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Genetic diversity of giant clams (*Tridacna* spp.) and their associated Symbiodinium in the central Red Sea.

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Genetic diversity of giant clams (*Tridacna spp*) and their associated *Symbiodinium* in the central Red Sea.

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Abstract:

The biodiversity of the Red Sea remains relatively understudied, particularly for invertebrate taxa. Documenting present patterns of biodiversity is essential for better understanding Red Sea reef ecosystems and how these ecosystems may be impacted by stressors (such as fisheries and climate change). Several species of giant clams (genus *Tridacna*) are reported from the Red Sea, although the majority of research effort has occurred in the Gulf of Aqaba. We investigated the genetic diversity (16S rDNA) of the *Tridacna* species found in the central Saudi Arabian Red Sea. We also investigated the genetic diversity (ITS rDNA) of symbiotic dinoflagellates *Symbiodinium* associated with these clams. Samples were collected from nine reefs on a cross-shelf gradient near Thuwal, Saudi Arabia. Two species, *T. squamosa* and *T. maxima*, were recorded, with the latter being the most abundant. *Tridacna squamosina*, a species recently reported in the northern Red Sea, was not found, suggesting that this species is not present or is very rare in our study region. All tridacnids sampled were found to harbor *Symbiodinium* grouped in Clade A, considered an opportunistic, heat-tolerant symbiont group in anemones and corals. The consistent association with Clade A *Symbiodinium* in central Red Sea tridacnids may reflect the consequence of adaptation to the relatively extreme conditions of the Red Sea. This study contributes to an ever-growing catalog of Red Sea biodiversity and serves as important baseline information for a region experiencing dynamic pressures.

Introduction:

The Red Sea hosts a wide variety of marine life, a high percentage of endemic species, and one of the largest reef systems on earth (Berumen et al. 2013; DiBattista et al. 2016). The Red Sea fauna is exposed to relatively extreme conditions, such as high temperature and salinity (Ngugi et al. 2012; Raitzos et al. 2013), and likely have unique adaptations to these potential environmental stressors. In some ways, the current conditions within the Red Sea may reflect those predicted to occur at reefs worldwide with continued climatic changes, presenting an opportunity to examine the ecology of organisms and their responses to stressful environments under future ocean conditions (see Voolstra et al. 2015). To establish a foundation for future work and assessment of potential impacts, it is first necessary to establish an understanding of which species are present and their distributions throughout the Red Sea.

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4 The Red Sea's status as a hotspot of biodiversity is evidenced in the steady
5 stream of newly described species from the region (e.g., Gill et al. 2013; Terraneo et al.
6 2014; Reimer et al. 2015; Russell et al. 2015; Bogorodsky et al. 2016). Unfortunately,
7 however, several threats to biodiversity are present, including overfishing (Hasan 2005;
8 Richter et al. 2008; Spaet & Berumen 2015), habitat loss (Badr et al. 2009; Othman et
9 al. 2010), and pressures from tourism (Gladstone et al. 2013). Benthic organisms living
10 on reefs, including coral and other invertebrates, are some of the most vulnerable to the
11 above-mentioned threats. Although invertebrates constitute the majority of biodiversity
12 on reefs, they are among the least-studied organisms in almost all reef systems (Beru-
13 men et al. 2013). Research on non-coral marine invertebrates in the Red Sea is limited
14 and the majority of these studies focused on the Gulf of Aqaba, which represents < 2%
15 of the area of the Red Sea (Berumen et al. 2013). Richter et al. (2008) documented that
16 a species of giant clam (*Tridacna costata*, later synonymized as *Tridacna*
17 *squamosina*) (Huber & Eschner 2011) represented > 80% of the recent fossil record in
18 the Gulf of Aqaba, but has now been reduced to a local population of less than 13 indi-
19 viduals. The species is highly vulnerable to overfishing as it typically grows in easily ac-
20 cessible reef top habitats (Richter et al. 2008). In the south of the Red Sea, Gladstone
21 (2000) reported notable impacts on clams as a result of human harvesting. Although it is
22 not a primary Red Sea fishery, it is clear that Red Sea clams are subject to extractive
23 pressure, as is the case in other parts of the world (Lucas 1994; Ashworth et al. 2003).

24 The host's *Symbiodinium* type, diversity, and the ability to shift *Symbiodinium*
25 communities affect its overall fitness (DeBoer et al. 2012; Fay & Weber 2012; Baker
26 2003; van Open et al. 2001; Rowan 1998). Host-associated *Symbiodinium* have been
27 shown to differ in depth, irradiance, and temperature preference and corals may change
28 their *Symbiodinium* communities when these factors fluctuate (Rowan & Knowlton 1995;
29 Rowan et al. 1997; Glynn et al. 2001; Stat & Gates 2010). Clade A has been found in
30 shallow-dwelling corals exposed to high irradiance levels and has the ability to produce
31 UV-protective amino acids in culture (Rowan et al. 1997; Banaszak 2000, 2006). Clade
32 A is opportunistic as it is present following disturbances and common in corals post-
33 bleaching (Rowan et al. 1997; Toller et al. 2001). Additionally, in anemones hosting
34 *Symbiodinium*, Clade A is thought to have the highest heat tolerance and produce high
35 quality photosynthate (Venn 2008). Therefore, as a heat-tolerant, shallow-dwelling *Sym-*
36 *biodinium* clade, Clade A may prove to be abundant in Red Sea tridacnids. However,
37 the high diversity within each *Symbiodinium* clade does not allow for accurate compari-
38 sons at this taxonomic level (Savage et al. 2002) and physiological responses in corals
39 and anemones hosting Clade A may not be applicable to tridacnid hosts. This has been
40 shown in thermal experiments with tridacnids where those hosting Clade A were more
41 susceptible to bleaching under increased temperature compared to those hosting Clade
42 C (Sison 2003). In the same study, tridacnids hosting Clade A were better-suited for low
43 light environments, whereas those hosting Clade C were better-suited for high light en-
44 vironments (Sison 2003). Again, tridacnids in Indonesia were found to harbor Clades A,
45 C, and D with hosts of Clade A found in cooler water, suggesting that Clades C and D
46 are more thermally tolerant in tridacnid hosts (DeBoer et al. 2012). Even with these con-
47 tradictions, tridacnids have been found to harbor Clades A, C, and D (reviewed in Baker
48 2003) and many studies have found Clade A as the dominant clade (Carlos et al. 1999;
49 Baillie et al. 2000; Pinzón et al. 2011; Ikeda et al. 2017). Like corals, the clams are able
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4 to acquire and dispel various types from the surrounding environment, raising the possi-
5 bility of clams preferentially retaining *Symbiodinium* types well-suited for a given envi-
6 ronment (DeBoer et al. 2012; Belda-Baillie et al. 1999). Due to the high temperature and
7 light levels in the habitats of Red Sea tridacnids, it is likely that the *Symbiodinium* pre-
8 sent are thermally tolerant and are specifically adapted to this unique environment.

9
10 This paper aims to illustrate the diversity of giant clams in the genus *Tridacna*
11 and the diversity of their algal symbionts from reefs along the central Red Sea coast
12 near Thuwal, Saudi Arabia. We expect to find the following species of *Tridacna* giant
13 clams: *T. maxima*, *T. squamosa*, and *T. squamosina*, as these are previously reported
14 from the Red Sea (Bodoy 1984; Othman et al. 2010; Richter et al. 2008; Huber &
15 Eschner 2011). Samples were collected from nine reefs on a cross-shelf gradient, in-
16 cluding sampling from both the exposed and sheltered side of the reefs. Our sampling
17 design would allow us to explore potential effects of reef type on diversity of clams and
18 symbionts. This data will provide a baseline of tridacnid diversity on the central Saudi
19 Arabian coast and will allow for comparisons of biodiversity as coastal development
20 continues in the region.

21 22 23 24 25 Materials and Methods:

26 27 28 Collection:

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30 Nine reefs were chosen to represent the central Saudi Arabian coast of the Red
31 Sea (Fig. 1). Three reefs were considered inshore, three midshelf, and three offshore,
32 all characterized by relative distance from the coast. Collection of samples took place
33 during the month of August 2015.

