Coral Reefs (2017) 36:1245–1252
DOI 10.1007/s00338-017-1618-1

NOTE

Assessing the utility of eDNA as a tool to survey reef-fish communities in the Red Sea

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Received: 11 July 2017 / Accepted: 17 August 2017 / Published online: 23 August 2017

Abstract Relatively small volumes of water may contain sufficient environmental DNA (eDNA) to detect target aquatic organisms via genetic sequencing. We therefore assessed the utility of eDNA to document the diversity of coral reef fishes in the central Red Sea. DNA from seawater samples was extracted, amplified using fish-specific 16S mitochondrial DNA primers, and sequenced using a metabarcoding workflow. DNA sequences were assigned to taxa using available genetic repositories or custom genetic databases generated from reference fishes. Our approach revealed a diversity of conspicuous, cryptobenthic, and commercially relevant reef fish at the genus level, with select genera in the family Labridae over-represented. Our approach, however, failed to capture a significant fraction of the fish fauna known to inhabit the Red Sea, which we attribute to limited spatial sampling, amplification stochasticity, and an apparent lack of sequencing depth. Given an increase in fish species descriptions, completeness of taxonomic checklists, and improvement in species-level assignment with custom genetic databases as shown here, we suggest that the Red Sea region may be ideal for further testing of the eDNA approach.

Keywords Biodiversity · Coral reef · Environmental DNA · Metabarcoding · Next-generation sequencing

Introduction

Coral reefs represent less than 0.02% of the surface area of our oceans and yet host more than 25% of the recognized marine species (Spalding et al. 2001). In the Red Sea, coral reefs are characterized by their high and unique biodiversity (DiBattista et al. 2016b), historical isolation (DiBattista et al. 2016a), and extreme seasonal and spatial fluctuations in abiotic and biotic factors (Raitsos et al. 2013). Yet compared to other major global reef systems (e.g., Great Barrier Reef), the associated communities of the Red Sea are considerably understudied (Berumen et al. 2013).

Community assemblages of reef fishes have historically been documented through diver-operated underwater visual census methods (UVC; Roberts et al. 1992) or baited remote underwater video surveys (BRUVs; Harvey et al. 2012). Even though these standardized survey methods are ideal for identifying large, mobile, and conspicuous species, the smaller, more cryptic species are often missed (see Ackerman and Bellwood 2000). In recent years, genomic approaches have shown considerable potential for identifying diversity in marine environments, although many of these studies are based on fixed, benthic sampling methods (e.g., Leray and Knowlton 2015). Environmental DNA (eDNA, i.e., genetic material sourced from microbes or that which has been shed from multi-cellular organisms), on the other hand, has proven useful in monitoring the presence/

Communicated by Editor-in-Chief Prof. Morgan S. Pratchett

Electronic supplementary material The online version of this article (doi:10.1007/s00338-017-1618-1) contains supplementary material, which is available to authorized users.

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absence of invasive species and assessing biodiversity in aquatic environments (e.g., Thomsen et al. 2012).

The majority of macrobial eDNA investigations to date have been species-specific, but multi-species PCR in combination with high-throughput sequencing (metabarcoding) can extend these methods to reveal a greater diversity of species in our oceans (e.g., Miya et al. 2015). Even though the sensitivity of these high-throughput assays is likely influenced by the frequency of cell shedding, water flow (currents or tides), and rates of DNA degradation, in most cases this approach has advantages when combined with existing monitoring efforts, including operating at a reduced cost. Given that small volumes of water may contain sufficient eDNA to sequence and detect target aquatic organisms (Thomsen et al. 2012; Miya et al. 2015), our study is the first to apply this noninvasive survey method and assess its utility to document the diversity of fishes inhabiting Red Sea coral reefs.

