 collection)	Collioure, France	4	A

^a, by December 2010.

The PCR products were subjected to a diagnostic restriction digest using *TaqI* and *DpnII* and the zooxanthellae clades were deduced from the restriction pattern obtained from agarose gel electrophoresis (Bythell *et al.*, 1997; Savage *et al.*, 2002). One sample of zooxanthellae from *Acropora clathrata* which was prepared immediately after collection from Abu Dhabi (UAE) was analysed as control. The *TaqI* and *DpnII* restriction patterns place the zooxanthellae from *A. clathrata* within clade C (Table 2; Figure 1). The presence of this clade in corals from the Arabian Gulf is somewhat surprising since a dominance of clade D was suggested for animals in this particular environment (Baker *et al.*, 2004). Moreover, a recent study found clade D zooxanthellae on *A. clathrata* colonies from the Arabian Gulf off the Iranian coast (Ghavam Mostafavi *et al.*, 2007). This finding underlines that at least some coral species maintain variability in their association with zooxanthellae even in habitats experiencing extreme temperatures. Restriction analyses revealed that *Montipora* and *Acropora* species were found to harbour clade C symbionts. In contrast, clade A zooxanthellae were found in two *Anemonia sulcata* specimens. One of the individuals, *A. sulcata* from Elba, Italy, was already shown 9 years ago to contain clade A symbionts which are found in most cnidarians

Table 2. Fragment lengths of small subunit rDNA restriction with *TaqI* and *DpnII*.

Clade	<i>TaqI</i> (~length in base pairs (bp)) ^a	<i>DpnII</i> (~length in bp) ^a
A	700/600 ^a	850/580 ^a
B	850/500 ^a	750/500 ^a
C	880/700 ^a	860/500 ^a
C* (<i>Entacmaea quadricolor</i> green)	886/709 ^b	862/554 ^b
E	720 ^a	860/500 ^a

^a, data from Rowan & Powers (1991), Brown *et al.* (2002) and Savage *et al.* (2002); ^b, data deduced from sequencing results of the present study.

of the Mediterranean Sea and the European Atlantic coast (Savage *et al.*, 2002; Visram *et al.*, 2006). The present study reveals that the symbionts have not changed despite living for a decade under tropical temperature conditions (constant water temperatures between 23 and 26°C) and in proximity to cnidarians containing mostly clade C symbionts. The red colour morph of *Entacmaea quadricolor* (Wiedenmann *et al.*, 2002) which was co-cultured with the two *Anemonia* specimens for nearly ten years harboured clade C zooxanthellae. One representative of the green morph of *Entacmaea quadricolor* showed an unusual restriction pattern. Whereas the *TaqI* digest yielded fragments with the lengths of ~880 and 700 base pairs (bp) indicative of clade C symbionts, the smaller fragment of the *DpnII* restriction was significantly longer than expected (Figure 1A).

To resolve the uncertainty about the nature of these zooxanthellae, SSU rDNA sequences were again amplified using the ss5z and ss3z primers, cloned and sequenced. Selected *Acropora* and *Montipora* sequences were determined for comparison. The sequences were aligned and a phylogenetic tree was constructed applying the maximum likelihood method (Figure 1B). Sequence analysis revealed that the zooxanthellae of the green morph of *Entacmaea quadricolor* also belonged to clade C, however, a 'shift' in one *DpnII* restriction site resulted in an increase in the length of the smaller fragment from 499 bp to 554 bp, explaining the observed change in the restriction pattern (Table 2).

Although SSU sequences have a limited power to identify subclades in comparison to internal transcribed spacer (ITS) regions (LaJeunesse, 2001), the sequence analysis reveals well supported differences between the zooxanthellae from different individuals that suggest that each of them is associated with different clade C subtypes (Figure 1). The fact that the sequences could not be matched with known subclades of clade C is most probably due to the lack of appropriate SSU reference sequences in Genbank. A deeper classification at the level of subclades using ITS1/2 sequencing is currently underway.