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35 Samples were collected via SCUBA between depths of 3 to 18 meters. Samples
36 of mantle tissue between 2 and 3 cm were cut using scissors and forceps from approxi-
37 mately 30 individual clams on each reef. Half of the samples per reef were taken from
38 the exposed (windward) side, and half were taken from the sheltered (leeward) side.
39 The sampling design focused on a target number of samples and not a quantification of
40 density patterns. Samples were rinsed with Milli-Q water and cut in half. Half of the indi-
41 vidual sample was stored in liquid nitrogen for long-term storage at -80°C. The other
42 half was stored in 95% ethanol to be used for DNA extraction in this study. A photo of
43 each individual was taken *in-situ*, followed by recording depth, reef type, and reef side
44 where the clam was found (see Appendix: Table 1).

45 46 47 48 DNA Extraction:

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50 A subsample of mantle tissue about 2 mm² was used for DNA extraction. DNA
51 was extracted according to the QIAGEN DNeasy Kit blood and tissue kit (QIAGEN, To-
52 kyo, Japan) and extracted DNA was placed in 96-well plates and stored in -20°C prior to
53 polymerase chain reaction (PCR) processing. Previous studies examining the DNA of
54 tridacnids used a variety of primers to isolate the gene loci cytochrome C oxidase
55 subunit I (COI), 16S, 18S, and 28S (Su et al 2014; Richter et al. 2008; Schneider &
56 Foighil 1999). However, some studies reported that obtaining sequences using the pri-
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mer set for amplifying the COI locus was the most difficult and proved entirely unsuccessful for some samples (Nuryanto et al. 2007; Su et al. 2014). Thus, to determine the species of our samples, we used only the mitochondrial gene locus 16S, isolated using the primer set 16Sar (F) (5'-CGC CTG TTT ATC AAA AAC AT-3') and 16Sbr (R) (5'-CCG GTC TGA ACT CAG ATC ACG T-3') (Su et al. 2014).

QIAGEN Multiplex PCR Kit (QIAGEN, Tokyo, Japan) was used for the polymerase chain reaction. The protocol for isolating the 16S gene locus was as follows: initial 95°C for 15 min to activate the QIAGEN mix, 30 cycles of 94°C for 6 minutes to denature the DNA, 53°C for 1 min to anneal the DNA, 72°C for 1 min for elongation of the DNA, and a final elongation step at 72°C for 10 min. To determine the lineages or Clades of *Symbiodinium* living inside the clam tissue, there are multiple gene loci that have been successful in previous studies examining the diversity of endosymbiotic algae including the internal transcribed spacer region (ITS), the plastid psbA minicircle, ssrRNA region, and the mitochondrial marker 18S (Reimer et al. 2006; LaJeunesse et al. 2011; Carlos et al. 1999; Rowen et al. 1992). In our study, to determine the Clades of *Symbiodinium*, the ITS-rDNA gene region was isolated using the zooxanthellae specific primer set zITS (F) (5'-CCG GTG AAT TAT TCG GAC TGA CGC AGT-3') and ITS4 (R) (5'-TCC TCC GCT TAT TGA TAT GC-3') (Reimer et al. 2006). The PCR protocol for the ITS region was as follows: 95°C for 15 minutes (activation), 35 cycles of 94°C for 30 seconds (denaturation), 51°C for 45 seconds (annealing), 72°C for 2 min (elongation), and 72°C for 10 minutes (final elongation). A portion of a single DNA extraction from each samples was used for isolating both the 16S and the ITS regions.

Following PCR, samples for both tridacnids and symbionts were checked using UV light after running in 1% agarose gel under 90V for 45 minutes. Samples were then cleaned by incubating with exonuclease I and FastAP™ Thermo sensitive Alkaline Phosphatase (ExoFAP; USB, Cleveland, OH, USA) at 37 °C for 60 min., followed by 85 °C for 15 min, and then sent for Sanger sequencing at the KAUST Bioscience Core Laboratory.

Sequence Alignment and Phylogenetic Inference:

Sequences from a total of 207 samples were successfully isolated from both the 16S region and the ITS region. Sequences were analyzed, trimmed (445 bp for 16S and 658 bp for ITS), and edited using the program Geneious R8 (Biomatters Ltd., Auckland, New Zealand). A BLAST search on GenBank was performed to confirm species identity.

Haplotype networks for each organism were inferred using the Bayesian analysis PHASE 2.1 (Stephens and Donnelly 2003) implemented in DnaSP 5.0 (Librado and Rozas 2009). The 16S tridacnid network includes all 207 samples (Accession numbers: LT63007-LT630286) plus 15 reference sequences from GenBank (see Table 1). Figures for tridacna were created omitting some shorter sequences (225 bp) from Huelsken et al. (2015) to provide the reader with another representation of the data. Shorter sequences may skew results as they do not align well with our longer sequences (445 bp) (see Appendix Figures 1-4). The ITS network included all 207 samples (Accession numbers: LT634156-LT634360) plus 13 reference sequences from GenBank (see Table 3). Each run was performed with a burn-in of 100,000 generations, followed by 200,000 generations. All runs returned consistent results and were able to phase each

haplotype pair with > 90% posterior probability. The Individuals with double peaks in their chromatograms were considered to be heterozygotes (Flot and Tillier 2010). Sequences were delineated using SeqPHASE (Flot 2010) when both alleles were of identical length. After delineation, heterozygote individuals were represented by their two allelic sequences in the alignments used for network construction. Median-joining networks showing the relationships among the haplotypes were generated in NETWORK v4.6.1.3 (Bandelt 1999). The haplowebs (Flot et al. 2010) were derived from the median-joining networks by drawing curves connecting haplotypes found co-occurring in heterozygous individuals occurring in the *Symbiodinium* network only. Final editing of haplowebs was done with Adobe Illustrator (Adobe Illustrator CC version 2015.3). After haplowebs were created, all unique haplotypes were examined and samples from each haplotype, reef type, and species were chosen to be included in the following phylogenetic analyses (see Table 2 & 4).

Three phylogenetic trees were created for tridacnids and *Symbiodinium*, Bayesian inference, neighbor-joining, and maximum likelihood. The Bayesian inference (BI) method was applied to the 16S and ITS datasets to create trees for tridacnids and *Symbiodinium* using Mr. Bayes 3.1.2 (Huelsenbeck & Ronquist 2001). The evolutionary module GTR + I + G was selected using the program jModelTest-2.1.4 (Darriba et al., 2011). BI analysis utilized a Markov chain Monte Carlo search with 1 million generations with trees sampled every 100 generations. Tracer v1.6 allowed us to choose an appropriate burn-in and look for trends that would cause problems with convergence (Rambaut et al. 2014). Phylogenetic trees for both tridacnids and *Symbiodinium* following the neighbor-joining (NJ) method were also generated using the Jukes Cantor genetic distance model by the phylogenetic tree building plug-in of Geneious 8.1.3. One hundred replicates were resampled using the bootstrap method and the support threshold was set to 90. Maximum likelihood (ML) trees were created for both tridacnids and *Symbiodinium* using the program PAUP V.4 (Swofford 2003) and the bootstrap value threshold was set to 70 (see Appendix: Fig. 6). A consolidated tree using the BI tree topology, which showed the highest resolution of branch separation, was created for both organisms. The tree for tridacnids was rooted with *Hippopus porcellanus* and *Hippopus hippopus* as outgroups (Lizano et al. 2014; Schneider & Foighil 1999), reference sequences from GenBank (Table 1), and our selected sample sequences (Table 2). The consolidated tree for *Symbiodinium*, also created from the BI tree topology, was rooted with *Gymnodinium impudicum* as the out group (LaJeunesse et al. 2001), additional sequences from GenBank (Table 3), and our selected sample sequences (Table 4). Both trees were then edited in software FigTree V1.4.2 (<http://tree.bio.ed.ac.uk/>) with a branch support threshold of 90% and then final editing was done with Adobe Illustrator.

