Towards an in-depth characterization of Symbiodiniaceae in tropical giant clams via multiplex metabarcoding

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Abstract

High-throughput sequencing is revolutionizing our ability to comprehensively characterize free-living and symbiotic Symbiodiniaceae, a diverse dinoflagellate group that plays a critical role in coral reef ecosystems. Most studies however, focus on a single marker for metabarcoding Symbiodiniaceae, potentially missing important ecological traits that a combination of markers may capture. In this proof-of-concept study, we used a small set of symbiotic giant clam (*Tridacna maxima*) samples obtained from nine French Polynesian locations and developed a multiplex metabarcoding method that pools and simultaneously sequences multiple Symbiodiniaceae genes for in-depth biodiversity assessments. Our results showed that the technique effectively recovered very similar proportions of sequence reads and dominant Symbiodiniaceae clades among the three multiplexed genes investigated per sample, and captured varying levels of phylogenetic resolution enabling a more comprehensive assessment of the diversity present. Multiplex metabarcoding offers significant analytical cost savings while providing exceptional phylogenetic information and sequence coverage.

Keywords (6-10 words): Biodiversity, Marine Ecology, Multiplex Metabarcoding, High-Throughput Sequencing, South Pacific Ocean, symbiosis, *Tridacna*.

Introduction

Giant clams (Family Tridacnidae) play important roles in reef systems, acting as shelter for a number of organisms (Mercier and Hamel 1996), contributing to primary production through their symbiosis with dinoflagellates (Neo et al. 2015), and as effective filter feeders (Klumpp and Griffiths 1994). Due to their large size, relative abundance and longevity, giant clams can be considered as centennial barometers of reef health (Knop 1996). Unfortunately, as a highly prized resource throughout much of their Indo-Pacific range, giant clams also contain some of the most endangered species due to habitat degradation and overfishing, i.e. wild stock depletion and local extinctions (IUCN Red List, Version 2018).

Giant clams on shallow reefs allow for the establishment of a diverse *in-situ* reservoir of interacting fungal, bacterial, and micro-algal communities (Baker 2003; Neo et al. 2015). Importantly, they form obligatory symbioses with, and release living cells of, Symbiodiniaceae (*sensu* LaJeunesse et al. 2018), a group of dinoflagellates that are critical for the survival of a myriad of tropical invertebrates, including corals. Despite these dynamic interactions, very little is known about the extent of symbiont diversity within giant clams and the potential exchange with other reef invertebrates engaged in similar symbiotic associations (i.e. nudibranchs and corals; Wecker et al. 2015). Unlike traditional molecular techniques (e.g. PCR-based fingerprinting methods and Sanger sequencing) that have been extensively used to shed light on Symbiodiniaceae diversity in reef organisms (reviewed in Coffroth and Santos 2005; Stat et al. 2006), recent advances in High-Throughput Sequencing (HTS) technologies now enable unprecedented sequencing depth for global biodiversity assessments of symbiotic and free-living communities of Symbiodiniaceae (Boulotte et al. 2016; Cunning et al. 2015; Edmunds et al. 2014; Hume et al. 2018; Shinzato et al. 2018;

Thomas et al. 2014). Nevertheless, such studies usually focus on metabarcoding analyses of single molecular markers in isolation, in particular the *ITS2* marker (but see Thomas et al. 2014), potentially overlooking intrinsic phylogenetic differences known to occur between distinct Symbiodiniaceae genes (Pochon et al. 2012, 2014).

Here we conducted a preliminary assessment of a multiplex metabarcoding approach via the pooling and side-by-side HTS analysis of three commonly employed nuclear and chloroplastic Symbiodiniaceae markers. The ability to combine multiple gene amplicon targets per sample offers tremendous potential for analytical cost savings while providing exceptional phylogenetic information and sequence coverage. This study describes the conceptual multiplex metabarcoding approach using giant clam *Tridacna maxima* as a model and discusses future applications for improving analyses of coral reef holobionts.

Material and Methods

Sample collection and DNA extraction

For this study, twelve DNA extracts from *Tridacna maxima* biopsies, previously collected between February 1st 2011 and November 2nd 2013 from nine islands in the French Polynesian Archipelagos (Figure 1, Table S1) were used (Dubousquet et al. 2018).