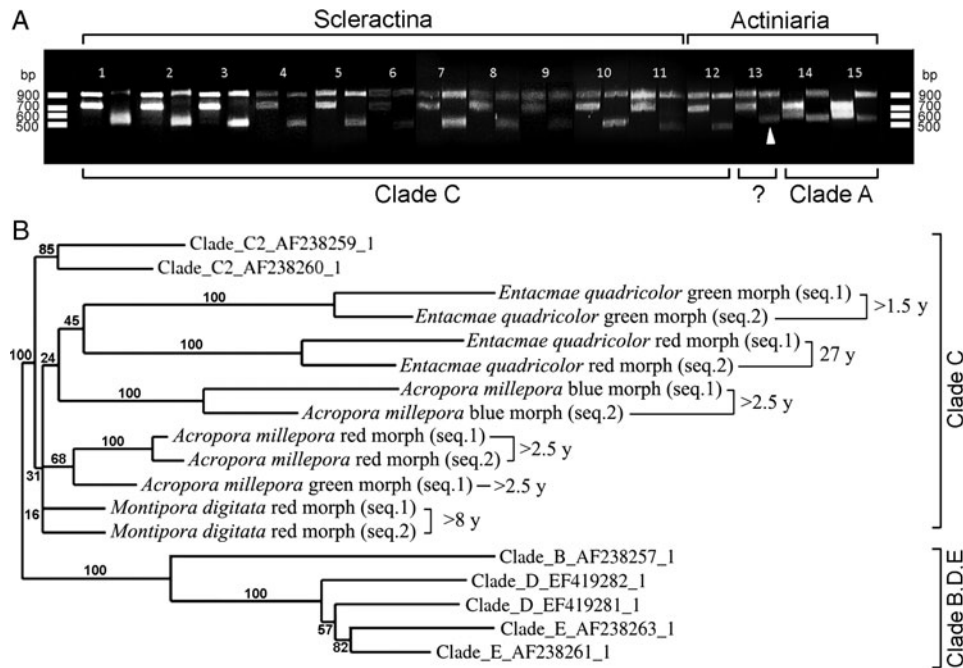


Fig. 1. Determination of zooxanthellae clades. (A) Ethidium bromide stained agarose gels show the restriction patterns of amplified *Symbiodinium* small subunit (SSU) fragments. Numbers indicate the species/colour morph under study. The DNA fragments of each individual were digested with *TaqI* (left lane) and *DpnII* (right lane). *Acropora microphthalma* (1), *Acropora millepora* pink morph (2), *A. millepora* green morph (3), *A. millepora* blue morph (4), *Acropora pulchra* (5), *Acropora clathrata* (6), *Montipora* sp. brown morph (7), *Montipora* sp. green-purple morph (8), *Montipora* sp. (red morph Ulm) (9), *Montipora* sp. (red morph Wilhelma) (10), *Montipora digitata* (11), *Entacmaea quadricolor* red morph (12), *E. quadricolor* green morph (13), and *Anemonia sulcata* collection 2000 (14), collection 2006 (15); (B) phylogenetic relationship of SSU sequences. Bootstrap values (% of 100 replicates) are shown next to the nodes. GenBank accession numbers are given for sequences that were not determined in the present study. Sequences derived from on host individual are numbered (seq. 1–seq. 2). The years (y) spent in aquarium culture are indicated at the end of the branch labels.

Sequences of zooxanthellae derived from the same individual are always most similar to each other (Figure 1B). Zooxanthellae of the red individual of *Entacmaea quadricolor* yielded sequences which are similar to those from the green individual, suggesting the presence of *E. quadricolor*-typical symbionts even after 27 years of aquarium culture. The divergence of sequences from representatives of different species with the same colour does not indicate an obvious connection between host pigmentation and the preference for a certain clade C subtype.

A high abundance of clade C symbionts in aquarium corals was also observed most recently by Smith *et al.* (2009). This correlation reflects the prevalence of clade C in Indo-Pacific reefs (Smith *et al.*, 2009; Van Oppen *et al.*, 2009) from which most of the studied corals originate, rather than a specific adaptation to aquarium conditions (Smith *et al.*, 2009). Importantly, no shift of the zooxanthellae complement to clade D is observed that could be interpreted as response to stressful conditions (Baker *et al.*, 2004) during transport or culture.

The results show that a diversity of zooxanthellae is maintained in corals and sea anemones over a long period of aquaculture. Therefore, well managed aquarium systems do not appear to lead to a selection for different symbiont types adapted to aquarium conditions, or a mixing of symbionts between hosts within the same aquarium. Such specimens may therefore be suitable for studying the physiological responses of hermatypic corals and allies to various environmental parameters over long periods of time. Moreover, the stability of symbiotic associations suggests that aquarium

systems can be helpful in *ex situ* conservation of threatened coral species.

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