Results:

After aligning and analyzing sequences for tridacnids and *Symbiodinium*, we found polymorphic sites corresponding to multiple haplotypes. For this reason, haplotype networks for each organism were created (Figs. 2 & 3). Tridacnid sequences belong to 31 unique haplotypes with all reference sequences belonging to separate haplotypes. Samples collected in this study represent 12 unique haplotypes with no trend

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4 based on reef type (Fig. 2). *Symbiodinium* sequences belong to nine unique haplotypes
5 with collected samples from this study making up four unique haplotype (Fig. 3). Se-
6 quences for phylogenetic trees for both tridacnids and *Symbiodinium* were chosen
7 based on these haplowebs and represent all unique haplotypes, reef type, and species.
8 During alignment for creating the haplowebs, all individual with double peaks were con-
9 sidered heterozygous at that loci. Results show that only ITS sequences showed heter-
10 ozygosity.
11

12 All of the samples of this study were *T. maxima* or *T. squamosa*, with *T. maxima*
13 being the most abundant. A BLAST search of the giant clam sequences on GenBank
14 confirmed that samples were from *Tridacna maxima* and *Tridacna squamosa*. The most
15 similar sequences from GenBank were included in our phylogenetic trees (Accession
16 numbers: DQ115320, AM909744, AM909753). Many studies have used morphological
17 characteristics to differentiate the tridacnid species, however, our identification was
18 solely based on genetic analyses. This was done due to the similar morphologies of the
19 species, which makes them difficult to identify, especially in the field (Fig. 6). Our con-
20 solidated phylogenetic tree is based on the BI topology as it has the highest resolution
21 of separation amongst species. The 16S BI topology shows *T. gigas* and *T. derasa* as
22 sister species with *T. mbalavuana* as a sister to the *T. gigas/derasa* clade (Fig. 4). This
23 topology also shows *T. crocea* and *T. squamosa* as sister species with *T. maxima* as a
24 sister to the *T. crocea/squamosa* clade (Fig. 4). The BI (Fig. 4) and ML trees (see
25 Appendix: Fig. 6) show a clear separation between references for both *T. maxima* and
26 *T. squamosa* and our collected sample sequences of those species respectively. Fur-
27 ther, both the BI and ML trees show slight variation amongst *T. maxima* collected sam-
28 ple sequences. *Tridacna sp.*, or the cryptic species from Huelsken et al. (2013) is a sis-
29 ter species to *T. maxima*, *T. squamosa*, and *T. crocea* in all tree topologies. The prelimi-
30 nary phylogenetic analysis, the NJ tree, was not able to separate *T. squamosa* and *T.*
31 *crocea* as sister species (supplemental material: Fig. 5), but this separation is clearly
32 seen in the BI and ML tree topologies.
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34 The tridacnid haplotype network and phylogenetic trees do not show a clear
35 pattern or trend of tridacnids based on reef type. The four *T. squamosa* samples did not
36 show any clear spatial distribution patterns differing from *T. maxima*. One individual was
37 found on the exposed side of an inshore reef, two were found on the exposed side of
38 offshore reefs, and one individual was found on the sheltered side of a mid-shelf reef
39 (see Appendix: Table 1). With this data, we can only assume that there is no effect of
40 reef type on tridacnid genetics; therefore no further analyses were performed.
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42 A search in GenBank confirmed that all of our ITS sequences were from
43 *Symbiodinium* of Clade A, a highly diverse clade containing many strains as shown in
44 Fig. 5. The high diversity in our consolidated tree supports previous phylogenetic trees
45 of *Symbiodinium* based on the ITS gene region (Lee et al. 2015; Ikeda et al. 2017). The
46 BI tree topology showed higher resolution than the NJ and ML trees (see Appendix
47 Figs. 5 & 6), and shows three distinct clades within Clade A of *Symbiodinium* from our
48 collected samples. However, only two of these clades have strong branch support
49 values of .94 and .92 Bayesian posterior probabilities. Although there seems to be
50 separation amongst *Symbiodinium* in the samples collected in this study as suggested
51 by the three clade separations, no spatial or host species pattern can be drawn from
52 this data.
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Discussion:

Our study of *Tridacna* giant clam diversity in the central Saudi Arabian Red Sea revealed unexpectedly low diversity. Although three species are reported from the Red Sea, we encountered only two despite sampling a variety of reefs and habitats. One species (*Tridacna maxima*) was far more common than a closely related congener (*Tridacna squamosa*) despite occurring in approximately equal numbers in other parts of the Red Sea. The third species may be extremely rare (or absent) in this region or it may have highly specific habitat preferences. Our study also explored the diversity of zooxanthellate symbionts harbored by the tridacnids; we found that all of our samples exclusively hosted *Symbiodinium* assigned to Clade A. This may indicate that Clade A *Symbiodinium* represent an optimal group for the local environmental conditions., although the full diversity that these assignments represent remains to be investigated.

Morphological identification of *Tridacna* spp. poses a problem for *in-situ* studies and surveys, and thus introduces a challenge for assessments of biodiversity. As part of our study, underwater photos of each individual were examined for morphological differences. Previous studies state that *T. maxima* varies greatly in color, usually has 4-5 ribs with round projections on the upper margins of its shell, and more commonly inhabits shallow reef tops (Jantzen et al. 2008; Su et al. 2014). *Tridacna squamosa*, commonly known as the fluted clam, has exaggerated scutes that open outward and individual clams are found deeper on the reef (Jantzen et al. 2008; Chan et al. 2008; Han et al. 2008). Following genetic identification of the species, our *in-situ* photographs of *T. squamosa* (Fig. 6) show slight variation in morphology, color pattern, and depth (7-17 m) indicating subtle differences from *T. maxima*. However, relying on morphological and ecological differences is not a reliable enough way to distinguish these two species *in-situ*, so we recommend that genetic analysis be used as the most reliable to assign individuals to species. It is noted that while these markers are useful for species differentiation, they may not be conserved enough for assessments of fine-scale population genetics.

Our results suggest distinct tridacnids within the *T. squamosa* and *T. maxima* species (Fig. 4). *Tridacna maxima* samples collected in this study differed from those collected in Indonesia (Huelsen et al. 2013) as indicated by the strong branch support values (Fig. 4). This may indicate a geographically distinct *T. maxima* in the Red Sea, which is possible due to the limited gene flow and unique characteristics of the region. *Tridacna squamosa* samples collected in this study also differ from reference sequences of *T. squamosa* (Fig. 4). Again, this separation may be due to a specifically adapted species in the Red Sea. Similar results are found in recent work by Hui et al (2016), which showed genetic differentiation between far north Red Sea samples (from the Gulf of Aqaba) and the rest of the Indo-Pacific using the COI locus.

Results from our tridacnid phylogenetic tree topologies are similar to those reported in previous studies that have examined tridacnid phylogeny based on molecular analyses. Strong branch support (1 or 100%) separates species into monophyletic groups of tridacnids within the tree and supports the early phylogenetic tree topology as

shown in Schneider & Foighil (1999) with *T. gigas*, *T. derasa*, and *T. mbalavuana* (originally *T. tevora*, but recently changed in Tiavouane et al. 2016) as sister species to *T. maxima*, *T. squamosa*, and *T. crocea*. An overview of all cockles in the family Cardiidae shows *T. gigas* and *T. derasa* as sister species with *T. mbalavuana* branching off from that clade (Herrera et al. 2015). However, when using the COI gene region, Nuryanto et al. (2007), were not able to distinguish a separation between *T. squamosa* and *T. crocea*. Our results show the cryptic tridacnid as a separate species from our tridacnid collection, but a sister species to *T. maxima*, *T. squamosa* and *T. crocea* similar to the tree from Huelsken et al. (2013). Overall, our consolidated tree shows a similar topology as those produced in molecular phylogenetic studies on tridacnids using 16S and COI gene regions.

Missing in our trees and sample collection is *T. squamosina* and only very few samples were from *T. squamosa* (4 out of 207); the majority of samples were from *T. maxima*. *Tridacna squamosina* was extremely rare (6 out of 1000 samples) in a previous study from the far north of the Red Sea (Gulf of Aqaba, Richter et al. 2008), so it is possible that our sample size was not large enough to detect this rare species. On the other hand, *T. squamosina* was not previously detected anywhere in the Red Sea prior to the work done by Richter et al. (2008), so it is unclear what its true distribution in the region is. In any case, its extreme rarity and susceptibility to harvesting (Richter et al. 2008) could lead (or may have already led) to local extinction in the central Red Sea. Further, the rarity of *T. squamosa* on the central coast of the Red Sea contrasts with the abundance in the Gulf of Aqaba where a ratio of 1:1 (*T. maxima* : *T. squamosa*) was found (Richter et al. 2008). The abundance of *T. maxima* supports previous suggestions that this species is more tolerant of environmental gradients and is the most geographically widespread (Othman et al. 2010; Jantzen et al. 2008). No reef type preference among *T. maxima* and *T. squamosa*, and therefore associated *Symbiodinium*, can be concluded from our data, and due to the difficulty of distinguishing these two species *in-situ*, a sampling bias is not likely to have been a factor. The exception to this may be sampling for *T. squamosina*, which appears to occur on shallow tops of reefs, which we did not specifically target.