Materials and methods

Study site

Sampling was conducted in the central Red Sea over a 3-d period in November 2015 near Thuwal, Saudi Arabia (Fig. 1). Seven sampling sites were located on inshore, midshore, and offshore reefs, with six replicate seawater samples (500 mL per sample) taken from both the exposed and sheltered sides of the reef (a total of 12 replicates from each reef). Four replicate samples were also taken from the King Abdullah University of Science and Technology (KAUST) boat harbor, where there are relatively fewer reef fish species. Multiple precautions were taken to minimize contamination with exogenous eDNA, including rinsing all water bottles and the filtering apparatus in a 10% bleach solution prior to use and between each sampling. Moreover, seawater was collected only at the surface (top 20 cm), on the windward side of the research vessel, and within 5 m of the reef, changing latex gloves in between samples. To minimize eDNA degradation, the samples were filtered immediately on the research vessel using a peristaltic pump (Sentino microbiology pump, Pall Life Sciences, MI) and 47 mm (0.45 μm) micronylon sterilized membranes (Pall Life Sciences), which were then placed in individual plastic bags and stored on ice prior to being frozen at –20 °C until eDNA extraction. All extractions were performed within 5 d of collection to limit the decay of template material on the membranes.

DNA extraction

Genomic DNA was extracted in a sterile environment at KAUST using a DNeasy blood and tissue kit (Qiagen Inc., CA) following the manufacturer's protocol with a few modifications: (1) 360 μL of ATL tissue lysis buffer and 40 μL proteinase K were added to 1/3 of the filter membrane that had been cut up in a UV hood; (2) the filter membrane was digested for 3 h; (3) the supernatant following digestion was removed and transferred to a sterile 2-mL tube where 400 μL of absolute ethanol and 400 μL of AL lysis buffer was added; (4) two separate microcentrifuge spins with the digested solution were used to pass all of the DNA through the filter column; (5) all samples were eluted in one step using 100 μL of AE buffer; and (6) extraction controls, for which all steps remained the same except for the addition of the filter membrane, were included for each set of 12 samples.

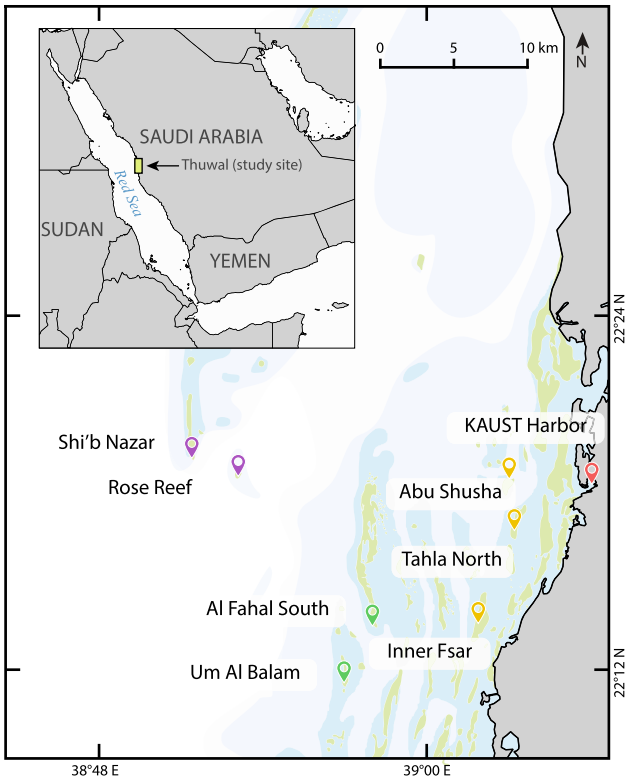

Fig. 1 Seawater sampling sites at inshore (*orange*), midshore (*green*), and offshore (*purple*) coral reefs, and a boat harbor (*red*) along the central Red Sea coastline near Thuwal, Saudi Arabia

Fusion-tag qPCR

Quantitative PCR (qPCR) experiments were conducted in a separate, dedicated laboratory at Curtin University in Australia where all benches and utensils were routinely cleaned with bleach and UV sterilized. This approach allowed us to: (1) recover fish 16S mitochondrial DNA barcode sequences of ~200 bp from mixed samples with the primers 16SF/D (5′-GACCCTATGGAGCTTTAGAC-3′) and 16S2R-degenerate (5′-CGCTGTTATCCCTADR

