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REPORT

Phylogenomics of *Palythoa* (Hexacorallia: Zoantharia): probing species boundaries in a globally distributed genus

'Ale'alani Dudoit[1,2] · Maria E. A. Santos[3] · James D. Reimer[3,4] · Robert J. Toonen[1]

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Abstract Zoantharians (Cnidaria: Hexacorallia: Zoantharia) of the genus *Palythoa* are ubiquitous species that occupy reef habitats in every tropical ocean. Disagreements among classifications based on morphology, reproductive traits, and molecular techniques have generated taxonomic challenges within this group. Molecular studies provide limited phylogenetic resolution between species, and discordance is frequently attributed to slow mitochondrial rates and lack of resolution among molecular markers. Here we conducted the first phylogenomic survey of *Palythoa*, using a reduced representation genomic approach (ezRAD) to resolve relationships among eight described and four putative *Palythoa* species ($N = 22$ plus two outgroups) across the Pacific and Atlantic Oceans. We constructed nearly complete mitochondrial genomes and assembled transcriptome loci datasets by reference mapping. A de novo assembly was performed for the holobiont dataset, and we compared a range of filtering strategies from unfiltered data down to 136 unlinked high-quality biallelic SNPs shared by all samples to resolve evolutionary lineages within *Palythoa*. Across all these datasets, the resulting Bayesian and ML trees revealed six highly concordant and well-supported clades, however, the phylogenomic data were inconclusive in resolving species relationships within the clades. We detected putative species complexes within two well sampled *Palythoa* clades (clades I and II), but species delimitation results were inconsistent in whether these clades contain multiple nominal species or represent a single variable species. Polyphyly in the broadly distributed species *Palythoa tuberculosa* and *P. mutuki* highlight the need for additional study. Consistency among nuclear and mitogenomic datasets points to a lack of biological understanding of species boundaries among these zoantharians rather than limitations of the molecular markers. More complete taxonomic sampling of nominal species across the geographic ranges of distribution is necessary to resolve species boundaries and evolutionary histories among members of this genus.

Keywords Next-generation sequencing · Systematics · Holobiont · Transcriptome · Mitochondrial

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✉ 'Ale'alani Dudoit
al.dudoit@hawaii.edu

[1] Hawai'i Institute of Marine Biology, School of Ocean and Earth Science and Technology, University of Hawai'i At Mānoa, Kāne'ohe, HI, USA

[2] Zoology Graduate Program, University of Hawai'i At Mānoa, Honolulu, HI, USA

[3] Molecular Invertebrate Systematics and Ecology Laboratory, Graduate School of Engineering and Science, University of the Ryukyus, Nishihara, Okinawa, Japan

[4] Tropical Biosphere Research Center, University of the Ryukyus, Nishihara, Okinawa, Japan

Introduction

Coral reef environments are some of the most biodiverse and productive ecosystems on Earth, providing essential cultural, economic, and ecological value (Cinner et al. 2012; Costanza et al. 2014). Although highly diverse, coral reefs have been impacted by anthropogenic stressors on a global scale (Pandolfi et al. 2003; Hughes et al. 2017) and are among the most vulnerable habitats to future climate

change (Hoegh-Guldberg et al. 2007). The combined stressors of human activities have prompted significant declines in scleractinian corals, which provide the foundations of these invaluable reef ecosystems (Eddy et al. 2018; Hughes et al. 2018). Zoantharians are common members of healthy coral reef communities, and although they are not reef-forming species, they are also threatened by climate change and other anthropogenic impacts (Rocha et al. 2020). Mortality from thermal stress events appears to tip the ecological balance in coral communities, such that many reefs formerly dominated by scleractinian corals are increasingly dominated by other taxa, such as opportunistic algae, sponges, and soft corals (Cruz et al. 2016; Heery et al. 2018; Lesser and Slattery 2020). The 2014–2017 global mass bleaching event impacted more than 75% of the coral reefs on the planet and reduced live coral cover by up to 51% on the Great Barrier Reef (Stuart-Smith et al. 2018). Yet no equivalent estimates are available for zoantharians as they are rarely included in reef surveys, due in part to taxonomic uncertainty in their identification. Further complicating the assessment of the conservation status of zoantharians, at least some species are among the non-scleractinian taxa that tend to increase on reef areas degraded by anthropogenic impacts (Cruz et al. 2015; Wee et al. 2017; Lachs et al. 2019). Given that species boundaries remain contentious, the identities of zoantharian species threatened by or benefitting from climate change remain uncertain.

Zoantharians are benthic cnidarians, generally colonial, including zooxanthellate genera are distributed worldwide in shallow tropical and subtropical waters, such as *Palythoa* and *Zoanthus*. The genus *Palythoa* is classified in the family Sphenopidae, and with the exception of three species known to occur in low-light environments or below 30 m depth (Irei et al. 2015), *Palythoa* species have symbioses with dinoflagellates of the family Symbiodiniaceae. Zooxanthellate *Palythoa* species can be commonly found on coral reefs, and often dominate coastal regions, and at least some species have been found to replace reef-building corals in response to anthropogenic stressors (Belford and Phillip 2012), especially nutrification (Cooke 1976; Karlson 1983; Costa et al. 2008; Lapointe et al. 2010; Cruz et al. 2014, 2015; Amato et al. 2016; Lachs et al. 2019). Further, models predict that increasing salinity and thermal bleaching projected by end-of-century emission scenarios favor increased dominance by the generalist species *Palythoa caribaeorum* (Kemp et al. 2006; Durante et al. 2018). Although some zoantharian species may benefit from a competitive shift away from scleractinian corals, such phase shifts tend to have cascading negative consequences with regards to both ecosystem services and coral reef biodiversity (Costa et al. 2008; Belford and Phillip 2012; Amato et al. 2016). Despite threats to coastal ecosystems that include zoantharians, this group remain highly understudied (Burnett et al. 1997; Reimer et al. 2019). As a consequence, identification of *Palythoa* species and higher taxonomic classification often remains challenging and unresolved (Burnett et al. 1995, 1997; Reimer et al. 2006, 2007; Hibino et al. 2014; Risi and Macdonald 2015; Mizuyama et al. 2018; Poliseno et al. 2020). The lack of taxonomic certainty among dominant species on reefs undergoing phase shifts from scleractinian to zoantharian dominance limits our ability to understand, predict and potentially reverse these ecosystem changes. This taxonomic morass prevents us from making precise statements about the proportion of species in each category and the risks to marine biodiversity in this complicated group.

Zoantharians of the genus *Palythoa* are popular in the saltwater aquarium trade due to their bright coloration, ease of propagation, comparatively low cost and wide availability (Deeds et al. 2011), but at the same time also produce one of the most potent marine toxins, palytoxin (PTX) (Moore and Scheuer 1971). Retailers frequently purchase unknown species of *Palythoa* based on coloration from suppliers who provide vague documentation of origin and rarely have robust species identifications (Deeds et al. 2011). This lack of information poses serious threats to aquarists because it is unknown what species are currently being distributed through the aquarium trade, their potential toxicity, or their human health risks. There have been multiple reports of accidental poisoning through the marine aquarium trade, however inconsistent identification and taxonomic uncertainty of *Palythoa* species impede our understanding of the risks to aquarium hobbyists (Hoffmann et al. 2008; Deeds and Schwartz 2010; Deeds et al. 2011; Tartaglione et al. 2016). Due to the current uncertain state of *Palythoa* taxonomy, it is unclear which species are being exported and imported around the world, along with the distribution of PTX among these species. Hence, there is also a strong human health concern linked to *Palythoa* taxonomy.

Formal taxonomic descriptions of *Palythoa* species have included polyp shape and size, colony shape, tentacle count, position and characters of the sphincter muscle, and nematocyst distribution, sizes, and abundances (Pax and Mueller 1957; Walsh and Bowers 1971; Ryland and Lancaster 2003; Shiroma and Reimer 2010). However, many of these morphological characters also appear to be plastic (Ryland and Lancaster 2003; Ong et al. 2013), and species redescriptions, particularly among shallow-water *Palythoa* spp., are likely common (Burnett et al. 1994; Reimer et al. 2004). Thus, the true number of species in the genus *Palythoa* is believed to be much lower than the more than 200 nominal species currently described in the literature (Burnett et al. 1994; Low et al. 2016).