Preparation of Multiplex High-Throughput Sequencing Libraries

Three sets of Symbiodiniaceae-specific primers with Illumina™ adapter tails (Table S2) were used to amplify each sample (S141-S152; Table 1) in separate Polymerase Chain Reactions (PCR). Three markers were amplified: (*i*) the Internal Transcribed Spacer 2 (*ITS2*) of the nuclear ribosomal RNA array using primers ITSD_illu and ITS2rev2_illu, (*ii*) the D1-D2 region of the 28S large subunit (*LSU*) nuclear ribosomal

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RNA gene using the newly designed primers LSU1F_illu and LSU1R_illu, and (*iii*) the hyper-variable region of the chloroplast (23S) ribosomal RNA gene using primers 23SHyperUP_illu and 23SHyperDN_illu (Manning and Gates 2008; Pochon et al. 2010). In order to sequence the three genes per sample in multiplex using HTS, individual purified products for each marker originating from the same giant clam were pooled together to enable the attachment of the same Illumina index (i.e. 12 samples). This was achieved by quantifying, diluting to 1 ng/μL and mixing 5 μL of each gene amplicon from the same giant clam together. To assess the levels of cross-contamination between samples potentially arising during the library indexing step, nine unmixed amplicon products (i.e. *ITS2*, *LSU* and *23S* amplicons from three haphazardly selected giant clams; samples S141-S143; Table 1), each with their own unique index to be added, were also prepared. More details on PCR and sequencing conditions are provided in Supplementary File 1.

Bioinformatics

For phylogenetic assignments of Symbiodiniaceae, three distinct in-house reference databases (for *ITS2*, *LSU* and *23S*) were generated and included sequence representatives from each of the nine existing Symbiodiniaceae clades (A to I), with (*i*) 409 unique sequences of *ITS2* types from GeoSymbio (Franklin et al. 2012), (*ii*) 37 representative sequences of *LSU* from Pochon et al. (2012), and (*iii*) 104 sequences of *23S* from Takabayashi et al. (2012). Amplicons recovered from sequencing were dereplicated using the USEARCH pipeline (Edgar 2010), and Symbiodiniaceae assignments were performed using a novel algorithm called 'Kallisto' (Bray et al. 2016) which only retained reads with 100% match to sequences present in the in-house databases. For sequences that did not result in exact matches, a second comparison using BLASTn against the National Center for Biotechnology Information (NCBI)

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nucleotide databases was performed and the accession numbers yielding exact matches were retained for downstream analyses. The number of unique sequences matching genotypes in the reference databases and GenBank was recorded (Table S3). Raw sequence data were submitted to the BioProject Archive under accession PRJNA471926 (SRR7181922-SRR7181942). More details on sequence processing and the bioinformatics pipeline used in this study are provided in Supplementary File 1.

Sequence Diversity Analyses

Unique sequence genotypes found at or above a 0.05% threshold from the total sequence abundance per sample were scored (Table S3), and reference genotypes identified retained for sequence diversity and phylogenetic analyses (Supplementary File 1).

Results and Discussion

A total of 1,590,047 sequences were obtained from the 21 samples (75,716 +/- 41,576 sequences per sample), which included 12 amplicon samples (S141-S152) each containing three multiplexed gene products (*23S*, *ITS2*, and *LSU*) and nine amplicon samples from three selected giant clam isolates (S141, S142, and S143) which only contained a single gene amplicon as internal controls (Table 1; Table S3). Following filtering, the proportion of total reads (Table 1) between the three genes was well-balanced with 398,442 reads (*23S*), 339,780 reads (*ITS2*), and 359,768 reads (*LSU*). In contrast, unique reads varied between 23,779 sequences for the *23S* gene and 71,776 sequences for the *LSU* gene (Table S3). The inclusion of nine positive controls, representing three amplicon products per gene sequenced in isolation, revealed the presence of low levels of sequence cross-contamination between samples (mean of 4.5 sequences ± 4.6 SD) (Table 1). This low-level of background contamination (1 to 23

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sequences per sample) represented <0.003% of the total reads per sample (Table S3). Therefore, as a conservative measure, we chose to remove sequences that represented < 0.05% of the total sequence abundance per sample.

Our bioinformatics pipeline identified 43 Symbiodiniaceae 23S chloroplast genotypes, including 16 that matched the 23S reference database and another 27 that matched sequences in GenBank. After exclusion of genotypes represented by less than 0.05% of the sequence abundance in each sample (Table S3), the number of Symbiodiniaceae genotypes retained for phylogenetic analysis was eleven (Figure S1). Similarly, analysis of the *ITS2* and *LSU* datasets led to the identification of 117 and 93 unique genotypes when using the original datasets, and to 46 and 51 unique genotypes following the 0.05% filtering threshold, respectively.