GTAACT-3′) (modified from Deagle et al. 2007); (2) add multiple forward ($N = 8$) and reverse ($N = 25$) fusion tags to the qPCR products that each contained a unique 10-bp Illumina adaptor sequence; and (3) estimate final concentrations of the DNA amplicons using SYBR Green as a reporter of fluorescence. All qPCRs were run in triplicate and included "no template" controls to check for sample cross-contamination. qPCR reactions (25 mL) consisted of the following: 10 × PCR Buffer (Bioline, London, UK), 0.25 mM $MgCl_2$, 10 mM dNTPs, 0.4 mg mL^{-1} bovine serum albumin, 1.25 U Ampli-Taq Gold (Applied Biosystems, CA), 20 mM primers, and 2 µL undiluted DNA extract. Cycling conditions were as follows: 95 °C for 5 min, followed by 45 cycles of 95 °C for 30 s, 54 °C for 30 s, and 72 °C for 45 s, with a final step of 72 °C for 10 min. Failed amplifications were not repeated, resulting in fewer than 12 replicates per sampling station. DNA amplicons from each sample were pooled in equimolar concentration based on qPCR endpoints and quantification on a LabChip GX Touch (PerkinElmer Health Sciences, MA), size-selected using a Pippen Prep (Sage Science, MA), and then purified using a QIAquick PCR Purification Kit (Qiagen Inc., CA) following the manufacturer's protocol. The final library was quantified on a LabChip GX Touch followed by sequencing in the TrEnD Laboratory at Curtin University on an Illumina MiSeq platform using 300 cycle MiSeq V2 reagent kits and nanoflow cells. All unfiltered sequence data are accessible from DRYAD (doi:10.5061/dryad.1pm20).

Bioinformatic filtering

Sequences were assigned to samples, quality filtered, and trimmed in Geneious Pro v 4.8.4 (Drummond et al. 2009) using the following workflow: (1) reads containing imperfect Illumina adaptor sequences were discarded; (2) reads containing imperfect forward or reverse fish 16S primer sequences were discarded; (3) reads were de-multiplexed based on the combined forward and reverse adaptor sequences; (4) reads with imperfect adaptor barcode sequences were discarded; (5) reads smaller than 160 bp were discarded; (6) singletons were discarded; and (7) chimeric sequences were flagged and discarded using USEARCH v 8.0.1623 (Edgar 2010).

Taxonomic assignment

We interrogated the NCBI BLASTn database (March 2016) on the Magnus Cray XC40 system located at the Pawsey Supercomputing Centre at Technology Park in Western Australia, with our 16S sequences in FASTA format. BLASTn results were imported into MEtaGenome ANalyzer (MEGAN) v 5.11.3 (Huson and Weber 2013), and taxonomic identities were assigned to genus or species (where possible) based on the lowest common ancestor (LCA) algorithm using the following settings: minimum number of reads = 2; minimum bit score = 300; top percent to be considered = 10%; and minimum complexity = not considered. A similarity of 95% was accepted for a genus-level match and 98% for a species-level match (Meyer and Paulay 2005). Taxonomic assignments to species with LCA were further evaluated against expert knowledge of species distributions and the most up-to-date checklist of Red Sea fishes (DiBattista et al. 2016b and references therein). To be conservative, matches at the species level were not accepted if that taxon was not known from the Red Sea or if 16S sequences from all species within that genus were not available on NCBI or in our custom genetic database.

Given multiple hits to poorly archived fish 16S sequences on NCBI, an issue raised by Pleijel et al. (2008), in addition to hits to fish species not found in the Red Sea (16 of the 25 species assigned), a modified BLASTn search was performed against a curated custom genetic database of Red Sea fishes, within which we selected a single representative 16S sequence from all reef-fish species found in the Red Sea that were available on NCBI. Where possible, sequences were extracted from datasets published by trusted sources and those that had vouchered their whole specimens at museums (Electronic supplementary material, ESM4. fasta). We additionally generated 16S sequences for 38 fish species sampled in the Red Sea to add to the custom database, which provided a total of 474 of the 1071 known reef-fish species from the region (DiBattista et al. 2016b).