Given this taxonomic uncertainty, a variety of studies have combined morphological assessments with molecular genetic markers to evaluate taxonomic validity of species within *Palythoa* with varying success (Burnett et al. 1995, 1997; Reimer et al. 2006, 2007, 2012; Hibino et al. 2014). For example, using both morphological and molecular data, Shiroma and Reimer (2010) reported on the formally undescribed *Palythoa* sp. 'yoron' from southern Japan, and hypothesized a hybrid origin for this putative species originating from *P. tuberculosa* and *P. mutuki*. Mizuyama et al. (2018) analyzed Japanese specimens of *P. tuberculosa*, *P. mutuki*, *P.* aff. *mutuki* and *P.* sp. 'yoron' using multiple independent criteria including morphology, habitat preference, genetic data, and spawning periods, noting incongruencies in apparent relationships among characters. Based on reproductive timing, *P.* sp. 'yoron' and *P.* aff. *mutuki* appeared to be reproductively isolated from *P. tuberculosa*, however phylogenetic analyses provided support for a monophyletic clade including *P. tuberculosa* and *P.* sp. 'yoron.' Discordance between morphological and marker-based DNA systematics have left species boundaries in this group unresolved. Slow rates of sequence evolution in the mtDNA of anthozoans (Shearer et al. 2002; Hellberg 2006), confounding signals from the multicopy ITS sequences (Vollmer and Palumbi 2004; Reimer et al. 2007; Johnston et al. 2017), and signals from past hybridization events or ancestral polymorphisms in zoantharians (Reimer et al. 2007) have all been proposed as factors contributing to the disagreements between phylogenetic, morphological and reproductive analyses.

Reduced representation genomic surveys offer a powerful tool to resolve such disagreements, because it is the most cost-effective way currently available to interrogate many loci from across the genome and test hypotheses regarding each of these proposed factors. Among the suite of reduced representation genomic approaches, Restriction site Associated DNA Sequencing (RAD-Seq) has become one of the most common in the literature because it is relatively inexpensive and simple in comparison to some alternatives (Andrews et al. 2016a). Further, RAD-seq has shown unprecedented power to assess intrageneric phylogenies and resolve species relative to PCR-directed sequencing in such diverse taxa as *Drosophila* (Rubin et al. 2012), beetles (Cruaud et al. 2014), snails (Razkin et al. 2016), surfperch (Longo and Bernardi 2015), tunas (Díaz-Arce et al. 2016), bamboo (Wang et al. 2017), African rift lake cichlids (Wagner et al. 2013), and several groups of hard corals (Herrera and Shank 2016; Johnston et al. 2017; Iguchi et al. 2019; Terraneo et al. 2019; Arrigoni et al. 2020; Forsman et al. 2020; Wepfer et al. 2020).

To examine whether uncertainty in species boundaries can be explained by the peculiarities of any individual marker type, we provide among the first genomic dataset among zoantharians to reconstruct phylogenetic relationships. Here, we examine species boundaries among *Palythoa* individuals representing eight described and four putative species in the genus *Palythoa* to help shed light on the taxonomic uncertainty of the genus. Our sampling strategy intentionally targets a range of specimens that include some for which the taxonomy is comparatively well-investigated as well as some specimens for which conflicting results in previous studies leaves the nominal taxonomy a subject of debate (Ryland and Lancaster 2003; Reimer et al. 2007, 2011, 2012; Irei et al. 2015; Mizuyama et al. 2018; Santos et al. 2019). We also include some widely occurring species with broad geographic sampling to test for cryptic divergence and to obtain an estimate of the within-taxon divergence levels to use as a metric for comparison. We evaluated the relationships and species boundaries among these samples using multiple different phylogenomic datasets: (a) a total holobiont dataset based on de novo assembly; (b) a high-quality single nucleotide polymorphism (SNP) dataset with no missing data (shared by all *Palythoa* taxa); (c) assembled loci from contigs mapped to a *Palythoa* transcriptome reference (containing 1,327 SNPs); and (d) nearly complete mitochondrial genomes.

Methods and materials

Specimen sampling

Tissue specimens were collected across the tropical West Pacific Ocean, Hawai'i, and Atlantic Ocean (Fig. 1, Table 1). The dataset includes 22 individuals (representing eight described and four putative species) from the genus *Palythoa.* We also include two outgroup species, *Zoanthus sansibaricus* Carlgren, 1900 of the same suborder Brachycnemina, and *Terrazoanthus* sp. of the suborder Macrocnemina, for a total of 24 individuals sampled. Collections were made via snorkeling or SCUBA diving, and tissue samples were stored in either salt-saturated DMSO (dimethyl sulfoxide) buffer (Gaither et al. 2011) or $> 95\%$ ethanol until DNA was extracted.

DNA extraction and quantification

Genomic DNA was extracted using the E-Z 96 Tissue DNA kit (Omega Bio-Tek, Inc) with two 100 µl elutions in water instead of a single elution in 200 µl of the supplied elution buffer. We used HPLC grade water in rotary evaporation so that the DNA could be concentrated without altering buffer concentrations or impacting downstream applications. Extractions were visualized in a 1.5% agarose gel, using TAE buffer, GelRed (Biotum, Inc) gel stain, and

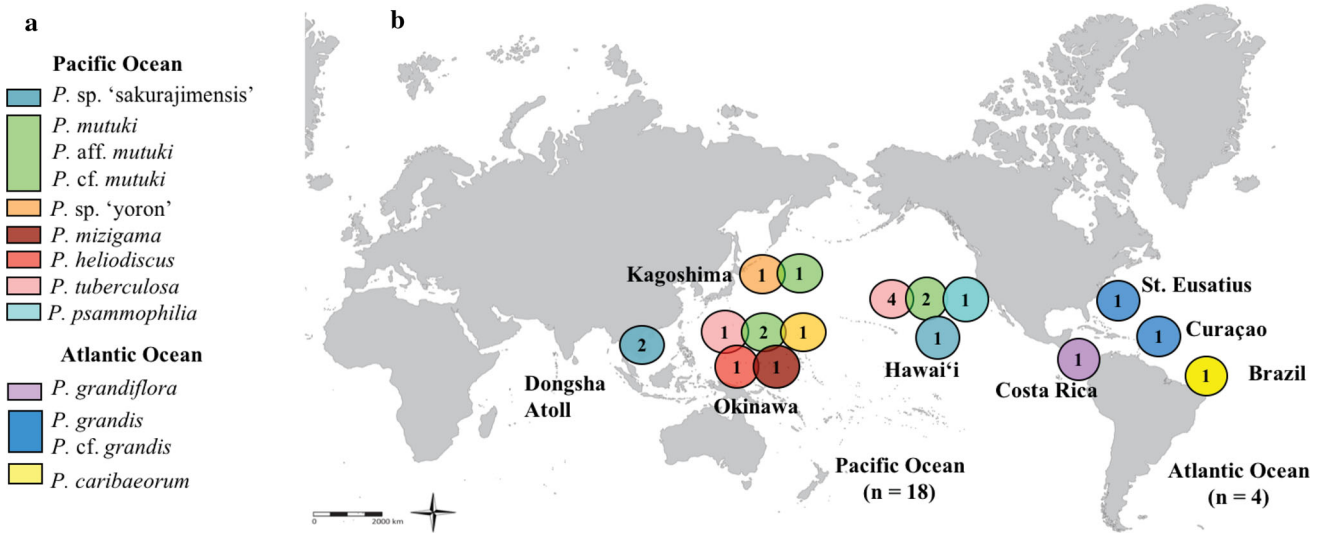

Fig. 1 Map of sample location for *Palythoa* species. **a** List of *Palythoa* species with the corresponding colors denoted in each ocean basin. **b** Circles in the map illustrates the number of individuals and colors correspond to species collected at each location

the 200–10,000 bp Hyperladder 1 (Bioline, Meridian Bioscience Inc). Most DNA extractions produced a high molecular weight band (> 10 kb), but some samples were partially degraded and were only included in the study if they yielded a smear with at least half the sample above 2.5 kb. Extractions were quantified using the AccuBlueTM High-Sensitivity dsDNA quantification kit (Biotium, Inc) with 8 standards and measured using a SpectraMax M2 microplate reader (Molecular Devices, LLC) at $\lambda_{Ex}/\lambda_{Em}$ 485/530 nm.

