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REPORT

Diversity and evolution in the zoanthid genus *Palythoa* (Cnidaria: Hexacorallia) based on nuclear ITS-rDNA

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Abstract Previous phylogenetic studies based on mitochondrial DNA markers have suggested that the zoanthid genus *Palythoa* may consist of both *Palythoa* species (*Palythoa tuberculosa*) and species formerly assigned to the genus *Protopalythoa* (*Palythoa mutuki, Palythoa heliodiscus*). In the present study various *Palythoa* spp. samples collected primarily from southern Japan with additional samples from the Indo-Pacific and Caribbean Sea were examined. The nuclear internal transcribed spacer of ribosomal DNA (ITS-rDNA) was sequenced and aligned for phylogenetic analyses to further investigate the relationship between *P. tuberculosa*, *P. mutuki*, and *P. heliodiscus*. ITS-rDNA analyses showed species groups forming monophylies with similar topology but with much higher resolution than seen for mitochondrial phylogenetic analyses. The results also confirmed the very close relationship of *P. tuberculosa* and *P. mutuki*. Some specimens appeared to be a potentially undescribed *Palythoa* species (designated *Palythoa* sp. sakurajimensis). Additionally, ITS-rDNA sequences of *P. mutuki* and *P. tuberculosa* showed additive polymorphic site, demonstrating for the first time a potential history of reticulate evolution in *Palythoa*.

Keywords Anthozoa · Reticulate evolution · ITS-rDNA · Zoanthid · Palythoa

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Introduction

The zoanthid genus *Palythoa* (Cnidaria: Hexacorallia) is common in shallow subtropical and tropical waters throughout the world. Although there are 193 species mentioned in the literature (Fautin 2006) it is likely that many of these nominal species have been re-described and that the true diversity of species in *Palythoa* is lower than this number (see Reimer et al. 2006c).

Traditional classification of *Palythoa* spp. has been largely based on morphological characteristics such as polyp shape, oral disk size and diameter, and tentacle number (see Ryland and Lancaster 2003). Additionally, *Palythoa* spp. were defined as having embedded "immersae" polyps and the related proposed genus *Protopalythoa* consisted of similar but "liberae"-polyped (non-embedded polyps) species (Pax 1910). However, using molecular markers (mitochondrial cytochrome oxidase subunit I [COI] and mitochondrial 16S ribosomal DNA sequences [mtDNA 16S rDNA]), it has recently been shown that *Palythoa* and *Protopalythoa* have a very close relationship up to the level of congeners (Reimer et al. 2006c), and that these two taxa should be combined into a single genus, *Palythoa*. In addition, data from the closely related genus *Zoanthus* have shown that polyp shape and other morphological characters are not necessarily good indicators of relatedness (Reimer et al. 2006b).

Up until now, molecular phylogenetic analyses of *Palythoa* have relied exclusively upon mitochondrial DNA markers (Sinniger et al. 2005; Reimer et al. 2006c), which have a slow rate of evolution in anthozoans compared to other animals (Shearer et al. 2002). For example, *Palythoa tuberculosa* and *Palythoa mutuki* only differ by 1–2 sites over 870 base pairs (bp) in mtDNA 16S rDNA (Reimer et al. 2006c). On the other hand, nuclear internal transcribed spacer of ribosomal DNA (ITS-rDNA) has been successfully used in a variety of other hexacorallian taxa to delineate boundaries between many species (e.g., see Hunter et al. 1997). ITS-rDNA, despite the potential presence of multiple copies (Marquez et al. 2003), has been particularly useful in exploring evolutionary patterns in closely related groups due to its extremely non-conservative nature (e.g., Hunter et al. 1997; Odorico and Miller 1997; Medina et al. 1999; van Oppen et al. 2000, 2002; Diekmann et al. 2001; Marquez et al. 2003; Fukami et al. 2004).

It has been proposed that the apparently unclear species boundaries in many genera of corals may at least partially be due to interspecific hybridization and subsequent reticulate evolution (Veron 1995). Many different species and genera of Hexacorallia have also been shown to reproduce in synchronous phase with the moon in mass spawning events (see Levitan et al. 2004; Penland et al. 2004; Ono et al. 2005), suggesting that hybridization, reticulate evolution and/or introgression (hybrids backcrossing with parents) in Anthozoa may be more widespread than it is currently known.

In this study, a nuclear marker (ITS-rDNA) was applied to: (1) obtain a phylogeny of this genus with improved resolution to confirm or refute the previous hypothesis that *Palythoa* and *Protopalythoa* are congeneric and (2) to examine the possibility of reticulate evolution in *Palythoa* spp.