Results and discussion

Our 16S eDNA metabarcoding approach at seven reef sites and a boat harbor in the central Red Sea revealed between 26 and 46 genera of fish (out of 511 known genera; DiBattista et al. 2016b) based on 250,145 total DNA sequences. These genera represent a diversity of conspicuous (e.g., *Chaetodon*), cryptobenthic (e.g., *Eviota*), numerically abundant (e.g., *Pseudanthias*), and commercially relevant shorefish (e.g., *Cephalopholis*, *Epinephelus*, *Lethrinus*, and *Lutjanus*) known to inhabit the Red Sea (DiBattista et al. 2016b). Our eDNA assignment approach using the NCBI database and our custom genetic database identified 12 or 6 out of the 21 most abundant reef fish genera identified by a recent UVC study (Roberts et al. 2016), respectively, and between 17 and 42% of these genera were only detected at a single site. This difference in detection capability may be due to the limited spatial scale of our sampling (<1% of the eastern Red Sea

coastline) or our lack of ability to resolve fish taxa based on short but amplifiable 16S fragments of DNA.

DNA sequencing was successful for 53 of the 88 replicate seawater samples collected at reefs in the central Red Sea (Fig. 1). Of these replicates, there was a modest bias toward amplification success on the sheltered side versus the exposed side of the reefs (31 vs. 22), which may indicate increased suspension of organic material or water residency time in the former; sampling effort should be focused here in future studies. However, given the lack of data for the exposed side of the reefs, we combined data from both sides of the reef for all downstream analyses. DNA degradation, primer biases, or a lack of template DNA may have all played a role in the samples that failed to amplify. Moreover, for eDNA surveys such as this, it is difficult to know a priori what the DNA template concentration will be in the water column. In some cases we recovered limited amounts of template, a finding that contrasts with other studies (Miya et al. 2015), but that is important to consider when designing eDNA surveys in an environment for the first time.

Our noninvasive survey method revealed a trend in sampling effort at some (but not all) reefs based on the number of cumulative fish genera assigned per replicate (Fig. 2a). For example, no new genera were assigned after as few as one replicate out of four total replicate seawater samples taken from KAUST harbor, whereas new genera continued to be assigned for two reefs that had at least eight or nine replicates included in the analysis. Indeed, taxonomic assignment to genus had not yet reached saturation at four of the eight reefs based on the upward trajectory of the accumulation curves. This suggests that 12 or more replicates, based on the volume of seawater that we filtered in this study (500 mL), may be required. In contrast, there was no clear bias in this "saturation trend" among inshore, midshore, or offshore reefs. No relationship was observed between the number of 16S sequences generated per reef and the number of genera assigned ($r^2 = 0.037$, $p = 0.17$; Fig. 2b), although when we removed two outlier data points that appeared to drive the regression (>2 SD, both from Al Fahal South), this relationship became significant ($r^2 = 0.45$, $p < 0.0001$), indicating a clear benefit of more replicates and increased sequencing coverage per replicate, also highlighted by rarefaction analysis of randomly selected samples (ESM Fig. S1).