The local rarity of *T. squamosa* and the absence of *T. squamosina* may be reasons for conservation concern. Tridacnids are easily accessible due to their occurrence in shallow reef areas and, without enforced protection, may be vulnerable to harvesting pressure. Despite the relatively extreme conditions present in the Red Sea, zooxanthellate organisms are not immune from thermal bleaching, with recent bleaching events impacting corals (Lozano-Cortés et al. 2016; Furby et al. 2013) and anemones (Hobbs et al., 2013), and the documentation of giant clam bleaching from other areas (Addessi 2001; Buck et al. 2002; Krishnan et al. 2011; Leggat et al. 2003; Norton et al. 1995; Wilkinson 1998). Wider surveys of tridacnid distribution patterns and potential anthropogenic threats would be necessary to more completely understand the stability of their populations in the central Saudi Arabian Red Sea. This study may serve as a valuable baseline as coastal development and climate change continue to threaten the reefs in the Red Sea region.

Due to the high diversity of Clade A *Symbiodinium*, it is possible that there are specific clades associated with Red Sea tridacnids that do not match to previously documented tridacnid associated *Symbiodinium* strains. This is further supported by the

clear separation of *Symbiodinium tridacnidorum* and our samples from the Red Sea tridacnids (.91 BP) on the same degree of separation as *Symbiodinium microadriaticum* and *Symbiodinium* sequences from this study. In contrast to previous studies, our tridacnid hosts did not harbor Clades C or D (DeBoer et al. 2013; Ikeda et al. 2017; Baillie et al. 2000). Because all clam individuals harbored Clade A symbionts and no separation trend was detected despite the three separate clades shown in the BI tree, no further formal analyses were performed to explore the relationship between clam species and *Symbiodinium* clade or the relationship between reef type and *Symbiodinium* clade.

The BI and ML tree topologies for *Symbiodinium* showed a clear separation between our samples and Clade A sequences from Lee et al. (2015) and other sources (see Table 3), suggesting a possible Red Sea-specific group of clam-associated Clade A *Symbiodinium*. Although we did see separation amongst collected samples, there was no difference resolved between the *Symbiodinium* of *T. maxima* and *T. squamosa*. Because *T. squamosa* do not rely on the photosynthetic performance of their symbionts as much as *T. maxima* (Jantzen et al. 2008), it could be hypothesized that they harbor different communities of *Symbiodinium* identifiable by future work.

Previous studies have found *Symbiodinium* from Clades A, C, and D in giant clams (Carlos et al. 1999; Baillie et al. 2000; reviewed in Baker 2003), but we found only *Symbiodinium* from Clade A, a clade shown to have high heat and irradiance tolerance (Baillie et al. 2000; Venn et al. 2008) but low tolerance to temperature stress in tridacnids (Sison 2003). The association with Clade A in Red Sea tridacnids most likely involves trade offs of heat tolerance, host growth, photosynthate production, etc, making it difficult to determine the exact role of this clade. Additionally, the presence of this clade as a symbiont may be purely due to geographic location, and could serve as an indicator of symbiotic hosts in the Red Sea. Recent work continues to highlight the amount of genetic and ecological diversity that may exist within each of the established clades (e.g., LaJeunesse et al. 2014), so future work could employ additional genetic markers to increase the resolution of potential differences in *Symbiodinium* communities of our samples.

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Figure Captions:

Figure 1. Map of the Red Sea with sampling reefs circled

Figure 2. Haplotype network of all tridacnid sequences in this study and those obtained from GenBank as references. Hash marks represent mutations between haplotypes and pie diagrams depict proportion of sequences from each reef type.

Figure 3. Haplotype network of all *Symbiodinium* sequences in this study and those obtained from GenBank as references. Hash marks represent mutations between haplotypes and pie diagrams depict proportion of sequences from each reef type.

Figure 4. Phylogenetic tree of 16S tridacnid sequences from this study and references from GenBank. Branch support values are Bayesian posterior probabilities/ neighbor joining bootstrap values/ maximum likelihood bootstrap values with the Bayesian inference tree topology.

Figure 5. Phylogenetic tree of ITS *Symbiodinium* sequences from this study and references from GenBank. Branch support values are Bayesian posterior probabilities/ neighbor joining bootstrap values/ maximum likelihood bootstrap values with the Bayesian inference tree topology.

Figure 6. Photographs taken *in-situ* depicting the morphology and color patterns of *T. maxima* (row A) and *T. squamosa* (row B).

Figure 1. Map of the Red Sea with sampled reefs circled.

Figure 2. Haplotype network of all tridacnid sequences in this study and those obtained from GenBank as references. Hash

Figure 3. Haplotype network of all Symbiodinium sequences in this study and those obtained from GenBank as references. Hash

Figure 5. Phylogenetic tree of ITS Symbiodinium sequences from this study and references from GenBank. Branch support values are Bayesian posterior probabilities/ neighbor joining bootstrap values/ maximum likelihood bootstrap values with the BI topology

Figure 6. Photographs taken in-situ depicting the

Table 1. Tridacnid reference sequences from GenBank included in the phylogenetic trees.

Accession number (GenBank)	Locality	Reference
<i>Hippopus porcellanus</i>		
AF122974	USA	Schneider and Ó Foighil, 1999
<i>Hippos hippopus</i>		
AF122973	USA	Schneider and Ó Foighil, 1999
<i>T. maxima</i>		
DQ115320	Taiwan	YC Tang, unpublished observations 2005
AM909744	Jordan	Richter et al. 2008
JX974849	Solomon Islands	Huelsken et al., 2013
JX974843	Solomon Islands	Huelsken et al., 2013
<i>T. squamosa</i>		
JX974841	Australia	Huelsken et al., 2013
JX974861	Solomon Islands	Huelsken et al., 2013
KJ508358	Philippines	Lizano and Santos 2014
AM909753	Jordan	Richter et al. 2008
<i>T. crocea</i>		
AF122980	Jordan	Schneider and Ó Foighil, 1999
<i>Tridacna sp.</i>		
JX974896	Solomon Islands	Huelsken et al., 2013
JX974895	Solomon Islands	Huelsken et al., 2013
<i>T. gigas</i>		
AF122975	USA	Schneider and Ó Foighil, 1999
<i>T. mbalavuana</i>		
AF122977	USA	Schneider and Ó Foighil, 1999
KU058676	New Caledonia	Tiavouane and Fauvelot, 2016
<i>T. derasa</i>		
AF122976	USA	Schneider and Ó Foighil, 1999

Table 2. Tridacnid sequences from this study included in the phylogenetic trees.

Sample ID	Accession number (GenBank)	Locality	Reef type	Species	Haplotype
1_3	LT630079	Saudi Arabia	Offshore	<i>T. maxima</i>	2
1_4	LT630080	Saudi Arabia	Offshore	<i>T. maxima</i>	1
1_6	LT630082	Saudi Arabia	Offshore	<i>T. maxima</i>	1
1_9	LT630085	Saudi Arabia	Offshore	<i>T. maxima</i>	3
1_11	LT630087	Saudi Arabia	Offshore	<i>T. maxima</i>	1
2_2	LT630098	Saudi Arabia	Midshelf	<i>T. maxima</i>	1
2_3	LT630099	Saudi Arabia	Midshelf	<i>T. maxima</i>	1
2_5	LT630101	Saudi Arabia	Midshelf	<i>T. maxima</i>	1
2_15	LT630111	Saudi Arabia	Midshelf	<i>T. maxima</i>	1
3_2	LT630118	Saudi Arabia	Inshore	<i>T. maxima</i>	1
3_3	LT630119	Saudi Arabia	Inshore	<i>T. maxima</i>	4
3_11	LT630127	Saudi Arabia	Inshore	<i>T. maxima</i>	5
4_15	LT630151	Saudi Arabia	Inshore	<i>T. maxima</i>	1
4_17	LT630153	Saudi Arabia	Inshore	<i>T. maxima</i>	6
4_18	LT630154	Saudi Arabia	Inshore	<i>T. squamosa</i>	7
5_10	LT630166	Saudi Arabia	Offshore	<i>T. maxima</i>	1
5_12	LT630168	Saudi Arabia	Offshore	<i>T. maxima</i>	4
5_14	LT630170	Saudi Arabia	Offshore	<i>T. maxima</i>	1
5_16	LT630172	Saudi Arabia	Offshore	<i>T. maxima</i>	8
6_3	LT630179	Saudi Arabia	Midshelf	<i>T. maxima</i>	1
6_7	LT630183	Saudi Arabia	Midshelf	<i>T. maxima</i>	9
6_11	LT630187	Saudi Arabia	Midshelf	<i>T. maxima</i>	1
6_20	LT630196	Saudi Arabia	Midshelf	<i>T. maxima</i>	1
7_5	LT630201	Saudi Arabia	Offshore	<i>T. maxima</i>	1
7_11	LT630207	Saudi Arabia	Offshore	<i>T. squamosa</i>	7
7_13	LT630209	Saudi Arabia	Offshore	<i>T. squamosa</i>	7
7_21	LT630217	Saudi Arabia	Offshore	<i>T. maxima</i>	10