Materials and methods

Sampling

Samples of *Palythoa* spp. were collected from several sites in Japan (Fig. 1) as well as from the Indian and Atlantic oceans between January 2004 and May 2006 (Table 1), and stored in 80–100% ethanol at −20°C. Samples (Fig. 2) included specimens both of the immersae '*Palythoa*' morphology (nominal *P. tuberculosa*, as well as a sample of *Palythoa* cf. *caribaeorum*) and the liberae '*Protopalythoa*' morphology (nominal *P. mutuki, Palythoa heliodiscus*, and unidentified specimens) (see Reimer et al. 2006c for species' details). As samples were collected, in situ photographs were taken to assist in identification and for collection of morphological data (oral disk/polyp diameter, color, tentacle count, polyp form). Sample nomenclature is explained in the notes in Table 1.

DNA extraction, PCR amplification, cloning, and sequencing

DNA was extracted from samples weighing 5–20 mg using a spin-column DNeasy Animal Extraction protocol (Qiagen, Santa Clarita, CA, USA). PCR amplification using the genomic DNA as a template was performed using HotStarTaq DNA polymerase (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. Mitochondrial 16S rDNA was amplified following procedures outlined in Sinniger et al. (2005). The ITS-rDNA region (the 3′ end of 18S rDNA, ITS-1, 5.8S rDNA, ITS-2, and the 5′ end of 28S rDNA) was amplified following procedures outlined in Reimer et al. (2007). The amplified products were visualized by 1.5% agarose gel electrophoresis. Some PCR-amplified DNA fragments were cloned and analyzed