Library preparation

The ezRAD (Toonen et al. 2013) libraries were prepared for high-throughput sequencing following the protocol of Knapp et al. (2016). Prior to digestion, samples were treated with AMPure XP purification beads (Beckmann Coulter, Danvers, MA, USA) at ~ 1:0.6 (DNA:beads) ratio to remove smaller DNA fragments, then adjusted to approximately 1 µg of total genomic DNA in 25 µl final volume. Genomic DNA was then restriction digested using the isoschizomers *Mbo*I and *Sau*3AI (New England BioLabs, Ipswich, MA), which both cleave at GATC recognition sites. Digestions were performed in a 50 µl reaction volume consisting of 25 µl dsDNA (~ 1 µg), 5 µl NEB Cutsmart Buffer (provided with restriction enzymes), 18 µl HPLC grade water, 1 µl *Mbo*I (10 units), and 1 µl *Sau3*AI (10 units) under the following thermocycler profile: 37 °C for 3 h, then 65 °C for 20 min. Digested samples were then cleaned using AMPure XP purification beads at a 1:1.8 (DNA/beads) ratio to remove fragments < 200 bp following the standard protocol and digested samples were visualized on a 1.5% agarose gel (as above).

Sequencing and trimming

Sequencing libraries were generated using the KAPA Hyper Prep DNA kit (Roche Sequencing and Life Science) following manufacturer protocols. Briefly, libraries were size-selected at 300-600 bp using purification beads following the protocol of Knapp et al. (2016) then amplified via PCR using the number of recommended cycles based on DNA quantifications for each library. Quality control of libraries included a Bioanalyzer (Agilent Technologies) run to examine fragment size distribution and qPCR to determine library concentration. Sequencing of acceptable libraries was performed at the Hawai'i Institute of Marine Biology (HIMB) Genetic Core Facility on the Illumina MiSeq platform using the V3 chemistry kit 2 × 300 bp paired-end reads. An average of 2 million reads per individual were trimmed to remove adapter sequences on both 5´and 3´ends using default settings in GENEIOUS v.11.0.5 (Biomatters Ltd) for mitochondrial genomes and trimmed, assembled, and genotyped using default settings in dDOCENT v.2.6.0 (Puritz et al. 2014) for the holobiont and transcriptome datasets.

SNP calling

We applied a comprehensive approach that compares datasets drawn from different subsets of the overall RAD dataset as outlined below. First, the quality of the raw sequence libraries was assessed for sequence quality scores, sequence length distributions, duplication levels, overrepresented sequences, etc., using FASTQC v0.11.9 (Andrews 2010). Sequencing libraries all passed this initial quality control and were then subsetted into holobiont

Table 1 Summary statistics for all species libraries of the mitochondrial, holobiont, and transcriptome datasets. Sample location coordinates (GPS), Depth in meters (m), number of reads from the sequencer (total # of reads), mean depth coverage (Avg. read depth), and percent coverage of reference sequence (ref. seq.)

Label	Species	Sample Location	GPS	Depth (m)	Total # of reads	Mt genome		Holobiont		Transcriptome	
						Avg. read depth	ref. seq	Avg. read depth	ref. seq	Avg. read depth	ref. seq
Pacific Ocean											
1MAsak	*Palythoa* sp. 'sakurajimensis'	Maui, Hawai'i	20.7575° N, 155.9884° W	Tidepool	1,314,179	156.4	90.2%	58.5	42.4%	2.4	6.8%
2MAZsa	*Zoanthus sansibaricus*	Maui, Hawai'i	20.7575° N, 155.9884° W	Tidepool	1,484,816	39.1	83.1%	36.7	48.0%	3.2	7.7%
27MAPmu	*Palythoa mutuki*	Maui, Hawai'i	20.7575° N, 155.9884° W	Tidepool	2,714,017	45.5	89.3%	21.2	62.8%	4.6	38.6%
29MAPmu	*Palythoa mutuki*	Maui, Hawai'i	20.7575° N, 155.9884° W	Tidepool	1,949,344	86.2	85.3%	102.5	93.4%	5.6	47.1%
226MAPtu	*Palythoa tuberculosa*	Maui, Hawai'i	20.7575° N, 155.9884° W	Tidepool	1,586,546	41.6	83.2%	200	76.5%	5.6	45.6%
117OAPtu	*Palythoa tuberculosa*	O'ahu, Hawai'i	21.5319° N, 158.2294° W	2	1,323,459	12.3	81.0%	90.2	66.0%	4.9	41.8%
135OAPtu	*Palythoa tuberculosa*	O'ahu, Hawai'i	21.2920° N, 157.8581° W	2	1,690,925	34.6	85.0%	75.9	85.7%	6.2	48.8%
143OAPtu	*Palythoa tuberculosa*	O'ahu, Hawai'i	21.2920° N, 157.8581° W	2	1,553,189	37.7	82.8%	149.7	87.5%	5.6	45.5%
psam_HIMB	*Palythoa psammophilia*	O'ahu, Hawai'i	21.4326° N, 157.7880° W	Intertidal	1,783,481	75.6	87.9%	45.6	83.9%	6.2	50.0%
Terr	*Terrazoanthus* sp.	O'ahu, Hawai'i	21.4443° N, 157.8098° W	1	1,665,886	29.5	64.1%	89.9	47.9%	4.7	7.2%
Z01_ptub	*Palythoa tuberculosa*	Okinawa	26.359722°N, 127.738889°E	5	1,646,095	74.4	85.7%	231.7	93.8%	6.0	47.0%
Z02_Phe	*Palythoa heliodiscus*	Okinawa	26.359722°N, 127.738889°E	5	1,520,169	22.5	81.2%	51.6	62.3%	3.3	15.9%
Z03_Pmiz	*Palythoa mizigama*	Okinawa	26.359722°N, 127.738889°E	7	1,690,582	36.5	86.1%	29.3	34.6%	2.9	23.8%
Z04_Pyoron	*Palythoa* sp. 'yoron'	Kagoshima	27.770278°N, 129.038333°E	Intertidal	2,262,230	46.2	87.5%	35.7	52.5%	2.1	20.3%
Z05_Pmut	*Palythoa mutuki*	Okinawa	26.0875°N, 127.708333°E	Intertidal	2,401,366	42.4	86.3%	99.9	92.2%	7.3	50.3%
Z06_Paffmut	*Palythoa* aff. *mutuki*	Kagoshima	27.770278°N, 129.038333°E	Intertidal	1,654,223	33.8	81.1%	107.4	92.0%	6.6	48.8%
Z07_Pcfmut	*Palythoa* cf. *mutuki*	Okinawa	26.846944°N, 128.286667°E	Intertidal	1,852,709	12.7	70.9%	104.3	96.9%	6.6	49.1%
Z08_Pyoron_deep	*Palythoa* sp. 'yoron'	Okinawa	26.444583°N, 127.768722°E	11	1,271,124	15.5	78.4%	78.2	87.6%	5.0	42.5%

Table 1 continued

Label	Species	Sample Location	GPS	Depth (m)	Total # of reads	Mt genome		Holobiont		Transcriptome	
						Avg. read depth	ref. seq	Avg. read depth	ref. seq	Avg. read depth	ref. seq
Atlantic Ocean											
Z09_Pgrandis	*Palythoa* cf. *grandis*	St. Eusatius	17.470444°N, 62.991722°W	11	2,010,137	21.3	77.2%	26.7	55.4%	4.0	19.5%
Z010_Pcar	*Palythoa caribaeorum*	Brazil	27.292222°S, 48.368806°W	10	1,874,568	26.3	85.6%	126.8	90.9%	6.9	49.8%
Z012_Psak	*Palythoa* sp. 'sakurajimensis'	Dongsha Atoll	20.766583°N, 116.897033°E	5	1,713,416	38.2	86.4%	16.9	35.0%	2.6	12.0%
Z013_Psak2	*Palythoa* sp. 'sakurajimensis'	Dongsha Atoll	20.766583°N, 116.897033°E	5	1,597,970	11.5	64.1%	268.8	90.6%	4.7	28.6%
Z014_Pgrandiflora	*Palythoa grandiflora*	Costa Rica	9.659333°N, 82.753472°W	1	1,777,003	34.9	83.5%	15.6	67.8%	6.8	51.9%
Z015_Pgrandis2	*Palythoa grandis*	Curaçao	12.060111°N, 68.846194°W	11	1,397,713	54.9	85.1%	26.9	52.3%	3.1	16.0%
				Average	1,738,964	42.9	82%	87.1	71%	4.9	34%

RAD datasets with a variety of filtering strategies, assembled transcriptome loci, and nearly complete mitogenomes as outlined below.

Holobiont dataset

The holobiont dataset was constructed via de novo assembly of individual read libraries using the recommended settings in dDOCENT (Puritz et al. 2014) to produce a total of 17,389 shared SNPs. First, reads from each sequencing library were trimmed using default settings in dDOCENT with an overlap (OL) assembly, then clustered based on overall sequence similarity using CD-HIT with a -c parameter of 90%. Clustered reads were mapped with BWA (mem algorithm) using default settings. SNPs were then identified within the dDOCENT pipeline using FREEBAYES (Garrison and Marth 2012) to call variants from merged bam files produced by dDOCENT. The dDOCENT pipeline produces SNPs in two variant call format files; (.vcf) and raw SNPs (TotalRawSNPs.vcf). The TotalRawSNPs file for each individual was imported into TASSEL v.5 (Bradbury et al. 2007) to examine the effects of missing data and explore similarities via PCA analyses.