Sample ID	Accession number (GenBank)	Locality	Reef type	Species	Haplotype
8_13	LT630239	Saudi Arabia	Midshelf	<i>T. maxima</i>	1
8_15	LT630241	Saudi Arabia	Midshelf	<i>T. maxima</i>	11
8_25	LT630251	Saudi Arabia	Midshelf	<i>T. maxima</i>	1
8_26	LT630252	Saudi Arabia	Midshelf	<i>T. squamosa</i>	7
8_30	LT630256	Saudi Arabia	Midshelf	<i>T. maxima</i>	1
9_2	LT630258	Saudi Arabia	Inshore	<i>T. maxima</i>	1
9_3	LT630259	Saudi Arabia	Inshore	<i>T. maxima</i>	13
9_10	LT630266	Saudi Arabia	Inshore	<i>T. maxima</i>	10
9_12	LT630268	Saudi Arabia	Inshore	<i>T. maxima</i>	14
9_18	LT630274	Saudi Arabia	Inshore	<i>T. maxima</i>	16
9_24	LT630280	Saudi Arabia	Inshore	<i>T. maxima</i>	1
9_28	LT630284	Saudi Arabia	Inshore	<i>T. maxima</i>	4
9_30	LT630286	Saudi Arabia	Inshore	<i>T. maxima</i>	17

Table 3. Symbiodinium reference sequences from GenBank included in the phylogenetic trees.

Accession Number (GenBank)	Locality	Reference
<i>Gymnodinium impedicum</i>		
AF131074	Not specified	Kim et al. unpublished observations 1999
<i>S. microadriaticum</i> Clade A		
LK934669 (strain CCMP828)	Korea	Lee et al. unpublished observations 2014
LK934672 (strain CCMP828)	Florida, USA	Lee et al. unpublished observations 2014
LK934667 (strain CCMP2458)	Gulf of Aqaba	Lee et al. unpublished observations 2014
<i>S. tridacnidorum</i> Clade A		
HQ896363 (strain A3-223)	Indonesia	DeBoer et al. 2012
HQ896366 (strain A3)	Indonesia	DeBoer et al. 2012
KM816410 (strain A3)	GBR, Australia	Lee et al. 2015
EU074857 (strain A3)	Not specified	Thornhill et al. 2007
<i>S. californicum</i> Clade E		
AF334659	California, USA	LaJeunesse 2001
<i>S. pulchrorum</i> Clade B1		
AF333511	Hawaii, USA	LaJeunesse 2001
<i>S. goreau</i> Clade C		
AF333515	Jamaica	LaJeunesse 2001

Table 4. Symbiodinium sequences from this study included in the phylogenetic trees.

Sample ID	Accession number (GenBank)	Locality	Reef type	Host species	Heterozygous	Haplotype
1_1	LT634156	Saudi Arabia	Offshore	<i>T. maxima</i>	N	1
1_14	LT634169	Saudi Arabia	Offshore	<i>T. maxima</i>	Y	1/3
1_15	LT634170	Saudi Arabia	Offshore	<i>T. maxima</i>	Y	1/3
2_2	LT634177	Saudi Arabia	Midshelf	<i>T. maxima</i>	N	2
2_3	LT634178	Saudi Arabia	Midshelf	<i>T. maxima</i>	N	1
3_1	LT634196	Saudi Arabia	Inshore	<i>T. maxima</i>	Y	1/3
4_6	LT634221	Saudi Arabia	Inshore	<i>T. maxima</i>	N	2
4_19	LT634234	Saudi Arabia	Inshore	<i>T. maxima</i>	Y	1/2
5_1	LT634236	Saudi Arabia	Offshore	<i>T. maxima</i>	Y	2/3
5_3	LT634238	Saudi Arabia	Offshore	<i>T. maxima</i>	N	2
6_5	LT634260	Saudi Arabia	Midshelf	<i>T. maxima</i>	Y	2/3
7_12	LT634288	Saudi Arabia	Offshore	<i>T. maxima</i>	Y	1/2
7_22	LT634298	Saudi Arabia	Offshore	<i>T. maxima</i>	Y	1/3
8_17	LT634323	Saudi Arabia	Midshelf	<i>T. maxima</i>	Y	1/2
8_26	LT634332	Saudi Arabia	Midshelf	<i>T. squamosa</i>	Y	2/4

Supplementary Material. Figure 1. Haplotype network of all tridacnid sequences in this study and those obtained from

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Supplementary Material

Figure 1_GCNJXhaplotype.pdf

Supplementary Material. Figure 2. 16S Bayesian Inference tree of sequences from this study and reference sequences excluding the

Supplementary Material. Figure 3. 16S neighbor joining tree of sequences from this study and reference sequences excluding the

Supplementary Material. Figure 4. 16S maximum likelihood tree of sequences from this study and reference sequences excluding the

Supplementary Material. Figure 5. 16S neighbor joining tree of sequences from this study and reference sequences from

Supplementary Material. Figure 6. 16S maximum likelihood tree of sequences from this study and reference sequences from

Supplementary Material. Figure 7. ITS neighbor joining tree of sequences from this study and reference sequences from

Supplementary Material. Figure 8. ITS maximum likelihood tree of sequences from this study and reference sequences from

Dear Dr Moritz Sonnewald,

Thank you for the opportunity to revise our manuscript for Marine Biodiversity. We have addressed the following points and now feel the manuscript is much improved. We hope you find it acceptable for publication in Marine Biodiversity at this time, although we would welcome any additional feedback you may have.

Our responses to each comment are provided below in colored text.

Reviewer comments:

Reviewer 1:

Genetic diversity of giant clams (*Tridacna* spp.) and their associated *Symbiodinium* in the central Red Sea.

Pappas et al. have investigated Tridacnid and *Symbiodinium* diversity in a poorly understood region of the Red Sea. By using a single gene marker (16S, ITS rDNA), the authors are able to identify multiple populations of *Tridacna* to the species level and which *Symbiodinium* strain is most prevalent. I found most of the manuscript to be concise and clearly written. However, there are some significant methodological and conceptual issues that must be addressed. I have the following comments that I think would strengthen the manuscript for publication.

Thank you for the positive and constructive feedback. We are grateful for the reviewer's time and we agree that the manuscript has been strengthened as a result of the suggested changes. Specific responses are below.

Figures:

The figures need to include the full figure legend.

We apologize for the issue with the figure legends not displaying fully in the figures in the PDF of this paper generated by the submission system. However, I believe this is a formatting error on the journal's submission page, which should be resolved in the final version. We have now included the figure captions following the references in the text to make them available to you.

Sequence Alignment and Phylogenetic Inference:

I found this section of the manuscript very confusing and in need of substantial clarification. The authors employ three methods to reconstruct a single gene tree for both Tridacnids and for *Symbiodinium* yet they only included two trees, the strict consensus trees from the UPGMA analysis. The additional four trees should be made available as part of the appendix if not provided in text.