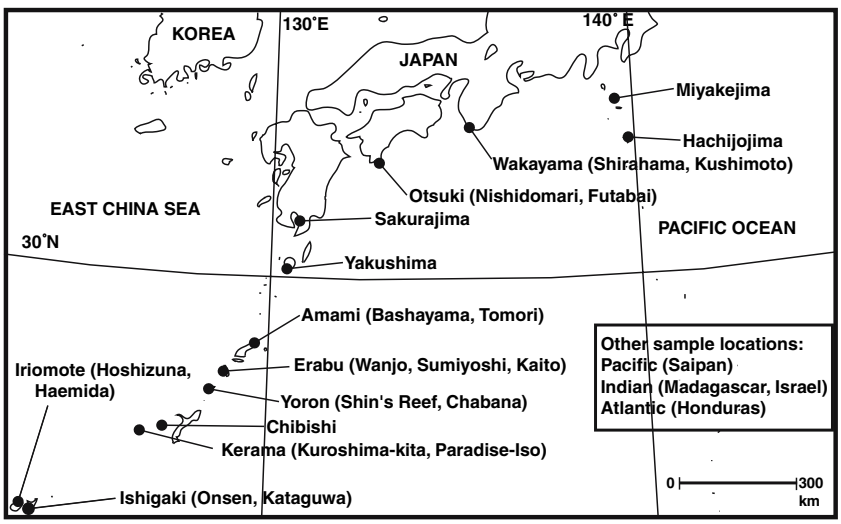

Fig. 1 Map showing sampling locations of *Palythoa* spp. in this study

Table 1 *Palythoa* samples used in this study

Sample name[a]	Location[b]	Depth	Sampling date	Collected by[c]	Morphological identification	mtDNA 16S rDNA Accession number	ITS-rDNA Accession number	Phylogenetic conclusion
PhErabuKaito1	Kaito, Erabu	−19.0	May 2005	JDR	*P. heliodiscus*	AB219224[d]	DQ997882	*P. heliodiscus*
PhIshigaki Kata2	Kataguwa, Ishigaki	−9.5	Feb 2005	JDR	*P. heliodiscus*	DQ997859	DQ997885	*P. heliodiscus*
PhIshigaki Kata3	Kataguwa, Ishigaki	−9.5	Feb 2005	JDR	*P. heliodiscus*	NA	DQ997884	*P. heliodiscus*
PhIshigaki Kata5	Kataguwa, Ishigaki	−10.0	Feb 2005	JDR	*P. heliodiscus*	DQ997843	NA	*P. heliodiscus*
PhIshigaki Kata11	Kataguwa, Ishigaki	−12.0	Dec 2005	JDR	*P. heliodiscus*	DQ997861	DQ997880	*P. heliodiscus*
PhSaipan LauLau1	Lau Lau, Saipan	−3.0	Dec 2004	JDR	*P. heliodiscus*	AB219223[d]	DQ997883	*P. heliodiscus*
PhSaipan LauLau2	Lau Lau, Saipan	−2.0	Dec 2004	JDR	*P. heliodiscus*	DQ997844	DQ997881	*P. heliodiscus*
PWakayama Shira1	Shirahama, Wakayama	+0.5	Apr 2006	JDR and HF	unknown *Palythoa* sp.	DQ997863	DQ997887	*P.* sp. sakurajimensis
PSakura Hakama1	Hakamagoshi, Sakurajima	−4.0	Feb 2006	JDR, SO, JT, and AI	unknown *Palythoa* sp.	DQ997842	DQ997886	*P.* sp. sakurajimensis
PEWanjo N1	Wanjo-north, Erabu	+0.5	May 2006	JDR	unknown *Palythoa* sp.	DQ997862	NA	*P.* sp. sakurajimensis
PmMiyakeIzuI1	Izushita, Miyakejima	0.0	June 2005	JDR	*P. mutuki* 1	AB219225[d]	DQ997889	*P. mutuki* 1
PmYakuSango1	Sangohama, Yakushima	0.0	June 2003	JDR	*P. mutuki* 1	AB219222[d]	DQ997890	*P. mutuki* 1
PmYaku Sango2	Sangohama, Yakushima	0.0	July 2004	JDR	*P. mutuki* 1	DQ997875	DQ997892	*P. mutuki* 1
PmAmami Tomori1	Tomori, Amami	+0.5	Aug 2004	JDR	*P. mutuki* 2	AB219220[d]	DQ997891	*P. mutuki* 2
PmAmami Tomori2	Tomori, Amami	+1.0	Aug 2004	JDR	*P. mutuki* 2	AB219221[d]	NA	*P. mutuki* 2
PmErabu Sumiyoshi1	Sumiyoshi, Erabu	+0.5	May 2006	JDR	*P. mutuki* 1	DQ997847	DQ997894	*P. mutuki* 1
PmIriomote Hoshi1	Hoshizuna, Iriomote	0.0	Feb 2006	JDR	*P. mutuki* 2	DQ997841	DQ997888	*P. mutuki* 2
PmIriomote Haemida3	Haemida, Iriomote	0.0	Feb 2006	JDR	*P. mutuki* 2	DQ997840	DQ997893	*P. mutuki* 2
PtMiyakeIzu1	Izushita, Miyakejima	−2.0	June 2005	JDR	*P. tuberculosa*	AB219218[d]	NA*	*P. tuberculosa*
PtHachijojima Bora2	Borahama, Hachijojima	−1.5	Jan 2006	JDR	*P. tuberculosa*	DQ997868	DQ997898	*P. tuberculosa*
PtWakayama Kushi1	Kushimoto, Wakayama	−1.0	Aug 2004	JDR	*P. tuberculosa*	DQ997874	DQ997899	*P. tuberculosa*
PtOtsuki Nishidomari1	Nishidomari, Otsuki	−6.0	Jan 2006	JDR	*P. tuberculosa*	DQ997857	NA	*P. tuberculosa*
PtOtsuki Nishidomari2	Nishidomari, Otsuki	−4.0	Jan 2006	JDR	*P. tuberculosa*	DQ997867	NA	*P. tuberculosa*
PtOtsuki Nishidomari3	Nishidomari, Otsuki	−3.0	Jan 2006	JDR	*P. tuberculosa*	DQ997853	DQ997939	*P. tuberculosa*
PtOtsuki Futabae1	Futabai, Otsuki	−12.0	Jan 2006	JDR	*P. tuberculosa*	DQ997865	DQ997918, 924-926, 944, 945[f]	*P. tuberculosa*
PtYakuSango3	Sangohama, Yakushima	−12.0	July 2004	JDR	*P. tuberculosa*	DQ997858	NA	*P. tuberculosa*
PtYakuSango4	Sangohama, Yakushima	−1.5	July 2004	JDR	*P. tuberculosa*	DQ997864	DQ997903, 928, 935-938[f]	*P. tuberculosa*
PtAmami Tomori2	Tomori, Amami	−1.5	Aug 2004	JDR	*P. tuberculosa*	DQ997839	DQ997897	*P. tuberculosa*
PtAmami Bashayama1	Bashayama, Amami	−2.0	Oct 2005	JDR	*P. tuberculosa*	DQ997845	DQ997923	*P. tuberculosa*
PtAmami Bashayama2	Bashayama, Amami	−2.0	Oct 2005	JDR	*P. tuberculosa*	DQ997851	DQ997900	*P. tuberculosa*