We also tested a variety of filtering stringency using the program VCFTOOLS v.1.13 (Danecek et al. 2011). These strategies ranged from the "raw" holobiont SNP dataset with no additional filtering (which included all shared SNPs), to the most stringent filtering used –mac 2 –minQ 30 with zero missing data among taxa. This most stringent filtering strategy is referred to as the "high quality" dataset and consisted of 136 unlinked, biallelic SNPs across all samples (–remove-indels –max-missing 1 –minQ 30 –mac 2 –recode –recode-INFO). The VCF file from each of the least and most stringent filtering strategies was converted to a fasta format using the program VCFKIT (https://github.com/Andersenlab/vcf-kit) using the phylo fasta command to generate maximum likelihood and Bayesian inference trees.

Transcriptome dataset

To generate the transcriptome dataset, sequencing libraries were assembled to the *Palythoa variabilis* transcriptome (accession number: GCVI00000000.1, Huang et al. 2016) retaining only those reads that mapped to the reference. This approach recovered a total of 1,460,914 SNPs and 77,225 contigs in dDOCENT. The contigs represent assembled loci that are equivalent to traditional PCR-based phylogenetic loci and can be analyzed either as loci or as an additional SNP dataset. Thus, we also filter these reads using VCFTOOLS (–remove-indels –max-missing 1 –minQ 30 –mac 2 –recode –recode-INFO) which resulted in a final dataset of 1327 unlinked, biallelic SNPs with no

missing data across all samples. Because both the assembled loci and SNP datasets reconstruct the same overall cladal structure, we present the SNP dataset here for consistency.

Mitochondrial genomes dataset

Each sequencing library was paired, trimmed, and assembled to the reference mitochondrial genome *Palythoa heliodiscus* (accession number: NC_035579; Chi and Johansen 2017) in GENEIOUS v.11.0.5 using the default parameters. As above, only reads that mapped to this reference were retained and genes that mapped to the mitochondrial reference are listed in Table S1. Consensus sequences were made from the reads assembled for each library (not including the reference sequence) using the 75% majority option, and Ns were called if coverage was less than 3X. Multiple sequence alignments (16,601 bp in length) were constructed using MUSCLE v3.8.425 (Edgar 2004) under default parameters with eight iterations, resulting in 654 SNPs.

Phylogenetic analyses

Substitution model selection

Substitution models were estimated by JMODELTEST v.2.1.10 (Darriba et al. 2012) to determine the model of evolution for each dataset. For the mitochondrial genome, TPM3uf + I + G was the best fit using the Bayesian Information Criterion (BIC). For the holobiont dataset, TPM3 + I + G was the best fit and for the transcriptome dataset TVM was the best fit. For analyses that could not incorporate the best model of sequence evolution, we applied the next best model that could be implemented in that program. We also calculated mean genetic distance (*d*) between clades for the mitogenomes and holobiont datasets in MEGA v.10.1.8 (Kumar et al. 2018). Percent sequence divergence was calculated for the mitochondrial genomes dataset in MEGAX v.0.1 (Kumar et al. 2018).

Phylogenetic tree reconstructions

Maximum likelihood reconstructions were computed using RAXML v.8.1.16 (Stamatakis 2014) for each of the datasets outlined above. For all our maximum likelihood analyses, we used the GTRGAMMA model of nucleotide evolution, conducted a rapid bootstrap analysis (*-f*), searched for the best scoring tree in a single run (*-a*), and used 1000 rapid bootstrap replicates to estimate clade support.

Bayesian inference trees were computed using BEAST v.2.6.0 (Bouckaert et al. 2019) with optimal substitution model calculated under BIC. For each dataset, parameters were set as default values except for the substitution model set to the best fit using BIC for each dataset, the molecular clock rate was changed to relaxed clock log normal (Drummond et al. 2006), and the priors were set to coalescent exponential population (Drummond and Rambaut 2007). A total of 10 million generations were run, with trees stored every 1000 generations, and the first 10% of trees discarded as burn-in. Ten independent runs were computed for each dataset to ensure convergence and log files were combined using the program TRACER v.1.7.1 (Rambaut et al. 2018). Analyses of all tree files were combined using the program LOG COMBINER v.2.6.2 and a maximum clade credibility tree was constructed using TREE ANNOTATOR v.1.6.0. The program FIGTREE v.1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/) was used to visualize the phylogeny.

A species tree was reconstructed from the most stringent filtered RAD dataset using the SNAPP package implemented in BEAST. For this analysis both outgroups were removed, which resulted in 245 high-quality informative SNPs shared across all *Palythoa* libraries. The program PGDSPIDER2 v.2.1.1.5 (Lischer and Excoffier 2012) was used to convert the VCF file to a binary nexus format. In BEAUTi, priors and mutation rates *u* and *v* were estimated from the data for all three models. All other settings were set as default. In BEAST, MCMC chains were run for 10 million generations, sampling every 1,000 generations. Convergence was assessed in TRACER and the first 10% of trees were discarded as burn-in. DENSITREE v.2.2.7 (Bouckaert and Heled 2014) was used to visualize posterior distributions of the topologies as cloudograms.

Species delimitation approaches

We considered a variety of species delimitation approaches as a potential way to shed light on these data. We settled on Poisson tree processes (PTP) species delimitation tests because this model tends to perform well given the number of taxa, loci and individuals per taxon in our study (Luo et al. 2018). We used the Bayesian implementation version (bPTP), available on the Species Delimitation Server (https://species.h-its.org/) run with the maximum allowed 500,000 MCMC iterations, thinning of 100 and burn-in of 0.1 as suggested (Zhang et al. 2013). Convergence of the model was confirmed by visual inspection of the likelihood plot of MCMC iterations after thinning.

Results

A total of 22 individual *Palythoa* and two outgroup (*Z. sansibaricus* and *Terrazoanthus* sp.) sequencing libraries were generated for the holobiont, transcriptome, and mitochondrial datasets (Table 1). The average number of reads across all libraries was 1,742,649 and average reference coverage was the highest in the mitochondrial dataset followed by the holobiont and transcriptome datasets (82%, 70%, and 33%, respectively). Samples Z07_Pcfmut, Z01_Ptub, and 29MAPmu had the greatest mean reference coverage across the holobiont, resulting in 96.9%, 93.8%, and 93.4%, respectively, and individuals Z03_Pmiz and Z012_Psak had the lowest percent coverage (34.6 and 35%, respectively; Table1). In contrast, the highest percent coverage across the transcriptome dataset was sample Z014_Pgrandiflora (51.9%) and individual 1MAsak had the lowest percent coverage (6.8%) (Table 1). Across the mitochondrial genome, coverage was the highest in samples 1MAsak, 27MAPmu, and psam_HIMB (90.2, 89.3, and 87.9%, respectively) and lowest coverage in samples Z013_Psak2 and Z07_Pcmut (64.1 and 70.9%, respectively, Table 1).

Holobiont and transcriptome datasets

We applied a variety of filtering methods to each the holobiont and transcriptome phylogenies, but comparison of results among the various filtering thresholds ranging from the raw unfiltered holobiont to the most highly filtered datasets always reconstructed the same six clades (Fig. 2). Although sister-group relationships among nominal taxa varied among each of the methods and many of the filtering strategies, reconstructed phylogenies were always congruent at the level of the six major clades (I–VI) reported here (Figs. S1–S3; S6, S7). Each of the holobiont and transcriptome datasets produced a congruent and well-resolved phylogeny at the level of the clades, with strong posterior probability (pp; ≥ 0.82) and maximum-likelihood support (bootstrap ≥ 78) regardless of the degree of filtering. Species relationships within clades, particularly clades I and II, differed among the datasets and filtering strategies, but typically with short branch lengths and highly variable support. Therefore, species were collapsed into clades for purposes of presentation in Fig. 2 but are examined in greater detail below.

Mitochondrial genomes

Reads from each sequencing library that mapped to the mitochondrial reference genome of *P. heliodiscus* (20,797 bp; Chi and Johansen 2017) resulted in an average 82% coverage of the reference genome (relative recovery of the complete mitogenome) across all taxa included here. Using only these mitochondrial genomes, we reconstructed the identical tree topology (other than inclusion of the reference mitogenome) with high branch support (BI ≥ 0.98; ML ≥ 0.84) for each of the six major clades (Fig. 2c).