We have addressed these concerns with a complete re-analysis of the sequences using the three methods as suggested by the reviewer. We have also realized the confusion caused by providing only one tree when the methods clearly stated that we created trees based on multiple methods for both tridacnids and *Symbiodinium*. In the manuscript, we now present two figures (Figure 2 and 3) representing the trees based on the BI method. The four other trees are now provided in the appendix as suggested by the reviewer.

Two of the methods (NJ and UPGMA) are not very robust methods of reconstruction and are either outdated (UPGMA) or typically used as a preliminary start point for more thorough analyses. It may be that all three analyses yielded an identical topology and, if so, the authors need to be explicit in conveying this information. Both NJ and UPGMA are distance based reconstructions so I question why the use of both. UPGMA is notoriously unreliable because it is very sensitive to unequal rates of molecular evolution (an assumption that is clearly violated in this dataset).

This issue raised by the reviewer is a valid concern and we addressed it by only including one distance-based method (NJ) to create trees. All reference to UPGMA have been removed. Also, because the reviewer pointed out that the NJ method is usually used as a preliminary starting point, we continued with the suggested methods of Maximum Likelihood and Bayesian Inference to create trees. Also, because all trees had similar topology for each organism, we created a consolidated tree based on the BI topology and included branch support values for each tree. This is now described in the Materials and Methods section. Separate trees (NJ and ML) can be found in appendix for both *Symbiodinium* and tridacnids.

It would also be useful for the authors to cite the software used to conduct the UPGMA analysis.

Due to the concerns that the reviewer raises about the UPGMA analysis, we did not include this in our revised version of the paper, and as mentioned above, all reference to UPGMA is removed. However, for all other models used, the software is cited.

While BI is much more computationally intensive, it allows the investigator to employ much more robust models of molecular evolution. I think the authors would greatly improve this manuscript if they conducted Maximum Likelihood analysis. ML analyses are fast, easy to implement, and will yield more accurate estimates of branch lengths. The authors could easily do an ML analysis using Garli, RAxML, or even PAUP*.

This is a great suggestion that ultimately strengthens the paper. We have decided to include a ML analysis using PAUP*. Branch support values for the ML trees are included in the consolidated tree and separate ML trees can be found in appendix.

How did the authors decide to make the “combined” tree? By combined, do they mean a reduced representation of the taxa? If so, how did they chose to reduce the sampled individuals?

The original trees showed consolidated trees of the following methods: NJ, BI, and UPGMA. However, revisions were made to include NJ, BI, and ML methods, which better represent the data, but again we consolidate the trees due to similar topologies from all methods. The reduction of the sampled individuals was originally done to represent each species at each reef type (inshore, midshelf, and offshore). However, after re-analysis of the sequences, we have decided to run haplotype analyses for both tridacnids and *Symbiodinium*. After examining the haplotypes of these organisms, sequences that represented unique haplotypes for each species and each reef type were chosen to represent all sampled sequences. (see Table 3 & 4).

Line 24: Sequences from a total of 207 samples were successfully isolated from both the 16S region and the ITS region. If you sequenced this many individuals and analyzed them in a phylogenetic context, you need to show these data. If you recovered large polytomies, you then be explicit in how you have chosen to represent your data. I think what the authors have done is sink polytomies by shelf location but it is not clear.

As described in the previous response, the reviewer is correct to say that we consolidated sampled sequences based on shelf location, however, with the revised trees, many more sequences were chosen to represent all unique haplotypes, species, and shelf locations. We have clarified this in the text. All of the sequences chosen to represent the haplotypes, species, and shelf locations are shown in the trees. A full list of the 207 samples can be found in the Appendix and the list of samples chosen to be used in the phylogenetic trees can be found in Table 2 and Table 4.

Bayesian analysis:

Did the authors assess stationarity and convergence in the BI analysis? This is a necessary step to identify and discard the burn-in for the analysis.

We were able to determine the burn-in by using Tracer V1.5, which analyzes trace files generated by Bayesian MCMC runs. The program is able to trace the parameter against state or generation number, which allows us to choose a suitable burn-in and look for trends that might suggest problems with convergence. This was not directly stated in the paper and so we have included this explanation with the description of the BI methods. We thank the reviewer for bringing this to our attention so that we could clarify the methods.

Trees and Branch support:

Figures 2 and 3:

It would be helpful to have the full figure legends included. Where do the node support values come from? Are they bootstrap proportions? Posterior probabilities? The authors need to be explicit with what these values represent. In figure 2 the split between *T. gigas* (AF 122975) and *T. tevora* (AF 233976) has very conflicting support values (100/0/91.8). Is this a mistake? Which method gives no support?

As mentioned above, the full figure legends are now found following the references and should also be resolved in the final version of the document. Each legend now specifies the branch support values as either bootstrap values or posterior probabilities depending on method. We greatly appreciate the reviewer's attention to detail in seeing the values for the separation between *T. gigas* and *T. tevora* (which has now been changed to *T. mbalavuana*, as it is described in Tiavouane & Fauvelot 2016). This was a typo, and the original node support here, represented the BI node values of 100.

Results

Line 57: Although previous studies have used morphological characteristics to differentiate the tridacnid species...

While many previous studies have focused on using gross morphology to define Tridacnids (and bivalves in general), several recent studies have utilized molecular data to assess the relationship within Tridacna (Schneider & Foighil 1999; Nuryanto et al. 2007; Huelsken et al. 2013), and among other marine bivalves (Herrera et al. 2015). This manuscript would greatly benefit from adding to the discussion and within the results how the current study compares, and possibly differs, to previous studies.

We agree with the reviewer and see the benefit of comparing our results to previous literature using molecular tools to create phylogenetic trees for tridacnids and *Symbiodinium* brings to the paper. For this reason, we have included a short comparison of our results to the suggested literature (Schneider & Foighil 1999, Nuryanto et al. 2007, Huelsken et al. 2013, and Herrera et al. 2015). The quoted text is from the discussion section.

"Results from our tridacnid phylogenetic tree topologies are similar to those reported in previous studies that have examined tridacnid phylogeny based on molecular analyses. Strong branch support (1 or 100%) separates species into monophyletic groups of tridacnids within the tree and supports the early phylogenetic tree topology as shown in Schneider & Foighil (1999) with *T. gigas*, *T. derasa*, and *T. mbalavuana* (originally *T. tevora*, but recently changed in Tiavouane et al. 2016) as sister species to *T. maxima*, *T. squamosa*, and *T. crocea*. An overview of all cockles in the family Cardiidae shows *T. gigas* and *T. derasa* as sister species with *T. mbalavuana* branching off from that clade (Herrera et al. 2015). However, when using the COI gene region, Nuryanto et al. (2007), were not able to distinguish a separation between *T. squamosa* and *T. crocea*. Our results show the cryptic tridacnid as a separate species from our tridacnid collection, but a sister species to *T. maxima*, *T. squamosa* and *T. crocea* similar to the tree from Huelsken et al. (2013). Overall, our

consolidated tree shows a similar topology as those produced in molecular phylogenetic studies on tridacnids using 16S and COI gene regions.”

Line 6-9: The separation of species grouped into separate subgroups is strongly supported by high bootstrap values (90 – 100 %)

How does this relate to previous research done on Tridacnids? Do you recover the same relationship as previous studies?

Yes, we did find similar results when we compared our trees to previous studies examining tridacnid phylogeny. Phylogenetic trees from Huelsken et al. 2013, Herrera et al. 2015, and Nuryanto et al. 2007 showed topologies very similar and even identical in some areas as our tree. We have added in a paragraph comparing our results with those of the literature suggested in the previous comment. To compare this result with previous literature, we describe our results with those suggested in the discussion section.

A minor point: bootstraps (BP) are not a percentage between species and variation amongst species was little as shown by the Bayesian Inference tree.

As noted earlier, this should be included in the supplemental.

We have clarified the wording for these details. Each tree is now described with the proper language depending on each method and all trees are provided either in the paper or in supplemental materials.

Line 11-14: *Tridacna maxima* is clearly separated from all other species of tridacnids...

Your results have recovered *T. maxima* as being sister to all other *Tridacna*. Being separated would imply that *T. maxima* is paraphyletic and possibly not a Tridacnid, however this phylogenetic analysis shows *T. squamosa* and *T. crocea* as closely related species...

Clarify the wording here:

T. squamosa is sister to *T. crocea* with a separation quantified by a bootstrap value of 52% in the BI tree.

Bayesian analyses do not give you bootstrap support, do you mean a posterior probability (PP) of 52? A PP/ BP of 52 is very weak support.

