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Short communication

Bacterial communities from corals cultured *ex situ* remain stable under different light regimes — Relevance for *in toto* aquaculture

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ABSTRACT

Microbial communities associated with corals are an important source of bioactive metabolites with great potential for drug discovery. However, culturing these symbiotic microbes is extremely complex and often impossible. *In toto* coral aquaculture performed *ex situ* (the culture of the holobiont–cnidarian host and associated microorganisms) has been suggested as a potential solution to solve the constraints of supplying metabolite biomass to fuel the drug discovery pipeline. In the present study we investigated if coral fragmentation and different light intensities (photosynthetically active radiation (PAR) of 50, 80 and 120 μmol quanta $m^{-2} s^{-1}$) significantly affect the diversity and structure of the microbial communities present in the leather coral *Sarcophyton* cf. *glaucum* assessed through polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE). The diversity and structure of the bacterial communities present in mother colonies and fragments stocked under identical conditions remained stable two months post fragmentation, as well as between coral fragments stocked under different PAR intensities. The observed stability in the microbial community supports that *in toto* coral aquaculture may be a suitable option to produce metabolite biomass from symbiotic microorganisms. The possibility to employ a low PAR intensity to grow coral fragments without significantly affecting their microbial diversity and structure is likely to decrease aquaculture production costs and improve its economic viability.

1. Introduction

Marine invertebrates display high microbial abundance and diversity (Olson and Kellogg, 2010; Shnit-Orland and Kushmaro, 2009; Sweet et al., 2010). Cnidarians, such as corals, harbour abundant, dynamic and structurally complex microbial communities (Ainsworth et al., 2010; Kooperman et al., 2007; Mouchka et al., 2010). Symbiotic microbes have an important role on coral health, as they produce bioactive secondary metabolites (Bourne et al., 2009; Garren and Azam, 2012; Shnit-Orland and Kushmaro, 2009). This feature has prompted bioprospecting efforts targeting chemical compounds produced by microbial communities associated with marine invertebrates (including corals) that were once attributed to their animal hosts (Hill and Fenical, 2010; Piel, 2004).

The increasing interest on new marine natural products (NPs) from corals and their microbiome (Leal et al., 2012) has promoted an intensification of coral harvest, an approach which can ultimately compromise the sustainability of bioprospecting (Montaser and Luesch, 2011) and its replicability (Li and Vederas, 2009). In order to address these issues, it has been recently recommended that future research on marine natural products targeting corals and/or their microbiome should rely on the use of specimens produced in captivity (Leal et al., 2013). Asexual propagation through fragmentation is considered as a simple solution for the sustainable and reliable supply of corals hosting photosynthetic endosymbionts (Leal et al., in press; Olivotto et al., 2011). While photosynthetically active radiation (PAR) intensity can affect coral nutrition and metabolism (Iglesias-Prieto and Trench, 1997), it can also affect the economical feasibility of coral aquaculture *ex situ* (Osinga et al., 2011; Rocha et al., 2013a).

To our best knowledge, no study has ever addressed if the diversity and structure of the microbial communities present in corals fragmented *ex situ* remains similar to that of their mother colonies, nor if different light regimes shift these microbial communities post fragmentation. In this way, our study tested the following hypotheses: 1) fragmentation does not affect the diversity and/or structure of microbial communities present in propagated corals stocked under the same conditions as mother colonies; and 2) different light regimes do not affect the diversity and/or structure of microbial communities present in fragmented corals propagated *ex situ*. The soft coral *Sarcophyton glaucum* (family Alcyonidae) was selected as a model organism for the present study given its recognized potential for the biodiscovery of new NPs (*e.g.* Badria et al., 1998; Fridkovsky et al., 1996; Tanaka et al., 2005), the suitability of fragmentation for its mass propagation *ex situ* (Calfo, 2007;

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Sella and Benayahu, 2010) and the current knowledge on its photobiology (Rocha et al., 2013b). We used a polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE) approach to screen for shifts in the structure of the bacterial communities associated with corals. PCR-DGGE is relatively less expensive and time-consuming as compared to other available approaches, such as the excision and sequence of bands, the analysis of clone libraries, or next-generation sequencing. Nevertheless, DGGE also provides an insight of the diversity and structure of microbial communities. Additionally, the rationale for selecting this methodological approach was further supported by its successful use in complex microbial communities, with next generation sequencing only confirming the structural shifts revealed by PCR-DGGE (*e.g.*, Cleary et al., 2012; Gomes et al., 2008).