SNAPP analyses

A species model SNP tree cloudogram was generated using a coalescent-based SNAPP analysis producing high posterior support for clades I–VI (Fig. 3a) and a maximum clade credibility tree (Fig. S8). As with the analyses above, species relationships within clades I and II was unclear, but species membership within each clade remained consistent and highly supported. Again, this most highly filtered set of high-quality SNPs recovered the same six distinct clades as seen in the other datasets, with only poorly sampled clades ($N = 1$ or 2) showing up as geographically distinct (Fig. 3).

Species relationships and species delimitation within clades

Bayesian phylogenetic analysis of the holobiont dataset recovered well-supported topologies that differentiated between conspecifics and nominal taxa alike (Fig. S4). For example, within clade I, all *P. tuberculosa* taxa (A and B; Fig. S4) showed high posterior support differentiating each colony from the others (pp = 1.0), and similarly, within clades II, III, and IV; *P. mutuki* species (A and B; Fig. S4), *P.* sp. 'sakurajimensis,' and *P. grandis* individuals yielded well-supported nodes (pp ≥ 0.98; Fig. S4).

Sequence divergences among the six clades for *Palythoa* samples ranged from 0.24 – 1.56% across the nearly complete mitochondrial genomes. Divergence of the *Palythoa* samples to the outgroups *Z. sansibaricus* and *Terrazoanthus* sp. ranged from 3–4.3% for the mtDNA dataset (Table 2).

The various datasets produced phylogenies in which species relationships differed, sometimes dramatically. Overall, species within clades I and II were characterized by short branch lengths with highly variable support and often sister-group relationships changed among the different datasets and filtering parameters. For example, in the holobiont phylogeny, *P. tuberculosa* was polyphyletic and sister to *P. caribeaorum* and *P.* sp. 'yoron,' whereas *P. mutuki* was polyphyletic with some individuals as sister to *P. psammophilia* while others appeared as sister to *P. grandiflora* (Fig. S4). In contrast, the transcriptome maximum-likelihood reconstructed an unresolved polytomy for all species in clades I and II (Fig. S2). Similar to the transcriptome dataset, the mitochondrial genomes

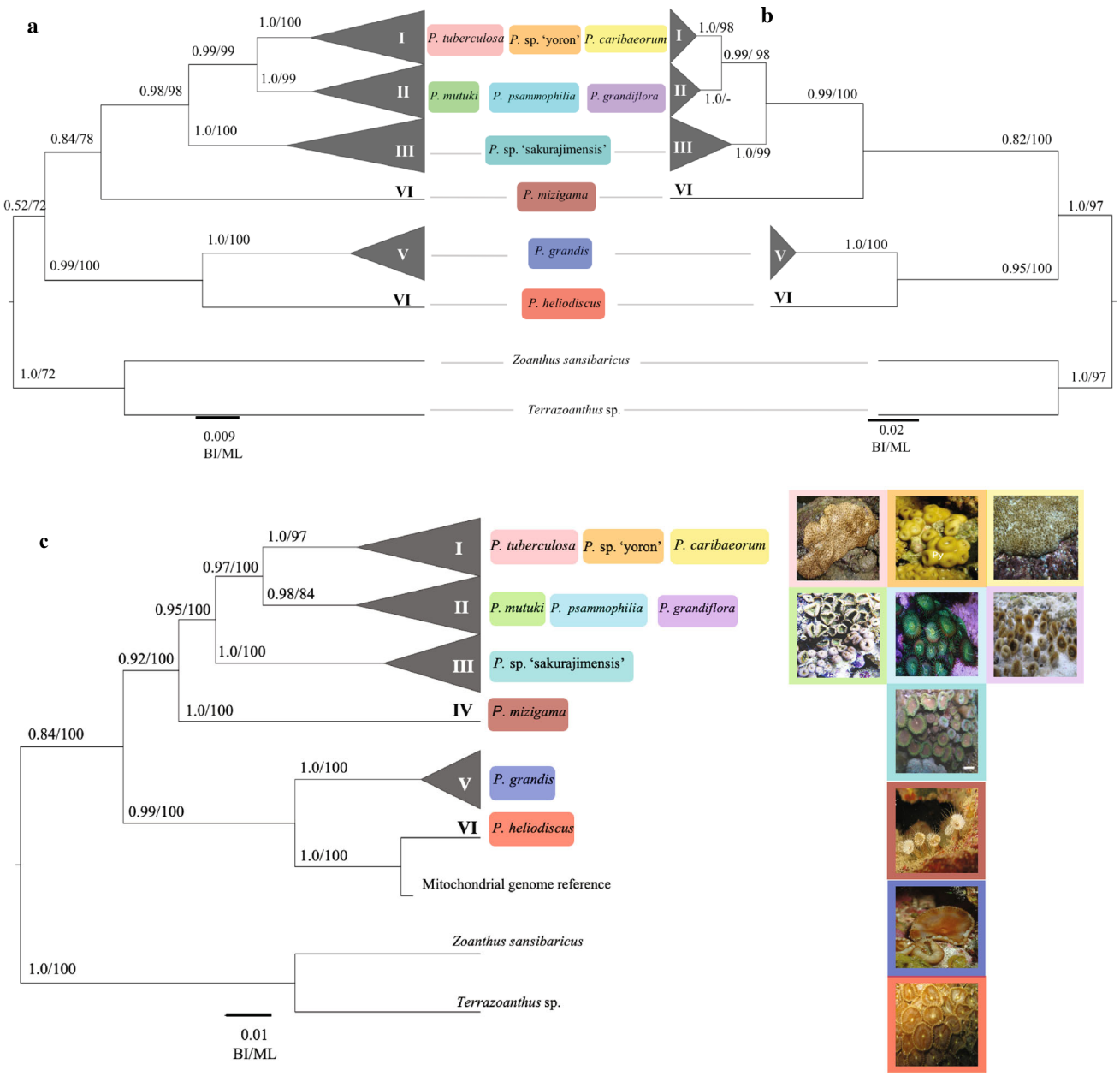

Fig. 2 Phylogenetic analyses of *Palythoa*. Bayesian and Maximum likelihood (BI/ML) phylogenetic analyses of the **a** Holobiont, **b** Transcriptome, and **c** Nearly complete mitochondrial ezRAD data for a total of 22 *Palythoa* species including eight nominal species and four putative or unknown species and two outgroups, *Zoanthus sansibaricus* and *Terrazoanthus* sp. Colors correspond to the denoted species and photos of each species. Clade I species: *P. tuberculosa*, *P. caribaeorum*, and *P.* sp. 'yoron'; Clade II: *P. mutuki*, *P.* cf. *mutuki*, *P.* aff. *mutuki*, *P. psammophilia*, and *P. grandiflora*; Clade III: *P.* sp. 'sakurajimensis'; Clade IV: *P. mizigama*; Clade V: *P. grandis* and *P.* cf. *grandis*; Clade VI: *P. heliodiscus*

maximum-likelihood phylogeny reconstructed an unresolved polytomy for the species in clade I, whereas the relationships with *P. mutuki* were more similar to the holobiont dataset and resolved as polyphyletic in clade II (Fig. S3).