The multiplexing approach yielded similar proportions of Symbiodiniaceae genera or clades (Figure 2), but with some notable differences. The genus *Symbiodinium* (Clade A) dominated in all three markers, particularly in *23S* (91.8%; dominant subclade type chvA2), with lower but similar proportions in *ITS2* (81.7%; dominant types A3/A6) and *LSU* (83.9%; dominant types A3/A13) datasets. The genus *Cladocopium* (Clade C) represented 7.9% (dominant type chvC1), 18.2% (dominant type C1), and 15.0% (dominant type C1) of sequence reads for the *23S*, *ITS2*, and *LSU* markers, respectively. *Gerakladium* (clade G) was only detected using the chloroplast *23S* gene (0.2% of reads), whereas the nuclear *LSU* gene displayed reduced specificity for Symbiodiniaceae as indicated by ~1% of sequence reads matching other organisms such as streptophytes (*Mitchella repens* and *Asclepias verticillata*), and the host giant clam *T. maxima*. Overall, the proportion of Symbiodiniaceae genera and sub-genera recovered between the multiplexed samples and the positive (single gene) controls were

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very similar (Table S4). More details on the Results are provided in Supplementary File 1.

The concept of multiplex metabarcoding, i.e. the tagging and pooling of distinct gene regions before simultaneous sequencing of pooled samples, has been used extensively in other research fields (e.g. De Barba et al. 2014; Fiore-Donno et al. 2018), but has never been applied to Symbiodiniaceae. In this proof-of-concept study, we show that the technique effectively recovered very similar proportions of sequence reads and dominant *Symbiodiniaceae* genera among the three multiplexed genes investigated per sample, providing more confidence that single gene primer biases did not occur. Another advantage is the ability to simultaneously visualize varying levels of phylogenetic resolution, enabling a more comprehensive assessment of the diversity present. For example, while the traditional 'species-level' *ITS2* marker enabled characterization of 46 Symbiodiniaceae sub-generic types, the *LSU* marker offered both high-specificity and resolution for Symbiodiniaceae (46 sub-generic types) in addition to identifying other host-associated organisms such as streptophytes, as well as the host *Tridacna*. The short *23S* marker used here is more conserved, but has been successfully used for specifically targeting free-living Symbiodiniaceae cells from water and sediment samples that are in low abundance (Manning and Gates 2008; Pochon et al. 2010). The unique detection of *Gerakladium* (clade G) using the *23S* marker highlights the added value of the multiplex approach for broader Symbiodiniaceae screening efficiency. Analytical cost is an important consideration for any research group aiming to monitor coral reef ecosystems, and the budget needed to include HTS for biodiversity assessments is highly variable. The cost ranges between AU $40-$100 per sample (Stat et al. 2018) and depends on the number of gene regions investigated, method of library preparation, sequencing depth, and whether multiplexing is employed as shown here.

This pilot project explored the use of multiplex metabarcoding for rapid, cost-effective and in-depth characterization of Symbiodiniaceae using the giant clam *T. maxima* as a model. We found that *Symbiodinium* and *Cladocopium* were the dominant genera in adult giant clams in French Polynesia, with similar sub-generic types (*ITS2* A3, A6, and C1) previously described as commonly associated with giant clams from around the world (see Supplementary File 1). Our approach paves the way for more comprehensive surveys of this important yet endangered group of reef invertebrates and its potential role as an important Symbiodiniaceae reservoir for declining coral reefs. Future investigations may also expand on this method to clarify species-level differentiation among Symbiodiniaceae taxa using other markers (e.g. nuclear Actin, chloroplast *psbA*, mitochondrial *COI* and *16S*), or simultaneously characterize all organisms (viruses, bacteria, fungi, and other eukaryotes) associated with a more diverse host range. Such holistic diversity assessments will improve our knowledge on the ecology and evolution of tropical holobionts and better predict the adaptation of coral reefs in a rapidly changing environment.

Conflict of Interest

Xavier Pochon is an Academic Editor for PeerJ.

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Table 1 Number of DNA sequences recovered from each sample (S141-S152), before and after quality filtration, and after demultiplexing into each gene. Samples S141 to S143 were used as control samples, each targeting only one of three PCR amplicons. Columns highlighted in grey show a low background contamination.