2. Materials and methods

2.1. Experimental design

As the taxonomic status of *S. glaucum* is still far from being consensual (McFadden et al., 2006; Steinke et al., 2012), the corals used on this experiment were classified as *Sarcophyton* cf. *glaucum*. Given the traceability issues associated with the trade of live corals for the marine aquarium industry (Cohen et al., 2013), special attention was paid to the origin of the mother colonies employed in our study. In this way, eight mother colonies of *S.* cf. *glaucum* were selected at an aquarium wholesale facility with accompanying documentation supporting that all specimens were harvested according to sustainable practices in Sumbawa, Indonesia 48 h before the purchase. The eight mother colonies were stocked for 1 month in a recirculated system described below. Mother colonies were illuminated with a 150 W metal halide lamp (BLV, Germany) with 12 h light:12 h dark photoperiod and a PAR intensity of 120 μmol quanta m^{-2} s^{-1}. Following the acclimation period, four mother colonies were fragmented using sterilized scalpels to produce three similar sized fragments (about 3 cm diameter) (n = 3 fragments × 4 mother colonies = 12 fragments). Fragments from each mother colony were individually attached with a rubber band to a labelled plastic coral stand (Coral Cradle®) and distributed by three light treatments with the following PAR intensities: 50, 80 and 120 μmol quanta m^{-2} s^{-1}. The rationale supporting the choice of these three PAR intensities was as follows: these PAR are within the range of values that are known to promote a high survival for *S. glaucum* cultured *ex situ* (Sella and Benayahu, 2010), they are known to promote a similar growth of *S.* cf. *glaucum* fragments cultured *ex situ*, as well as significantly conditioning the production area available (in terms of m^2) (Rocha et al., 2013b). Each tank stocking the coral fragments was illuminated from above with a 150 W metal halide lamp (BLV, Germany) with 12 h light:12 h dark photoperiod. The distance between each light system and the water surface was adjusted to obtain a homogeneous PAR intensity at the level of the coral fragments for each light treatment. PAR values were measured at the level of coral fragments with a Quantum Flux meter (Apogee, MQ-200) with a submergible sensor. Each light treatment was composed of 4 replicates (1 from each mother colony). The remaining four mother colonies were kept at 120 μmol quanta m^{-2} s^{-1} until the end of the experiment.

The experimental recirculated system employed was operated with synthetic saltwater (prepared by mixing Tropic Marin Pro Reef salt – Tropic Marine, Germany – and freshwater purified by a reverse osmosis unit) and composed by 90-L glass tanks (0.6 m × 0.6 m × 0.25 m) connected to a 150 L filter tank equipped with a protein skimmer (ESC150 ReefSet, Portugal), a biological filter (composed by approximately 30 kg of live rock), two submergible heaters (Eheim Jäger 300 W, Germany), a calcium hydroxide reactor (KM500 Deltec, Germany) connected to an osmoregulator (Deltec Aquastat 1000; that provided automatic compensation for evaporated water with freshwater purified by a reverse osmosis unit) and a submerged pump (EHEIM 1262, Germany; providing an approximate flow of 1000 L h^{-1} to each tank). Additionally, each tank was equipped with a single circulation pump (Turbelle nanostream — 6025 Tunze, Germany; approximate flow of 2500 L h^{-1}). For additional details on the experimental recirculated system employed, please see Rocha et al. (2015). Partial water changes (10% of total experimental system volume) were performed every week using synthetic saltwater. Water parameters were maintained as follows: temperature 26 ± 0.5 °C, Total Ammonia Nitrogen 0.050 ± 0.002 mg L^{-1}, NO_2^-–N 0.030 ± 0.002 mg L^{-1}, NO_3^-–N 1.000 ± 0.004 mg L^{-1}, PO_4^{3-}–P 0.010 ± 0.001 mg L^{-1}, pH 8.2 ± 0.1, alkalinity 4.0 ± 0.1 mEq L^{-1}, Ca^{2+} 410 ± 20 mg L^{-1}, Mg^{2+} 1320 ± 20 mg L^{-1} and salinity 35 ± 0.5.

The experiment was finished after 2 months, with propagated coral fragments being collected and preserved in 96% ethanol and stored at −20 °C for later DNA extraction and determination of microbial communities' structure. Simultaneously, one fragment from each of the four mother colonies that were not initially fragmented was also collected and preserved as previously described.

2.2. DNA extraction and PCR amplification

Coral fragments were aseptically cut into pieces with a scalpel and homogenized for 1 min at high speed in a conventional rotor-stator type homogenizer equipped with a stainless steel probe (OMNI International TH, Warrenton, VA, USA). Coral sub-samples of 500 mg were submitted to a mechanical lysis with the FastPrep FP120 bead beating system (Bio 101 SAVANT) prior to DNA extraction. Total community DNA was extracted using the PowerSoil DNA extraction kit (MoBio Laboratories, Carlsbad, CA) following the manufacturer's instructions.