We have adopted the suggested wording. The original node value of 52 was produced by the BI method, but with revised trees, a different result is described. The new tree shows a BI bootstrap value of .96

Line 25-36: The NJ tree showed clear separation between *T. maxima* and *T. squamosa* with a bootstrap value of 98% and a clear separation between *T. squamosa* and *T. crocea* with a bootstrap value of 93.4%, which was a stronger separation when compared to the BI method. Again, the NJ method showed a stronger separation between *T. squamosa* samples and previously entered

sequences for *T. squamosa* collected in the Philippines (NJ bootstrap 95.2% vs BI bootstrap 71%). Like the BI method, all *T. maxima* grouped together with a strong separation of 98% in the NJ tree. The UPGMA method further supported the separation of all groups with 100% separation between *T. maxima* and *T. squamosa*. There was also a stronger separation between *T. crocea* and *T. maxima* shown by the bootstrap value of 94.6% in the UPGMA tree method.

Again, it is necessary to include all the trees from the different reconstruction methods if you are going to compare their results.

We agree that this is a necessary inclusion. As mentioned above, all trees are now included in the appendix.

There seems to be some confusion as to what support values are given by each type of analysis. Bayesian analyses allow you to assess posterior probabilities not bootstrap proportions. The support values and tree topology shown in Figure 2 suggest *T. maxima* and *T. squamosa* nested within a larger polytomy with no/ weak support. As presented here, the authors have found strong support (100/98/100) for the monophyly of *Tridacna*. *T. maxima* may be sister to all other *Tridacna* (as pointed out earlier) by a very short branch that is not discernable but it is not clear in the figure. The authors recovered *T. squamosa* as sister to *T. crocea* with strong support (90/98.6/94.6). *T. gigas*, *T. tevora*, and *T. derasa* form a well supported clade, but their placement seems uncertain in respect to other *Tridacnids*. Line 37-40: *Symbiodinium* samples were all from clade A with a slight separation as shown by the BI method (bootstrap values between 67 and 96%) amongst the six samples shown (Figure 3).

As mentioned earlier, all wording has been changed to better describe results from each method for each tree. Again, thank you for ensuring that we use the proper vocabulary.

Line 19-20 in Results: no formal analysis on its spatial patterns were conducted. What were the formal spatial analyses conducted? There is this one statement about spatial analysis but I did not see where this was done or its results.

We see the confusion here, as we did not specify how we were examining spatial patterns. We have added clarification on the spatial analysis and the decision to not investigate into it further. For the clams, one species was rarely encountered, precluding any meaningful analysis of its spatial distribution. For the more common species (*T. maxima*), there was no apparent spatial segregation in the phylogenetic trees, so we did not pursue any formal analysis for this. Likewise for the symbionts, all the samples were found to have the same clade, so no further analyses of spatial patterns were conducted. This has been clarified in the Results section for clams and symbionts.

We have added text in the Results section:

“The tridacnid haplotype network and phylogenetic trees do not show a clear pattern or separation trend of tridacnids based on reef type. The four *T. squamosa* samples did not show any clear spatial distribution patterns differing from *T. maxima*.”

And:

“Although there seems to be separation amongst *Symbiodinium* in the samples collected in this study as suggested by the three clade separations, no spatial or host species pattern can be drawn from this data.”

Clade A (green) is sister to Clade A1 (orange) with strong to moderate support, depending on what the node values represent in Figure 3, rather than being “slightly separated”.

During the revision process, the results section was largely rewritten. We have taken care to be more precise with the vocabulary used when describing the relationships shown by the trees.

Discussion:

I would like the authors to expand on the discussion by including the conservation implications of this work. It is clear Tridacnids are in danger of experience drastic reductions due to several factors such as over collecting and climate change. How do the authors think Tridacnid diversity has shifted due to these threats?

We see the importance of discussing the conservation implications of this study and agree with the reviewer’s comment. We have expanded the Discussion to explicitly address these concerns. A paragraph has been added at the end of the Discussion to highlight the potential threats arising from over-collection and bleaching. There is also Discussion material about the rarity of *T. squamosina* and the uncertainty about its true distribution throughout the Red Sea. Our sample size may not have been large enough to detect its presence in the central Red Sea or it may have a restricted regional or habitat distribution.

The authors point out that morphology is not a useful feature for identifying species of Tridacna and therefore the use of molecular data is necessary. Given the large lack of resolution seen within Tridacna using 16S, is this actually a more robust alternative? While 16S has been successfully used in other studies (Schneider & Foighil 1999; Herrera et al. 2015) to determine Tridacnid phylogenetics, it does not seem like an ideal marker for population level studies.

We agree with the reviewer that the 16S gene region is not ideal to identify population structure within one species of tridacnids. However, because the aim of this study was to determine the species present and not to determine population genetics, 16S was ideal and provided a high enough resolution to the species level for all samples. The reviewer is correct that we were unable to resolve spatial patterns within our study, for example, but we were able to use the method to discriminate the species reliably. We have added a note in the discussion to address the issue of using 16S to detect fine-scale changes in populations.

Added in the text:

“However, it is noted that while these markers are useful for species differentiation, they may not be conserved enough for assessments of fine-scale population genetics.”

Herrera, N.D., ter Poorten, J.J., Bieler, R., Mikkelsen, P.M., Strong, E.E., Jablonski, D. & Stepan, S.J. (2015) Molecular phylogenetics and historical biogeography amid shifting continents in the cockles and giant clams (Bivalvia: Cardiidae). *Molecular Phylogenetics and Evolution*, 93, 94–106.

Huelsken, T., Keyse, J., Liggins, L., Penny, S., Trembl, E.A. & Riginos, C. (2013) A novel widespread cryptic species and phylogeographic patterns within several giant clam species (cardiidae: Tridacna) from the Indo-Pacific ocean. *PLoS ONE*, 8.

Nuryanto, A., Duryadi, D., Soedharma, D. & Blohm, D. (2007) Molecular Phylogeny of Giant Clams Based on Mitochondrial DNA Cytochrome C Oxidase I Gene. *HAYATI Journal of Biosciences*, 14, 162.

Schneider, J. a & Foighil, D.O. (1999) Phylogeny of giant clams (Cardiidae: Tridacninae) based on partial mitochondrial 16S rDNA gene sequences. *Molecular phylogenetics and evolution*, 13, 59–66.

The studies cited by the reviewer add very important comparisons and information related to the present study and so were included in the paper appropriately. They can be found in the literature cited pages.

Reviewers' comments:

Reviewer #2: Reviewer 2

Manuscript Number: MARB-D-16-00384

Melissa K. Pappas, Song He, Royale S. Hardenstine, Hana Kanee, Michael L. Berumen, « Genetic diversity of giant clams (Tridacna spp.) and their associated Symbiodinium in the central Red Sea.»

This study contributes to round out the picture of mtDNA (16S) variation in giant clams and their associated Symbiodinium (ITS) across the world, adding new data from Red Sea.

The major criticism of this study is the use of old databases for phylogenetic inferences, both for giant clams and Symbiodinium. Huelsken et al. provided 54 unique haplotypes for 16S in 2013 (Huelsken et al. 2013). Doubtless, the separation of giant clam species in this study will be improved, especially for *T. maxima* spp. For Symbiodinium, even if the authors used ITS rDNA, studies highlighted that ITS2 provides sufficient resolution to discriminate Symbiodinium spp (Arif et al. 2014). Because ITS2 is included in the ITS-rDNA region, the authors can use the ITS2 databases to discriminate their Symbiodinium species.

We appreciate the suggestion of the reviewer to add sequences from recent publications on tridacnids and *Symbiodinium* to the trees in this paper. We have now included these in our analyses. The addition of these sequences provides a more thorough and descriptive relationship of both organisms. Multiple sequences generated from Huelsken et al. (2013) were added in the tridacnid trees (see Table 2). Although the tridacnid tree structure and supports did not change much with the addition of these sequences, our process of creating the phylogenetic trees was improved by consulting the haplotype information of our sequences as Huelsken et al. (2013) has done. As noted above in response to Reviewer #1, we have done a thorough re-analysis of our dataset and have described those approaches above.