Sample ID	**Source reads**	**Filtered reads**	**23S reads**	**ITS2 reads**	**LSU reads**
Multiplexed					
S141	75731	53654	22072	17813	13435
S142	89975	65312	26504	24395	14040
S143	78009	48881	21061	10256	17321
S144	172319	126860	48941	39131	38128
S145	147293	104743	31048	34457	38662
S146	72548	51886	23268	16817	11537
S147	118815	79339	29870	32449	16332
S148	50176	34810	12577	11695	10264
S149	4728	3381	2400	366	599
S150	88926	59387	20788	22068	16216
S151	53016	38314	15964	12882	9298
S152	60107	42239	17075	13108	11707
Controls					
ITS2 only					
S141	85824	52588	8	52335	1
S142	81924	52270	10	51988	6
S143*	130	13	5	6	2
LSU only					
S141	56565	31134	8	7	30758
S142	92110	62629	23	0	62129
S143	114431	69823	9	0	69318
23S only					
S141	77522	66763	66399	3	3
S142	42004	36422	36263	3	9
S143	27894	24239	24149	1	3
Total reads	**1590047**	**1104687**	**398442**	**339780**	**359768**

*One control sample (S143 ITS2) failed at sequencing, resulting in only 130 raw reads.

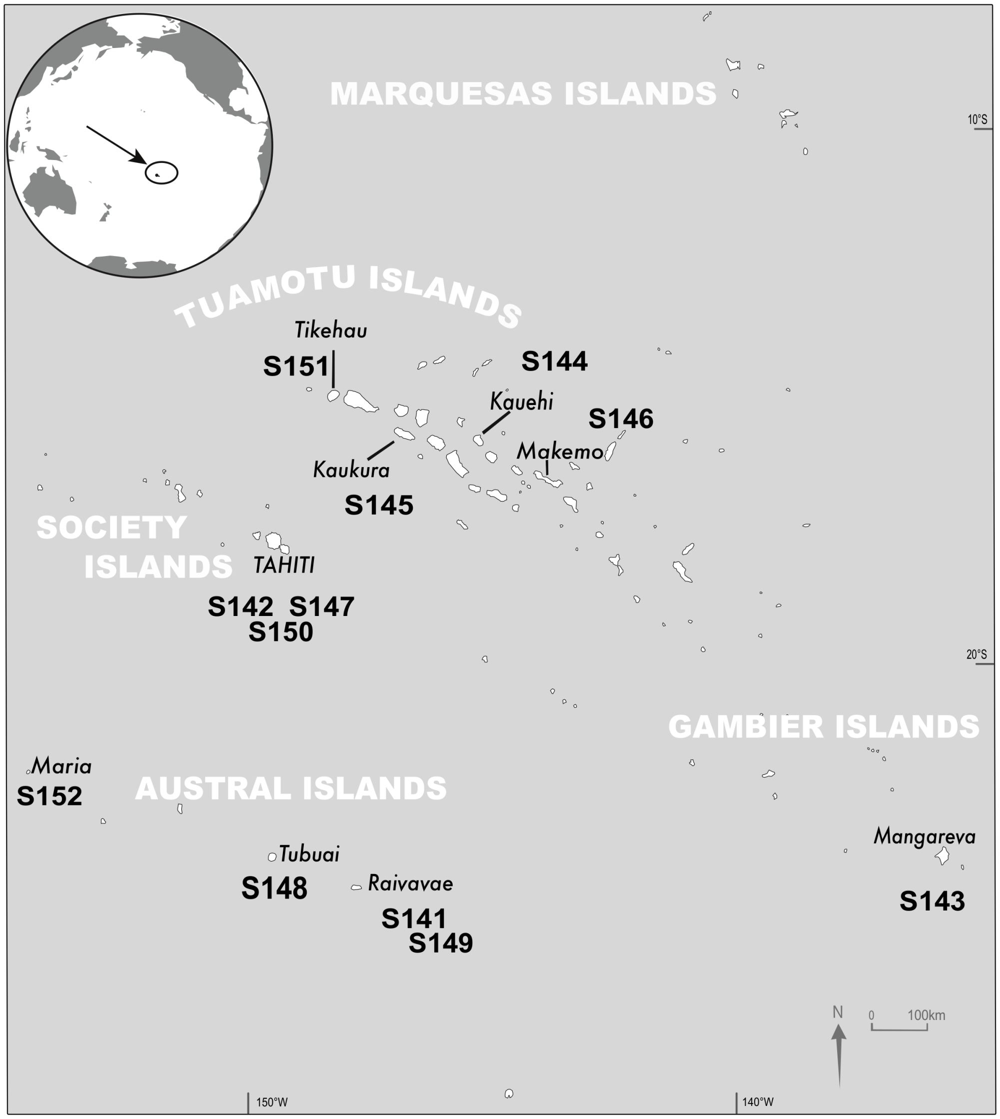

Figure 1 Location and sample identification for the twelve *Tridacna maxima* samples investigated in this study (credit to R. Canavesio).