Species delimitation using the full dataset recovered the same six major clades as each of our other analyses. Ideally, species delimitation tests should not include outgroups, but what constitutes an outgroup is difficult to decide when we have consistent phylogenomic cladal structure and uncertain species boundaries. We found that the species delimitation test results differed among each of the marker class datasets (SNP holobiont, mtDNA, transcriptome), and even within the same dataset based on species delimitation approach (data not shown). For example, when we restricted our analyses to only clades I,

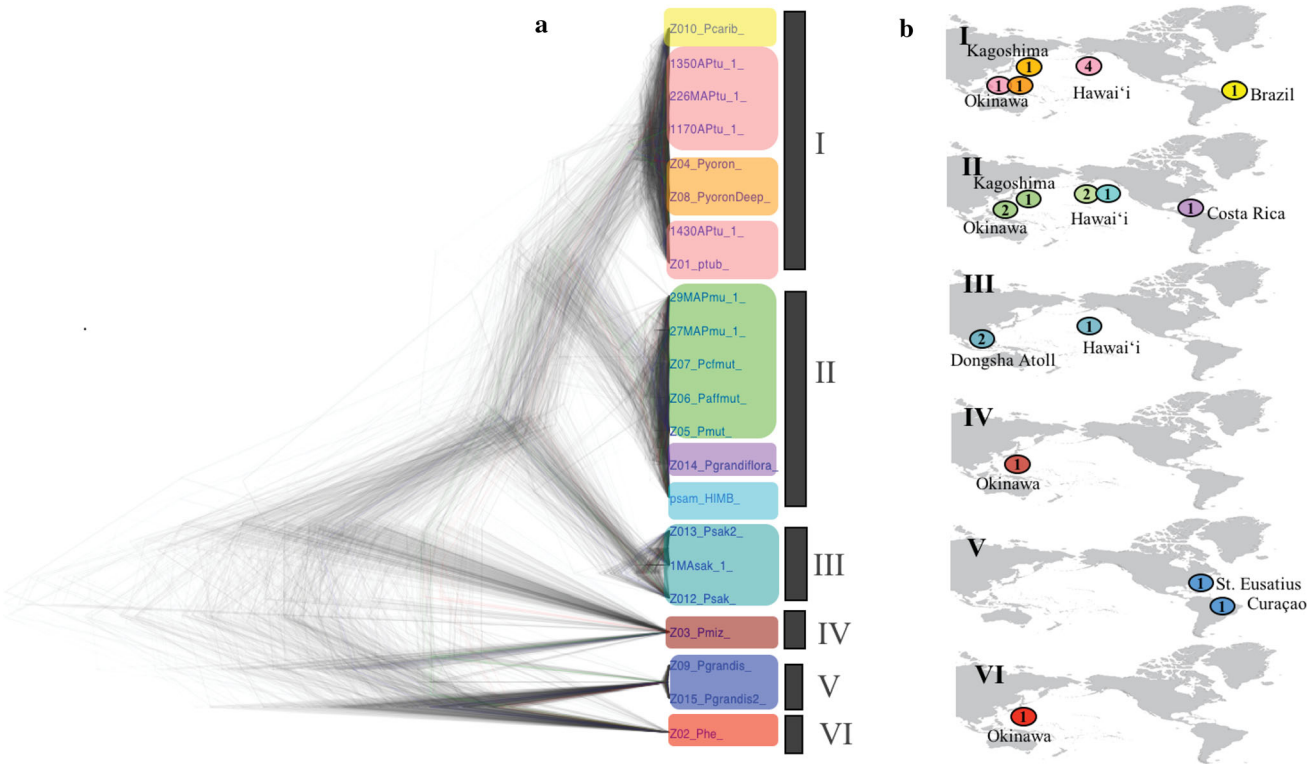

Fig. 3 Species tree of *Palythoa.* **a** Depicted is a cloudogram from the Bayesian phylogenetic analysis program SNAPP based on the posterior distribution of 245 unlinked biallelic SNPs. Colors of the SNAPP tree topology represent different agreements; darker grey areas indicate greater tree topology credibility and lighter grey areas are disagreements in tree topology. Species groups are represented by distinct color groups. Clade I: *P. tuberculosa*, *P. caribaeorum*, and *P.* sp. 'yoron'; Clade II: *P. mutuki*, *P.* cf. *mutuki*, *P.* aff. *mutuki*, *P. psammophilia*, and *P. grandiflora*; Clade III: *P.* sp. 'sakurajimensis'; Clade IV: *P. mizigama*; Clade V: *P. grandis* and *P.* cf. *grandis*; and Clade VI: *P. heliodiscus* **b** Map of sampling location for each clade of species. Map image provided by Richard Coleman

II, and III, species delimitation using maximum likelihood collapses clades I and II into a single species group with 15 members but supported the three *P.* sp. 'sakurajimensis' in clade III as a distinct species from all the others. In contrast, the highest Bayesian support solution for this same dataset identified 16 species, with each of our samples as a distinct species from every other, except for the two *P.* sp. 'yoron' (Z04_Pyoron & Z08_PyoronDeep) and two *P. mutuki* (27MAPmu & 29MAPmu) samples each being collapsed into single species (Fig. S5).

Discussion

We present the first genomic dataset to evaluate phylogenetic relationships and species boundaries in the zoantharian genus *Palythoa.* We incorporated specimens from eight described and four putative species to evaluate how well current taxonomy is supported by phylogenetic analyses. Species were sampled from across the distributional range of the genus to include a range of well-resolved to uncertain nominal species, and two species with broad geographic sampling to test whether relationships matched the currently accepted taxonomy. Our results do not support the current taxonomy of these groups, but instead revealed six highly concordant and well-supported clades among trees reconstructed using all datasets: mitochondrial genomes, assembled transcriptomic loci, highly filtered and unfiltered holobiont, and SNP-based phylogenies. These data, particularly the mitochondrial genomes, can easily be combined with existing and future datasets to expand the current study. There appears to be evidence for species complexes in the most well sampled clades (I and II), and some nominal taxa are polyphyletic within these clades. However, our lack of geographic and taxon sampling beyond these groups limits our ability to draw conclusions about the other clades.

These six clades are well supported by every facet of the genetic data, yet it remains somewhat unclear whether these clades represent species or genera. Results from species delimitation approaches are inconsistent among datasets. Further, the maximum likelihood solution combines clades I and II into a single variable species putting clades at a largely species level, whereas the Bayesian solution retains each of the nominal taxa as distinct species, suggesting clades represent higher level subgeneric or

Table 2 Percent divergence level comparisons for the nearly complete mitochondrial genome in zoantharians

	Clade I	Clade II	Clade III	Clade IV	Clade V	Clade VI	*Z. sansibaricus*	*Terrazoanthus* sp.
Clade I	–	0.27%	0.28%	0.77%	1.04%	1.56%	3.28%	3.87%
Clade II		–	0.24%	0.73%	1.00%	1.53%	3.20%	3.83%
Clade III			–	0.60%	0.81%	1.32%	3.03%	3.75%
Clade IV				–	0.88%	1.41%	3.08%	3.70%
Clade V					–	0.86%	3.04%	3.55%
Clade VI						–	3.28%	3.84%
Z. sansibaricus							–	4.38%
Terrazoanthus sp.								–

Comparison species groups are as follows: Clade I: *P. tuberculosa*, *P. caribaeorum*, and *P.* sp. 'yoron'; Clade II: *P. mutuki*, *P.* cf. *mutuki*, *P.* aff. *mutuki*, *P. psammophilia*, and *P. grandiflora*; Clade III: *P.* sp. 'sakurajimensis'; Clade VI: *P. mizigama*; Clade V: *P. grandis, P.* cf. *grandis*; Clade VI: *P. heliodiscus;* and Outgroups: *Zoanthus sansibaricus* and *Terrazoanthus* sp

perhaps even generic distinctions. Despite the volume of data and approaches employed here, these results echo the uncertainty of previous work where low resolution among nominal taxa remains. While our study can reject the hypothesis that inconclusive species boundaries result from low resolution molecular markers, we still failed to determine whether each clade represents a single or many species. Given that uncertainty, we are unable to resolve this issue with the data in hand, and we have elected to use the term 'species complex' to refer to the members of clades that are consistently resolved among datasets but poorly resolved among approaches or with differing datasets herein.

Recent studies on clades I and II in southern Japan provide hints to help solve this species dilemma. Mizuyama et al. (2018, 2020) collected phylogenetic (mtCOI, mt16S rDNA, ITS-rDNA, ALG11), reproductive, morphological, and Symbiodiniaceae data from *P. tuberculosa* and *P.* sp. 'yoron' (clade I), *P. mutuki* and *P.* aff. *mutuki* (clade II), and the majority of non-genetic data strongly suggests the presence of four species, distinguishable by reproductive timing and morphology. Similar to our current results, the groupings observed by Mizuyama et al. (2018) were not clearly delineated by molecular data, indicating a very shallow evolutionary history for these putative species, and pointing toward possible recent speciation events. Overall, our study reconfirms that additional studies with a variety of datasets are needed to resolve species delineation questions, and to determine where the boundaries are between species within the genus *Palythoa* (Mizuyama et al. 2018, 2020).

The species complexes we observed contained specimens from two distinct ocean basins, the Atlantic and Pacific, in both clades I and II. Previous research has reported zoantharian sibling species between oceans in the genera *Palythoa* and *Zoanthus*, and these sibling species have similar morphologies and are phylogenetically closely related (Reimer and Fujii 2010; Reimer et al. 2012; Santos et al. 2016; López et al. 2019). Such patterns have not been observed in other Atlantic–Indian/Pacific anthozoans such as in scleractinian hard corals (Scleractinia; Fukami et al. 2004) or in hydrocorals (*Millepora*; Arrigoni et al. 2018); these two groups both have deep genetic divergences for shallow-water congeneric species from different oceans, suggesting previous theories on speciation of coral reef anthozoans may not be applicable to zoantharians.