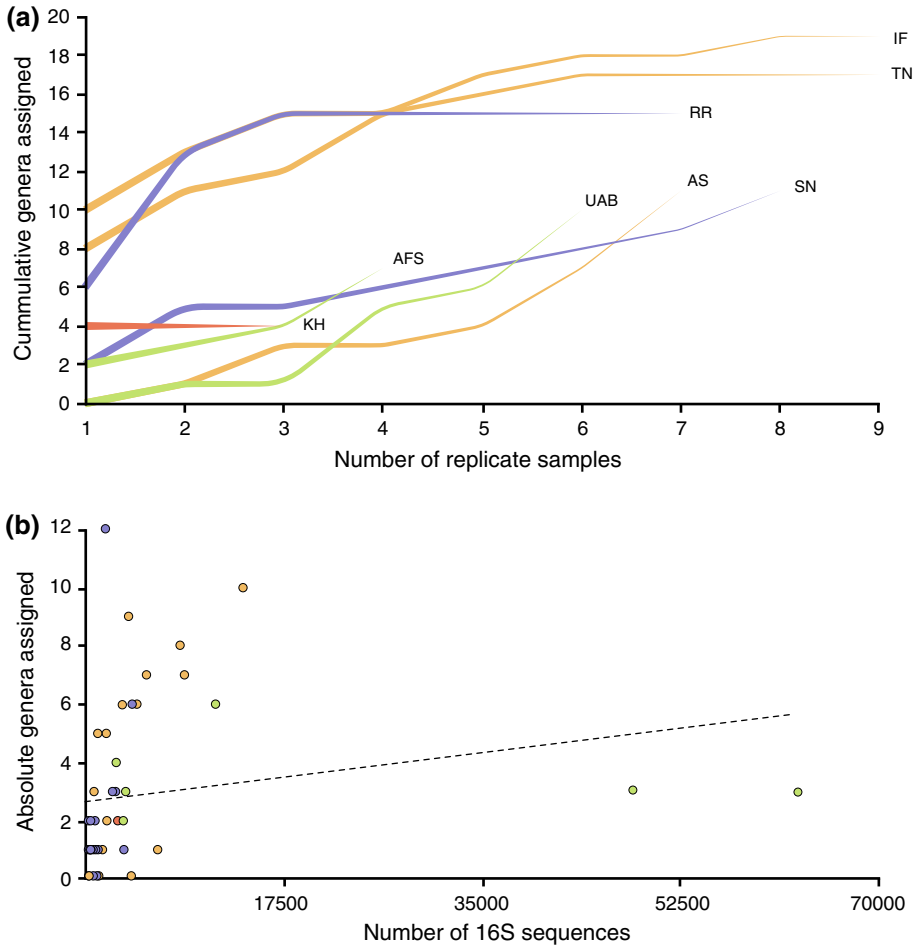

Fig. 2 Number of cumulative (**a**) or absolute (**b**) genera assigned per replicate as a function of amplifiable 16S mitochondrial DNA sequences detected at seven inshore (*orange*), midshore (*green*), and offshore (*purple*) coral reefs, and a boat harbor (*red*) along the central Red Sea coastline near Thuwal, Saudi Arabia. *SN* Shi'b Nazar; *RR* Rose Reef; *UAB* Um Al Balam; *AFS* Al Fahal South; *IF* Inner Fsar; *TN* Tahla North; *AS* Abu Shusha; *KH* KAUST Harbor