Sequences from Arif et al. (2014) were also added to the phylogeny of *Symbiodinium*, giving us a higher resolution, which has helped us explain the diversity within Clade A alone. Sequences were labeled with strain or species where applicable.

The ITS sequences of this study chosen to be included in the phylogenetic tree represent each haplotype, each reef type, and each strain present in the samples. However, the final phylogenetic tree does not show separation of our samples based on any of these variables.

It is important to note that in the appendix, we have also included trees and a haplotype network for tridacnids omitting some of the suggested sequences from Huelsken et al. (2013). Both tridacnid trees and haplotype networks were created with and without the addition of the “cryptic” species of *Tridacna* to determine which was a better representation of our data. The cryptic tridacnid sequences from Huelsken et al. (2013) were much shorter (225 bp) than our sequences (445 bp) and thus to preserve the integrity of our data and alignment, we created figures omitting the shorter sequences for reference to the readers. In the text of the paper, the methods, results, and discussion sections are based on the tridacnid trees and haplotype network including the “cryptic” tridacnid species (Figures 2 and 3). Because of the discrepancy in sequence length available for the “cryptic” species, we have included a haplotype network and trees based only on our longer sequences (i.e., excluding the “cryptic” species) as supplemental material (Figures 1-4).

Moreover, Lee et al. in 2015 provided ITS rDNA sequences, specific of giant clams, with the characterization of a new species named *S. tridacnidorum* (Lee et al. 2015). Do not use these sequences is a major negative point to this study in 2017. Unfortunately, a new study with ITS-rDNA region of *Symbiodinium* harbored in giant clams has just been published (Ikeda et al. 2017). In consequence, it would be appreciated to analyse the data with appropriate and with latest updated sequences.

We added sequences from the Lee et al. (2015) paper to see if there was a relationship between the *Symbiodinium* in our samples and the *Symbiodinium* described as *S. tridacnidorum*, however as shown by Figure 5, our *Symbiodinium* do not match to the *Symbiodinium* described in the Lee et al. (2015) paper. The fact that our samples did not match this *Symbiodinium* type is interesting and those implications are discussed in the results section.
Added text:

“Due to the high diversity of Clade A *Symbiodinium*, it is possible that there are specific clades associated with Red Sea tridacnids that do not match to previously documented tridacnid associated *Symbiodinium* strains. This is further supported by the clear separation of *Symbiodinium tridacnidorum* and our samples from the Red Sea tridacnids (.91 BP) on the same degree of separation as *Symbiodinium microadriaticum* and *Symbiodinium* sequences from this study.”

Ikeda et al. (2017) reports tridacnids from Japan hosting *Symbiodinium* from Clades A, C and D, however they do not provide the readers with any of their own sequence accession numbers. They do give accession numbers to references that are identical sequences for some of their samples, but these sequences only represent Clade A3 and A6, which are already included in our tree from other sources. We did not feel the need to add more of these strains to the tree as they group within the *Symbiodinium tridacnidorum* paraphyletic group.

Other points by line

Page 2:

Fig 1.: A zoom out of the Red Sea, locating the collected region, would be appreciated.

We agree with the reviewer that a map depicting the Red Sea is very helpful and so it was added in Figure 1.

Page 10:

43: Pinzon et al 2011 and not 2010.

This mistake has been corrected.

43-48: The authors associate the presence of clade A in giant clams with its heat resistance in anemones (Venn 2008). This is in contradiction with the analysis of DeBoer et al. on giant clams (DeBoer et al. 2012). This point should be developed and discussed.

We appreciate this comment from the reviewer as it helped us develop a better explanation of *Symbiodinium* in Clade A. As the reviewer points out, there is contradictory evidence from studies looking at *Symbiodinium* in Clade A from different hosts. We comment on the diversity and unpredictability of Clade A both in the introduction and in the discussion. Added text is as follows:

Introduction:

“Additionally, in anemones hosting *Symbiodinium*, Clade A is thought to have the highest heat tolerance and produce high quality photosynthate (Venn 2008). Therefore, as a heat-tolerant, shallow-dwelling *Symbiodinium* clade, Clade A may prove to be abundant in Red Sea tridacnids. However, the high diversity within each *Symbiodinium* clade does not allow for accurate comparisons at this taxonomic level (Savage et al. 2002) and physiological responses in corals and anemones hosting Clade A may not be applicable to tridacnid hosts. This has been shown in thermal experiments with tridacnids where those hosting Clade A were more susceptible to bleaching under increased temperature compared to those hosting Clade C (Sison 2003). In the same study, tridacnids hosting Clade A were better-suited for low light environments, whereas those hosting Clade C were better-suited for high light environments (Sison 2003). Again, tridacnids in Indonesia were found to harbor Clades A, C, and D with hosts of Clade A found in cooler water, suggesting that Clades C and D are more thermally tolerant in tridacnid hosts (DeBoer et al. 2012).”

Discussion section:

“Previous studies have found *Symbiodinium* from Clades A, C, and D in giant clams (Carlos et al. 1999; Baillie et al. 2000; reviewed in Baker 2003), but we found only *Symbiodinium* from Clade A, a clade shown to have high heat and irradiance tolerance (Baillie et al. 2000; Venn et al. 2008) but low tolerance to temperature stress in tridacnids (Sison 2003)....Recent work continues to highlight the amount of genetic and ecological diversity that may exist within each of the established clades (e.g., LaJeunesse et al. 2014), so future work could employ additional genetic markers to increase the resolution of potential differences in *Symbiodinium* communities of our samples.”

Page 12:

26: What's the length of the sequences after cleaning to perform phylogenetic trees?

The length of the sequences post-editing for 16S was 445bp and for ITS was 658bp. The shorter sequences added in the triadcnid tree from Huelsken et al. (2013) were

225bp long. We overlooked the inclusion of this information and thank the reviewer for bringing it to our attention and it has now been added.

42 & 46: Figure 2 and 3 should be cited in the result section, not in the materials and methods.

Thank you to the reviewer for pointing this out. All mentions of these figures are now in the results section and not in the methods.

Page13:

40: How many blast hits were analysed? What are the parameters?

It is important to include this information. The Results section now includes these details:

Added text:

“All of the samples of this study were *T. maxima* or *T. squamosa*, with *T. maxima* being the most abundant. A BLAST search of the giant clam sequences on GenBank confirmed that samples were from *Tridacna maxima* and *Tridacna squamosa*. The most similar sequences from GenBank were included in our phylogenetic trees (Accession numbers: DQ115320, AM909744, AM909753).”

And:

“A search in GenBank confirmed that all of our ITS sequences were from *Symbiodinium* of Clade A...”

References:

Arif C, Daniels C, Bayer T, et al (2014) Assessing Symbiodinium diversity in scleractinian corals via next-generation sequencing-based genotyping of the ITS2 rDNA region. Mol Ecol 23:4418-4433. doi: 10.1111/mec.12869

DeBoer T, Baker A, Erdmann M, et al (2012) Patterns of Symbiodinium distribution in three giant clam species across the biodiverse Bird's Head region of Indonesia. Mar Ecol Prog Ser 444:117-132. doi: 10.3354/meps09413

Huelsken T, Keyse J, Liggins L, et al (2013) A novel widespread cryptic species and phylogeographic patterns within several giant clam species (Cardiidae: Tridacna) from the Indo-Pacific Ocean. PLoS One 8:e80858. doi: 10.1371/journal.pone.0080858

Ikeda S, Yamashita H, Kondo S, et al (2017) Zooxanthellal genetic varieties in giant clams are partially determined by species-intrinsic and growth-related

characteristics. PLoS One 12:e0172285. doi: 10.1371/journal.pone.0172285

Lee SY, Jeong HJ, Kang NS, et al (2015) *Symbiodinium tridacnidorum* sp. nov., a dinoflagellate common to Indo-Pacific giant clams, and a revised morphological description of *Symbiodinium microadriaticum* Freudenthal, emended Trench & Blank. Eur J Phycol 50:155-172. doi: 10.1080/09670262.2015.1018336

All literature cited by the reviewer has been added to the paper appropriately and all literature has added important information to this study. All literature cited by both reviewers can be found in the paper and in the literature cited section.

Once again, we thank the reviewers and the editor for your time and input. We hope that you now find the manuscript suitable for publication in Marine Biodiversity. Please let us know if you require any additional feedback or changes.

On behalf of the co-authors,

MP