Shallow Atlantic and Indian/Pacific coral reef species are generally thought to have been isolated from each other for at least 3MY, since the closing of the Isthmus of Panama (IP; O'Dea et al. 2016). Closely related sibling species have been documented on either side of the IP as a result of the land barrier for many marine taxa (Knowlton and Weigt 1998; Lessios 2008; Marko and Moran 2009), as well as for some of the *Palythoa* species examined in this study (Fig. 3b). Populations of *P. caribaeorum* are found on the Western African coast (Wirtz and d'Acoz 2008) and *P. tuberculosa* has been reported from the Southeastern African coast (Risi and Macdonald 2016), but warm water species in the Atlantic and Indian oceans are separated by the Benguela Upwelling barrier, located in the southern tip of Africa, and present for more than 2 million years (Hutchings et al. 2009). Thus, given their isolation from each other for millions of years, these *Palythoa* taxa should be different species. Nevertheless, some marine species may have been able to maintain limited genetic flow between these regions during interglacial periods with the cessation of the cold-water upwelling, or through leakage of gyres of the Agulhas Current from the Indian to the Atlantic Ocean (Peeters et al. 2004; Bowen et al. 2006; Andrews et al. 2016b; Dudoit et al. 2018). Future research on the phylogeography of *Palythoa* will hopefully elucidate the relationships and genetic flow within and between ocean basins.

Clade I: *P. tuberculosa*, *P. caribaeorum*, and *P.* sp. 'yoron' species complex

In our study, this clade included two described species and one unknown, putative species. Both described species have extensive distributions, with *P. tuberculosa* being the most widespread *Palythoa* species in the Indian/Pacific oceans, ranging from the Red Sea to the Galapagos (Burnett 2002; Reimer et al. 2007, 2017b; Reimer and Hickman 2009). Similarly, *P. caribaeorum* is the *Palythoa* species with the most extensive distribution across the Atlantic Ocean (Santos et al. 2019), reported from the Caribbean (Sebens 1982; Montenegro et al. 2020) to Brazil (Santos et al. 2016) and Africa (Wirtz and d'Acoz 2008), including oceanic islands such as Ascension (Reimer et al. 2017a), Cape Verde (Reimer et al. 2010) and Saint Helena (Santos et al. 2019). A long-living larval stage of more than 5 months has been recorded for *P. tuberculosa* (Polak et al. 2011), and the larvae of *Palythoa* spp. are regularly reported from plankton trawls in oceans above 20 °C (Ryland et al. 2000). Additionally, a high frequency of fertile polyps and continuous gametogenesis has been observed in *P. caribaeorum* in Brazil (Boscolo and Silveira 2005), as well as many asexual reproductive modes (Acosta et al. 2005). Zoantharian species also have the potential to disperse by rafting (Santos and Reimer 2018). Although these varieties of reproduction and dispersal could promote gene flow among populations of each species, there have been very few studies investigating genetic connectivity of *Palythoa* populations (Burnett et al. 1994). Moreover, unexpectedly, in some of our analyses (e.g., Fig. S4), some *P. tuberculosa* specimens from Hawai'i were more closely related to *P. caribaeorum* than to other specimens of *P. tuberculosa*. Questions have surrounded the identity of many *Palythoa* spp. (e.g. Burnett et al. 1995, 1997; Ryland and Lancaster 2004), and our genomic results here indicate again that more work is needed.

In contrast to the extensive distribution of *P. caribaeorum* and *P. tuberculosa* in the Atlantic and Pacific oceans, respectively, the putative species *P.* sp. 'yoron' thus far has a restricted distribution confined to the Ryukyu Archipelago of southern Japan, and is found there in sympatry with *P. tuberculosa* and *P. mutuki* (Shiroma and Reimer 2010; Mizuyama et al., 2018). Previous studies have suggested that *P.* sp. 'yoron' may be a hybrid between *P. tuberculosa* and *P. mutuki* based on ITS-rDNA sequences and intermediate morphological characteristics (Reimer et al. 2007; Shiroma and Reimer 2010). Mizuyama et al. (2018) conducted a morphological and phylogenetic comparison of *P. tuberculosa*, *P. mutuki* species, and *P.* sp. 'yoron' revealing morphological and reproductive differences between these three lineages whereas phylogenetic data united *P. tuberculosa* and *P.* sp. 'yoron' in a monophyletic clade.

Our analyses similarly grouped *P. tuberculosa* and *P.* sp. 'yoron' together within the same clade, but they did not form clearly resolved sister groups in any of our analyses. In all our phylogenetic datasets, the two specimens of *P.* sp. 'yoron' (Z04_Pyoron collected from the intertidal in Kagoshima and Z08_PyoronDeep collected at 11 m in Okinawa) always grouped together. Except for analyses in which the entire clade was an unresolved polytomy, the two *P.* sp. 'yoron' formed a well-resolved sister pair distinct from both *P. tuberculosa* and *P. caribaeorum* (albeit not sister to either species). *Palythoa* sp. 'yoron' until now has been reported from comparatively high in the intertidal zone (Shiroma and Reimer 2010; Mizuyama et al., 2018), and the genetic distances between our intertidal specimen and the deeper specimen are at the same level as distances between other recognized species, indicating the deeper specimen may belong to a different, closely related species.

Clade II: *P. mutuki* group, *P. psammophilia* and *P. grandiflora* species complex

Similar to clade I, clade II contains species from both the Atlantic and Pacific oceans. Each of our analyses grouped *P. mutuki*, *P. psammophilia*, and *P. grandiflora* and the two putative sympatric species *P.* aff. *mutuki* and *P.* cf. *mutuki* within clade II (Figs. 2, 3, 4). *Palythoa mutuki* is widely distributed across the Pacific (Ryland and Lancaster 2003; Reimer et al. 2007) and Indian oceans (Kumari et al. 2016) and in the Red Sea (Reimer et al. 2017b), while *P. grandiflora* is also broadly distributed across the Atlantic Ocean with an amphi-Atlantic distribution (Santos et al. 2019). In contrast, *P. psammophilia* has a distribution limited to Hawai'i (Walsh and Bowers 1971). The external morphologies of *P. psammophilia* and *P. mutuki* are generally similar with regard to polyp shape and color patterns in the oral disk. Additional analyses would be needed to resolve if the former species is a junior synonym of *P. mutuki*; a high number of inadvertent redescriptions of the same *Palythoa* species from different localities has previously been suggested (Burnett et al. 1997b; Reimer et al. 2010; Hibino et al. 2014). Previous phylogenetic analyses of *P. mutuki* and *P.* aff. *mutuki* found that they formed a separate and paraphyletic clade from other *Palythoa* species, but each had clear morphological differentiation and distinct spawning periods (Mizuyama et al. 2018). Here, *P. mutuki* species were clearly polyphyletic with Z05_Pmut coming out as sister to *P.* aff. *mutuki* and being more distantly related to the other *P. mutuki* samples (27MAPmu & 29MAPmu) than either group was to the *P. psammophilia* or *P. grandiflora* specimens (Fig. S4).

Clade III: *P.* sp. 'sakurajimensis'

This putative undescribed species has a broad distribution throughout the Pacific, being reported from southern Japan (Reimer et al. 2007) and recently found in the Red Sea (Reimer et al. 2017b). Despite extensive sampling throughout this broad geographic range, *P.* sp. 'sakurajimensis' is rarely observed, indicating that it is more cryptic or less common than other *Palythoa* species. *Palythoa* sp. 'sakurajimensis' specimens are typically found as a single polyp or small colonies (Reimer et al. 2017b) and appear to able to tolerate cooler water temperatures, below 15 °C (Ono et al. 2008), which other zooxanthellate members of this genus cannot. Previous phylogenetic analyses based on ITS and 16S rDNA data support *P.* sp. 'sakurajimensis' as a well-supported and distinct clade from other *Palythoa* groups (Reimer et al. 2007, 2017b). Our findings confirm these previous studies, although the sister-group relationships among samples from Hawai'i to the South China Sea varied among datasets. Regardless, specimens of *P.* sp. 'sakurajimensis' consistently formed a highly supported monophyletic clade among all datasets (Figs. 2, 3, 4), and are distinct in species delimitation approaches supporting the validity of recognizing this clade as a species.