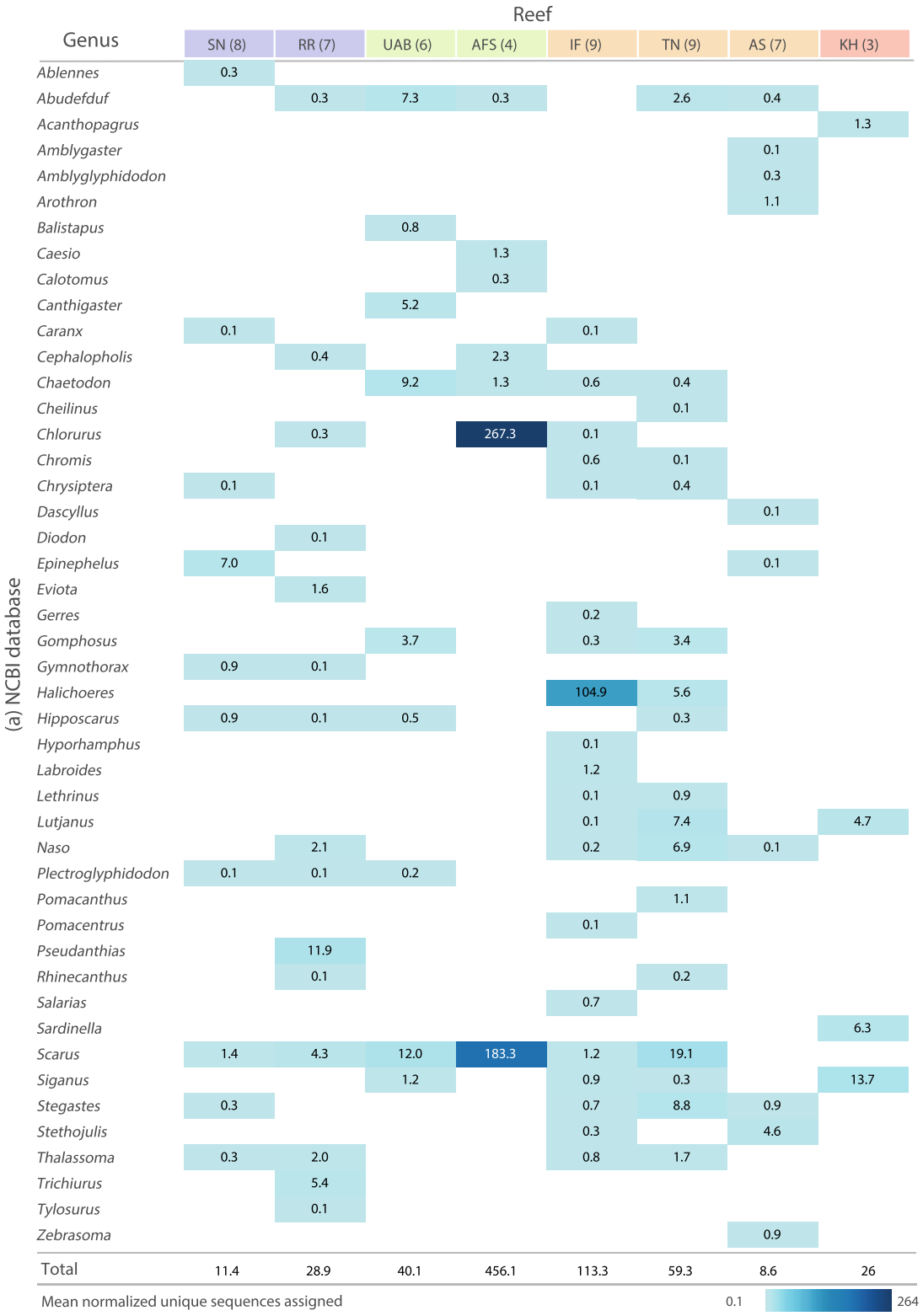

Fig. 3 Heatmap showing the mean number of unique 16S mitochondrial DNA sequences for each genus (**a**, **b**) detected at seven inshore (*orange*), midshore (*green*), and offshore (*purple*) coral reefs, and a boat harbor (*red*) along the central Red Sea coastline near Thuwal, Saudi Arabia. Analyses were independently conducted using the National Center for Biotechnology Information (NCBI) database (**a**) or our custom genetic database (**b**). *Numbers in parentheses* are the number of seawater replicates that amplified per sample site. See Fig. 1 for summary of sample sites; abbreviations as in Fig. 2