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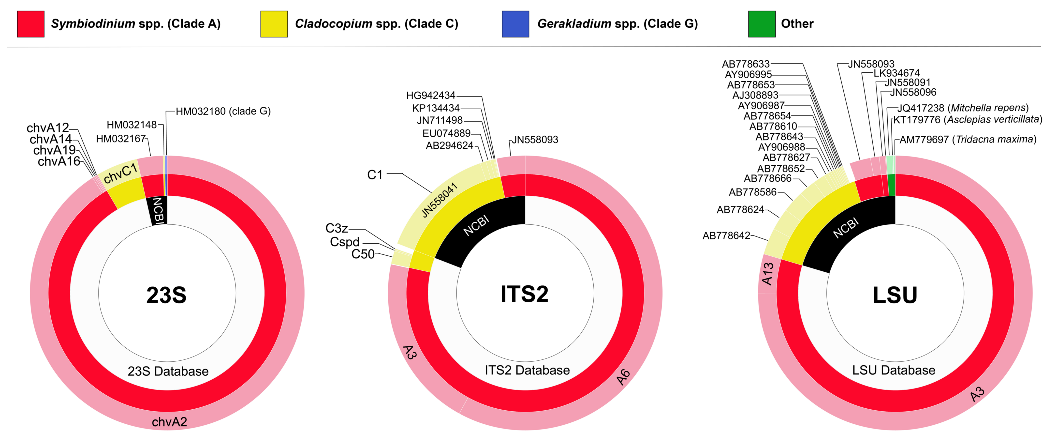

Figure 2 Global Symbiodiniaceae diversity charts obtained from each of the three datasets (left to right: *23S*, *ITS2*, and *LSU*). The proportion of sequences matching one of the three in-house reference databases or NCBI (inner circles) and their corresponding phylogenetic affiliation at genus (i.e clade; middle circles) and sub-generic (i.e. subclade; outer circles) levels. Sequence reads representing <0.1% of total read abundance are not included.

Supplementary Information

Table S1 Identification numbers, collection localities and date collected for the twelve samples of *Tridacna maxima* investigated in this study.

Table S2 List of primers used for generating PCR amplicons. Illumina adaptors are shown in bold.

Table S3 Sequence counts and blast annotations for the 21 amplicon samples analysed in multiplex and individually (controls) over three distinct genes (*23S*, *ITS2*, *LSU*). Sheet 1 includes the merged counts and dereplicated data; Sheet 2 includes exact *23S* sequence matches against the Takabayashi et al. (2012) database and NCBI; Sheet 3 includes retained *23S* genotypes following the 0.05% abundance threshold; Sheet 4 includes exact *ITS2* sequence matches against the GeoSymbio database and NCBI; Sheet 5 includes retained *ITS2* genotypes following the 0.05% abundance threshold; Sheet 6 includes exact *LSU* sequence matches against the Pochon et al. (2012) database and NCBI; and Sheet 7 includes retained *LSU* genotypes following the 0.05% abundance threshold.

Table S4 Percentage comparison of each Symbiodiniaceae sub-generic type recovered using the three markers in 'Multiplex' versus single 'Control' markers (see Table 1). The proportion of each sub-generic type between 'Multiplex' and 'Control' is almost identical for the *23S* marker, but shows some minor differences for the *ITS2* and *LSU* markers. For example, four *ITS2* types were detected in the 'Multiplex' but not in the 'Control' samples, and there were five instances where *LSU* types were detected in the 'Control' but not in the 'Multiplex' samples. These minor differences are likely attributable to PCR or sequencing biases.

Figure S1 Unrooted circled trees of Symbiodiniaceae genotypes inferred using the Neighbor-Joining method, with (**A**) 11 *23S* sequences, (**B**) 46 *ITS2* sequences, and (C) 51 *LSU* sequences.

Figure S2 Distribution of Symbiodiniaceae genera (i.e. clades) in *Tridacna maxima* obtained from each of the three datasets (left to right: *23S*, *ITS2*, and *LSU*) per sample identification (S141-152).

Supplementary File 1 Extended Materials & Methods, and Results & Discussion sections.

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