Clade IV: *P. mizigama*

The species *P. mizigama* occurs in low-light environments such as coral reef caves or crevices, and has been reported from the Central Indo-Pacific, Japan and New Caledonia (Irei et al. 2015). There has yet to be any studies on the biology or life history of this species since its original description (Irei et al. 2015). This is one of three known azooxanthellate *Palythoa* species, along with *P. macmurrichi* and *P. umbrosa*. Thus, *P. mizigama* is an important lineage to better understand the evolution of symbioses between hexacorals and dinoflagellates. Previous phylogenetic analyses using fragments of mitochondrial and nuclear markers showed incongruent tree topologies and were not able to clearly solve the phylogenetic positions of *P. mizigama*, *P. umbrosa* and the azooxanthellate genus *Sphenopus* (Irei et al. 2015). In our analyses, this species was clearly genetically distinct from the other *Palythoa* species, and was a sister lineage of clades I, II and III, but additional geographic and taxon sampling within this clade is needed.

Clade V: *P. grandis*

Palythoa grandis is only known from the Caribbean, with little information on its biology and life history (Ryland and Lancaster 2003; Reimer et al. 2012). Our two specimens grouped as sister taxa within a well-supported clade across all the phylogenetic datasets examined in this study. The divergence between these specimens was of approximately equal magnitude to those of some groups within clade I and clade II, indicating the need to examine this group more closely with broad geographical sampling in the future (Fig. S4).

Further, we originally included a sample putatively identified as *P. variabilis* in our analyses for comparison, however, initial results showed this specimen to be very closely related to a confidently identified specimen of *P. grandis*, which led us to re-examine the specimen. Upon re-examination, it was determined that this putative *P. variabilis* specimen was misidentified and was in fact a juvenile single-polyp specimen of *P. grandis*. Thus, we lost the inclusion of *P. variabilis* in our study, but it is noteworthy that there is virtually no branch length between our re-examined *P. grandis* specimen and the reference *P. variabilis* transcriptome. Based on our taxonomic examination and available genetic data, the question of whether the reference transcriptome is also drawn from a misidentified individual (i.e., the correct species name for the transcriptome reference is *P. grandis*) deserves careful examination. Alternately, the taxonomic relationship between *P. grandis* and *P. variabilis* may need to be phylogenetically re-examined in the near future.

Clade VI: *P. heliodiscus*

This species is distributed throughout the Pacific Ocean; in Australia (Ryland and Lancaster 2003), Japan (Reimer et al. 2007), Palau (Reimer et al. 2014) and Taiwan (Reimer et al. 2011). Additionally, individuals of *P.* cf. *heliodiscus* have recently been reported from the Red Sea (Reimer et al. 2017b), indicating that this clade may be quite widespread. *Palythoa heliodiscus* is found in deeper areas ($> \sim 10$ m) with lower sunlight (Reimer et al. 2007). Future sampling of additional Red Sea specimens is needed to confirm whether *P. heliodiscus* is a single broadly distributed species, or also represents a possible species complex as in clades I and II. Previous phylogenetic analyses based on COI, 16S and ITS separated *P. heliodiscus* as a highly supported monophyletic clade relative to other *Palythoa* species (Reimer et al. 2006, 2007, 2011). Our study confirms these past results, and *P. heliodiscus* formed a highly supported monophyletic clade sister to *P. grandis* (clade V, Fig. 2).

What constitutes a species in *Palythoa*?

Although species relationships within the clades varied between datasets and methods, all were highly congruent in recovering six well-supported clades within *Palythoa*. However, even with genomic level data, clear species

boundaries in this group remained elusive, and the question remains with regard to what level of taxonomic resolution these clades represent. Species delimitation approaches provided conflicting results depending on the data used and whether maximum likelihood (collapsing clades I and II into a single species) or Bayesian (nominal taxa are all distinct species) evidence was favored. Likewise, branch support with each class of data was equally high between some of the described species within a clade as it was among the clades. For example, specimens of *P. tuberculosa* A and B were distinct and as well-supported as any of the clades (pp = 1; Fig. S4). A similar trend was observed where *P. mutuki* A is more closely related to some Atlantic *P. grandiflora* than to the other *P. mutuki* B sampled in this study (pp $\geq$ 0.93; Fig. S4). This raises the question of what constitutes species-level differences in our genomic dataset, and what are the thresholds for divergences observed within, versus between, species for the genus *Palythoa*.

Thresholds of sequence divergence have been proposed as one method to delineate species across a broad array of understudied marine taxa, though appropriate values must be determined for each marker and each group (Costa et al. 2009; Zemlak et al. 2009; Radulovici et al. 2010; Bucklin et al. 2011; Chen et al. 2011; Layton et al. 2014). For the *Palythoa* species included in this study, we calculated the percent sequence divergences between clades based on the nearly complete mitochondrial genome, and compared our values to mtDNA data available in the literature for zoantharians. Among *Palythoa* species studied to date, among-species mtCOI sequence divergences spanned from 0–1.14%, while divergence among genera have been reported as typically in the range of 3–4% (Sinniger et al. 2005, 2008; Reimer et al. 2006, 2007, 2012). Across nearly complete mitochondrial genomes, we observed a sequence divergence range from 0.24 to 1.56% among *Palythoa* clades, whereas sequence divergences to the outgroups *Zoanthus sansibaricus* and *Terrazoanthus* sp. ranged between 3.0–4.3% (Table 2). These values are remarkably concordant, and our results suggest that the entire mitochondrial genome provides roughly the same resolution as the COI for *Palythoa* species (also see Poliseno et al. 2020). However, with the lack of a threshold consensus metric among zoantharians, the question of what threshold of molecular divergence constitutes a species in *Palythoa* still remains.

It is possible that some of the taxonomic signal, or alternatively some of the noise in that signal, may result from differences in the symbiont communities present within holobiont sequencing libraries. Zoantharians possess endosymbiotic zooxanthellae (Symbiodiniaceae) with various levels of symbiont specificity and flexibility among species (Reimer et al. 2008). For example, Forsman et al. (2020) show coevolution among hosts and symbionts in the scleractinian coral genus *Porites* that reinforces the observed phylogenetic pattern. Alternatively, symbiont flexibility within the same host species could result in divergent symbiont communities that would introduce noise into the phylogenomic pattern of holobiont datasets (Magalon et al. 2006; Sawall et al. 2015). Here, we compared results from the symbiont-containing holobiont libraries to both mitogenome and host-specific transcriptomic datasets which showed identical branching patterns to that of the holobiont at the clade level (Fig. 2). Thus, although we were unable to determine the extent to which differences in the relationships among species within clades were impacted by inclusion or exclusion of Symbiodiniaceae reads, we are confident that they did not overwhelm the phylogenetic signal of the six clades recovered by all datasets.

Future directions and conclusions

Our study presents the most complete phylogenomic analyses of a zoantharian genus to date, but still fails to resolve a clear species-level phylogeny for the genus *Palythoa*. The consistency of findings across mitochondrial genomes, assembled transcriptomic loci, highly filtered and essentially unfiltered RAD holobiont data, and high-quality SNP tree reconstructions rejects the hypothesis that low-resolution molecular data are to blame for uncertainty and point to a more complicated biological explanation for taxonomic uncertainty in this group. Across all methods and datasets, we recovered six distinct clades within the genus *Palythoa*, although sister relationships among species varied among datasets and approaches. Membership within the clades was remarkably consistent and well-supported among all datasets; however, the appropriate taxonomic level of these clades remains open to debate. Species delimitation approaches provided inconsistent support for any conclusion, instead ranging from the clades being polymorphic single species to each of the nominal taxa being distinct species within clades, depending on the dataset and approach used. Our results highlight that future broad geographic and taxonomic examination, rather than superficial taxon sampling, will be required to resolve this uncertainty. For example, extensive geographic sampling of widely distributed taxa such as *P. tuberculosa* together with its sister taxa *P. caribaeorum* will improve our understanding of the geographic distribution of clades, level of within-species genetic variation, and help to resolve the polyphyly of currently accepted species. Surprisingly, our data reconstructed close relationships between *Palythoa* lineages of the Atlantic and Pacific oceans in two out of the six species complexes recovered here that merit further attention. Thus, future research should focus on a greatly expanded effort in both species-

level taxon sampling for the genus and with multiple individuals per species across as much of the geographic range of each species as is possible. Until we can quantify the amount of variation expected across geographic ranges within species relative to that among species, it remains unclear exactly what constitutes a species within the genus *Palythoa*. Given the taxonomic complexity of this group is biological rather than methodological, it is now clear that increasing sampling effort, both taxonomically and geographically for all nominal taxa, and combining phylogenetic sampling with more detailed life history studies is necessary to resolve taxonomic uncertainties and clearly define species boundaries.

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Declarations

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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