A nested PCR approach was used to amplify the V6–V8 region of bacterial 16S rDNA fragments using the bacterial primer pairs 27F/1492R (Weisburg et al., 1991) for the first PCR and 968GC/1401R (Nubel et al., 1996) for the second PCR. The PCR laboratory methodology was performed as described by Gomes et al. (2008). PCR amplicons were verified by electrophoresis using 5 μL of PCR mixtures in an agarose gel at 2% using 1× Tris-Acetate-EDTA (TAE) at 90 V for 25 min.

2.3. Denaturing gradient gel electrophoresis (DGGE)

DGGE was performed using a DCode Universal Mutation Detection System (Bio-Rad, Hercules, CA, USA). Amplified 16S rRNA gene sequences were applied on a polyacrylamide gel at 8% (w/v) containing a 40-58% denaturant gradient. The run was performed in 1× TAE electrophoresis buffer for 16 h at 60 °C and a constant voltage of 160 V. DGGE gels were silver-stained according to Heuer et al. (2001).

2.4. Data processing and statistical analysis

The analysis of DNA fingerprints obtained from the 16S rRNA banding patterns on the DGGE gels was performed using the software package GELCOMPAR 4.0 (Applied Maths, Ghent, Belgium), as described by Smalla et al. (2001). The diversity of DGGE 16SrDNA gene profiles obtained from coral samples was assessed with the Shannon index of diversity (H') being calculated based on the number of DGGE bands (diversity) and its intensity (relative abundance). The rationale supporting the use relative band intensities over a presence–absence analysis relied on the fact of: 1) this approach not obscuring the relationship between bacterial community composition and explanatory variables (Muylaert et al., 2002); and 2) the fact of relative band intensity being stable and reproducible in the same sample, with the inclusion of weaker bands decreasing the bias associated with statistical analysis (when compared to a presence/absence approach) (Schauer et al., 2000). A Student's t-test was used to determine the existence of significant differences between the H' from mother colonies and fragments stocked under the same light regime of mother colonies (120 μmol quanta m^{-2} s^{-1}), while a one-way analysis of variance

(ANOVA) was used to test for significant differences among the H' recorded for coral fragments stocked under each light treatment. One-way ANOVA and Student's t-test were performed using the software Statistica 10 (StatSoft Inc., USA) after verifying the assumptions of normality and homogeneity of variance.

The raw data matrix retrieved from GELCOMPAR 4.0 analysis was processed to relative abundances and log (x + 1) transformed, as this procedure: 1) places more emphasis on compositional differences among samples rather than on quantitative differences; 2) decreases the influence from highly dominant taxa (in our study operational taxonomic units, OTUs) with regards to that of less abundant ones; and 3) the " + 1" allows for the comparison of all bands (even absent bands) between samples (as the log transformation requires non-zero data) (Anderson, 2008). After this transformation, similarity/difference matrixes were constructed using the Bray–Curtis distance and employed to perform analysis of similarities (ANOSIM) to test for significant differences in the structure of bacterial communities: 1) between mother colonies and fragments stocked under the same light regime, and) 2) among coral fragments propagated under different PAR intensities. The output statistic of ANOSIM, R, can take a value of 0 if there are no differences of community structure or a value of 1 when a perfect dissimilarity occurs. ANOSIMs were performed using the software package PRIMER 6 (Primer-E Lta, Plymouth UK) (Clarke and Gorley, 2006).

3. Results and discussion

The DGGE profile employed to assess the structure of the bacterial communities revealed a high number of equally abundant bands in mother colonies and all fragments of *S.* cf. *glaucum* analyzed in the present work (Fig. 1). The diversity of bacterial communities of *S.* cf. *glaucum* was not affected by the process of coral fragmentation, as H' values recorded for coral fragments and mother colonies stocked for 2 months under the same PAR (120 μmol quanta m^{-2} s^{-1}) were not significantly different ($t = 0.690$; $df = 6$; $P = 0.516$) (2.10 ± 0.19 and 1.99 ± 0.20 for fragments and mother colonies, respectively; values are means of four replicate samples ± SD). The ANOSIM further confirmed that coral fragmentation did not significantly affect ($R = 0.302$; $P = 0.086$) the structure of the bacterial community recorded in the mother colonies and coral fragments stocked under the same PAR intensity (120 μmol quanta m^{-2} s^{-1}). These results are relevant to support *in toto* aquaculture (the culture of the holobiont–cnidarian host and associated microorganisms) as a tool to supply microbial biomass associated with marine invertebrates, namely corals, for drug discovery (Leal et al., 2013, 2014). Indeed, if no significant shifts occur in the structure of the bacterial community present in coral fragments, *in toto* aquaculture may contribute to solve the "supply problem" (see Leal et al., 2014), as microorganisms producing target metabolites in mother colonies are more likely to have been retained during fragmentation and/or grow-out. Well established protocols for the propagation *ex situ* of *Sarcophyton* are already available (Calfo, 2007; Sella and Benayahu, 2010), which allow the production of coral biomass using simple and economically feasible zootechnical procedures. Researchers may therefore use *in toto* aquaculture to assure a regular supply of coral biomass and their associated microbiome to fuel the initial steps of the marine drug discovery pipeline.