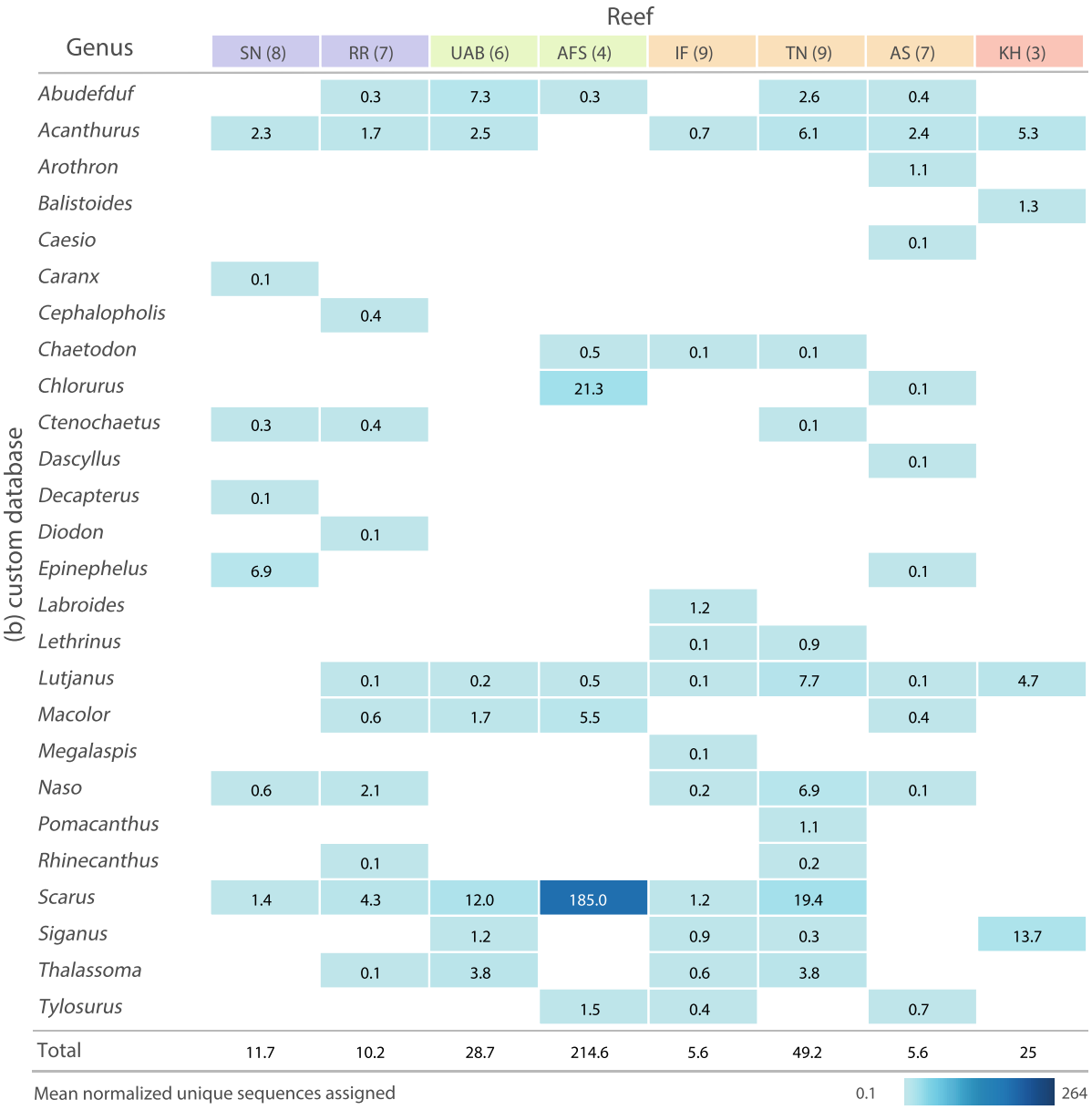

Fig. 3 continued

When the number of unique sequences per genus was averaged across each reef we identified over-represented genera (e.g., *Chlorurus*, *Halichoeres*, *Scarus*) at some, but not all, reefs based on assignments made using the NCBI database (Fig. 3a) and our custom genetic database (Fig. 3b). This may indicate biases in the workflow that preferentially target these genera or increased DNA concentrations of these genera present in seawater samples. Moreover, some of the common genera in the Red Sea were notably absent from our data (e.g., *Apogon, Coris, Gobiodon, Pseudocheilinus, Pygoplites*; Roberts et al. 2016). Low values for the average number of unique sequences in most cases (Fig. 3a: mean = 8.08 ± 3.65 SE, median = 0.67; Fig. 3b: mean = 5.02 ± 2.65, median = 0.65) further suggest that there may be a clear benefit of increased coverage per reef by either increasing the number of replicates (>12 replicates), increasing the volume of water filtered (>500 mL), decreasing the pore size of the filter membrane to capture smaller particles (<0.45 µm), or generating higher numbers of metabarcoding reads. The benefit of generating higher numbers of metabarcoding reads is supported by our rarefaction analysis of randomly selected samples from a single reef, where there was considerable variability in the level of

Table 1 Summary of reef fish detected (presence/absence) at the species level by seawater sampling at seven coral reefs and a boat harbor along the central Red Sea coastline near Thuwal, Saudi Arabia