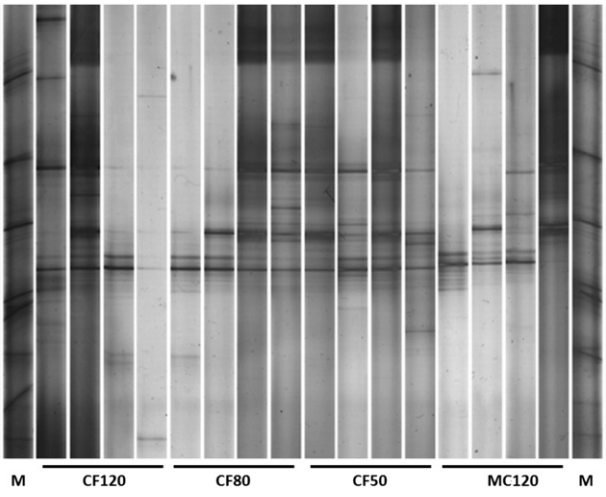

Fig. 1. DGGE fingerprint patterns of bacterial communities displayed by fragments (*CF*) of *Sarcophyton* cf. *glaucum* stocked under PAR intensities of 50, 80 and 120 μmol quanta m^{-2} s^{-1} (CF50, CF80 and CF120, respectively) and mother colonies stocked under a PAR intensity of 120 μmol quanta m^{-2} (MC120). M — molecular weight marker.

The present study also revealed that no significant differences in diversity were recorded for corals stocked under different PAR intensities ($H' = 2.10 \pm 0.19$, 1.98 ± 0.26 and 2.18 ± 0.17 for 50, 80 and 120 μmol quanta m^{-2} s^{-1}, respectively; values are means of four replicate samples ± SD; $F = 0.643$; $df = 2$; $P = 0.548$). No significant differences (ANOSIM; $R = 0.067$; $P = 0.221$) were recorded among the bacterial communities displayed by coral fragments grown under different light regimes (50, 80 and 120 μmol quanta m^{-2} s^{-1}). Light plays a key role on the physiological condition of the photosynthetic endosymbionts of cultured corals (Osinga et al., 2011). According to Rocha et al. (2013b), shifts in light intensity identical to those here investigated do not affect the photobiology or growth performance of *S.* cf. *glaucum* fragments. As already discussed by Rocha et al. (2013b), this information is economically important. The previous authors refer that the use of a low PAR intensity, such as 50 μmol quanta m^{-2} s^{-1}, decreases nearly 66% of electrical costs associated with illumination for biomass production *ex situ* of *S.* cf. *glaucum* than when using a PAR of 120 μmol quanta m^{-2} s^{-1}. Moreover, Rocha et al. (2013b) also report that by using a PAR of 50 μmol quanta m^{-2} s^{-1} it is possible to increase the production area for this species *ex situ* in nearly 66%, when compared to the use of a PAR of 120 μmol quanta m^{-2} s^{-1}.

The composition of the microbial communities associated with mother colonies and coral fragments used in this study were not determined (this task was beyond the scope of the present study; see Introduction). Nonetheless, it was possible to demonstrate that fragmentation and different light regimes (different PAR intensities) played no significant effect on their diversity (H') and structure. These findings offer goods perspectives for the use of *in toto* aquaculture to supply the biomass of target microorganisms associating with corals, particularly those known to be obligate symbionts that are extremely difficult to culture using conventional techniques (Singh and Macdonald, 2010). As highlighted by Khalesi et al. (2009), the fine tuning of stocking conditions may be used by researchers to optimize the aquaculture of coral fragments and maximize the production of target metabolites. Our study supports that, at least for *S.* cf. *glaucum*, this fine tuning of stocking conditions does not compromise the diversity and structure of its microbial community. This finding supports the suitability of this coral species for *in toto* aquaculture. Future studies addressing the supply issue of target microorganisms associating with other coral species (as suggested by Leal et al., 2013, 2014) should survey potential shifts in their microbial communities during initial culture trials. While PCR-DGGE may present a number of pitfalls when addressing band identification (for a detailed discussion on these issues please see Sekiguchi et al., 2001; Kušar and Avguštin, 2012; Neilson et al., 2013), researchers may still rely on the use of this rather inexpensive molecular approach to monitor significant shifts in microbial community structure (Neilson et al., 2013) among *a priori* defined groups (Cleary et al., 2012). In this way, we recommend the use of PCR-DGGE analysis when evaluating the suitability of *in toto* aquaculture for biotechnological purposes targeting the microbial community of corals.

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