Family	Species	NCBI 16S database	Custom 16S database
Acanthuridae	*Ctenochaetus striatus* (Quoy & Gaimard, 1825)		√
Acanthuridae	*Naso brevirostris* (Cuvier, 1829)		√
Acanthuridae	*Naso unicornis* (Forsskål, 1775)	√	
Balistidae	*Balistoides viridescens* (Bloch & Schneider, 1801)		√
Balistidae	*Balistapus undulatus* (Park, 1797)	√	
Balistidae	*Rhinecanthus rectangulus* (Bloch & Schneider, 1801)		√
Belonidae	*Ablennes hians* (Valenciennes, 1846)	√	
Belonidae	*Tylosurus crocodilus* (Lesueur, 1821)	√	√
Blenniidae	*Salarias fasciatus* (Bloch, 1786)	√	
Clupeidae	*Amblygaster sirm* (Walbaum, 1792)	√	
Diodontidae	*Diodon hystrix* Linnaeus, 1758	√	
Diodontidae	*Diodon liturosus* Shaw, 1804		√
Labridae	*Labroides dimidiatus* (Valenciennes, 1839)	√	√
Labridae	*Scarus collana* Rüppell, 1835		√
Labridae	*Thalassoma lunare* (Linnaeus, 1758)		√
Labridae	*Thalassoma rueppellii* (Klunzinger, 1828)		√
Lutjanidae	*Macolor niger* (Forsskål, 1775)		√
Pomacanthidae	*Pomacanthus maculosus* (Forsskål, 1775)		√
Pomacentridae	*Abudefduf vaigiensis* (Quoy & Gaimard, 1825)		√
Pomacentridae	*Dascyllus trimaculatus* (Rüppell, 1829)		√
Siganidae	*Siganus luridus* (Rüppell, 1829)		√
Siganidae	*Siganus rivulatus* (Forsskål, 1775)		√
Sparidae	*Acanthopagrus bifasciatus* (Forsskål, 1775)	√	

Matches were obtained using BLASTn searches in the NCBI mitochondrial DNA 16S database or using a custom 16S database (see Materials and methods). A similarity of 98% was accepted for a species-level match; to be conservative, matches at the species level were not accepted if that taxon was not known from the Red Sea and matches were not accepted if 16S sequences from all known Red Sea species within that genus were not available in either database

sequencing at which saturation was reached for assignment of genera (ESM Fig. S1). Based on heatmaps (Fig. 3), there was also an indication that different reefs had different compositions of fish. Although previous aquatic studies have attempted to infer the abundance of taxa from the number of sequence reads (amphibians: Pilliod et al. 2013; fish: Mahon et al. 2013; Lacoursière-Roussel et al. 2016), we chose to avoid this inference due to confounding factors potentially skewing the proportions of reads, including fluctuating environmental conditions, variable rates of DNA degradation (Dejean et al. 2011), and low template numbers (Murray et al. 2015).

Even with our custom genetic database, less than half of all Red Sea species were represented with a 16S barcode sequence (474 of the 1071 species). Indeed, some speciose genera were under-represented in our custom database, with $\leq$10% of the species in their respective genera with a 16S barcode sequence (ESM File S1; ESM Fig. S2). Nonetheless, almost twice as many taxa were confidently assigned to species using our custom database ($N = 16$) versus the NCBI database ($N = 9$), despite the same conservative criteria being used in both cases (Table 1).

Caveats to consider when barcoding seawater samples based on 16S fish DNA include misidentification in the NCBI reference database, incomplete lineage sorting or hybridization between species, or (in our case) a dearth of vouchered sequences from reef fish sampled in the Red Sea. Also, because 16S is a much shorter read for eDNA applications, the taxonomic resolution at the species level is weak, particularly in the Red Sea. Indeed, we have previously shown that intraspecific divergence of reef fish from adjacent Indian Ocean populations can be comparable to interspecific comparisons (DiBattista et al. 2013) and that cryptic lineages are not uncommon (DiBattista et al. 2017). These caveats are not unique to our study or restricted to reef fish, but can be mitigated with increased regional barcoding initiatives, and may be less of an issue for better-characterized coral reefs (e.g., Great Barrier

Reef). The benefit of this approach despite its equivocal outcome is that it may prove to be more cost-effective than investing in expensive field time and also that eDNA studies are not limited by poor environmental conditions (e.g., visibility, hazardous conditions) or a lack of taxonomic expertise. These initial eDNA trials from the Red Sea show that the method has several potential applications but that surveys will need to be carefully designed given the number of potentially confounding factors.

Acknowledgements This study was funded by KAUST Award No. CRG-1-2012-BER-002 and baseline research funds to M.L.B., as well as a Curtin University Early Career Research Fellowship to J.D.D., M.S., and M.B. acknowledge the support of ARC Linkage Project (LP160100839) to explore marine metabarcoding applications. The authors would also like to acknowledge KAUST's Coastal and Marine Resources Core Lab for logistical support, as well as Matthew Power, Megan Coghlan, and Brendan Chapman for DNA sequencing assistance.

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