Table 1 continued

Sample name[a]	Location[b]	Depth	Sampling date	Collected by[c]	Morphological identification	mtDNA 16S rDNA Accession number	ITS-rDNA Accession number	Phylogenetic conclusion
PtErabuWanjo2	Wanjo, Erabu	−2.0	May 2006	JDR	*P. tuberculosa*	DQ997850	NA	*P. tuberculosa*
PtErabuWanjo3	Wanjo, Erabu	−2.0	May 2006	JDR	*P. tuberculosa*	DQ997846	DQ997902	*P. tuberculosa*
PtErabu Sumiyoshi2	Sumiyoshi, Erabu	−9.0	May 2006	JDR	*P. tuberculosa*	DQ997855	NA	*P. tuberculosa*
PtYoronShin1	Shin's Reef, Yoron	−1.0	May 2005	JDR	*P. tuberculosa*	AB219219[d]	DQ997921	*P. tuberculosa*
PtYoronShin2	Shin's Reef, Yoron	−2.0	May 2005	JDR	*P. tuberculosa*	DQ997877	NA	*P. tuberculosa*
PtYoron Chabana2	Chabana, Yoron	−2.0	May 2005	JDR	*P. tuberculosa*	DQ997879	DQ997922	*P. tuberculosa*
PtChibishi Naga1	Nagannu-kita, Chibishi	−3.0	June 2004	JDR	*P. tuberculosa*	DQ997860	DQ997896, 927, 930, 932-934[f]	*P. tuberculosa*
PtChibishi Naga2	Nagannu-kita, Chibishi	−4.0	June 2004	JDR	*P. tuberculosa*	DQ997869	NA	*P. tuberculosa*
PtKerama Kuro3	Kuroshima-kita, Kerama	−2.0	June 2004	JDR	*P. tuberculosa*	DQ997856	NA	*P. tuberculosa*
PtKerama Paradise1	Paradise-Iso, Kerama	−7.0	June 2004	JDR	*P. tuberculosa*	DQ997854	NA	*P. tuberculosa*
PtIshigaki Onsen1	Onsen, Ishigaki	−8.5	Feb 2005	JDR	*P. tuberculosa*	NA	DQ997919, 929[f]	*P. tuberculosa*
PtIshigaki Kata1	Kataguwa, Ishigaki	−10.0	Feb 2005	JDR	*P. tuberculosa*	DQ997866	DQ997920	*P. tuberculosa*
PtIshigaki Kata4	Kataguwa, Ishigaki	−10.0	Feb 2005	JDR	*P. tuberculosa*	DQ997873	NA	*P. tuberculosa*
PtIshigaki Kata6	Kataguwa, Ishigaki	−12.0	Dec 2005	JDR	*P. tuberculosa*	DQ997852	NA	*P. tuberculosa*
PtIriomote Hoshi1	Hoshizuna, Iriomote	0.0	Feb 2006	JDR	*P. tuberculosa*	DQ997848	DQ997904-917[f]	*P. tuberculosa*
PtSaipan LauLau1	Lau Lau, Saipan	−3.0	Dec 2004	JDR	*P. tuberculosa*	DQ997872	DQ997895	*P. tuberculosa*
PMadagascar 289	Piscine-Sakatia, Madagascar (Indian)	−10.0	July 2005	FS	*Palythoa* sp.	DQ997878	DQ997901	*P. tuberculosa*
PtIsrael1	Eilat, Israel (Indian)	−3.0	May 2006	OP	*P. tuberculosa*	DQ997849	DQ997931, 940, 941[f]	*P. tuberculosa*
PtIsrael2	Eilat, Israel (Indian)	−1.0	May 2006	OP	*P. tuberculosa*	DQ997876	NA	*P. tuberculosa*
PcHond1	Utila, Honduras (Atlantic)	−8.0	Feb 2004	FS	*P. caribaeorum*	NA	DQ997942, 943, 946[f]	*P. tuberculosa* group
ZsSH23	Hakamagoshi, Sakurajima	−9.0	July 2004	JDR	*Z. sansibaricus*	AB219187[e]	NA	*Z. sansibaricus*
ZsES1	Sumiyoshi, Erabu	0.0	May 2006	JDR	*Z. sansibaricus*	DQ997871	NA	*Z. sansibaricus*
ZgYS1	Sangohama, Yakushima	−1.5	July 2004	JDR	*Z. gigantus*	AB219192[e]	NA	*Z. gigantus*
ZkYS1	Sangohama, Yakushima	−1.5	July 2004	JDR	*Z. kuroshio*	AB219191[e]	NA	*Z. kuroshio*
ZSH50	Hakamagoshi, Sakurajima	−5.0	Feb 2005	JDR	*Zoanthus* sp.	DQ997870	NA	*Z. kuroshio* group

* NA data not acquired

[a] Sample names follow the convention of Genus-species-Location-Location-sample number, except for species from Israel and Honduras, where location is simply country name. Additionally, when only genus name was known, no species designation was added to the sample name

[b] Locations for samples from oceans other than the Pacific indicated in parentheses. All locations in Japan unless otherwise noted

[c] Name abbreviations: JDR = J. Reimer, HF = H. Fukami, SO = S. Ono, JT = J. Tsukahara, AI = A. Iwama, FS = F. Sinniger, OP = O. Polak

[d] From Reimer et al. (2006c)

[e] From Reimer et al. (2006a)

[f] Samples with more than one ITS-rDNA sequence Accession Number were cloned. Clones are indicated in Fig. 4 by sequences with hyphenated sample numbers

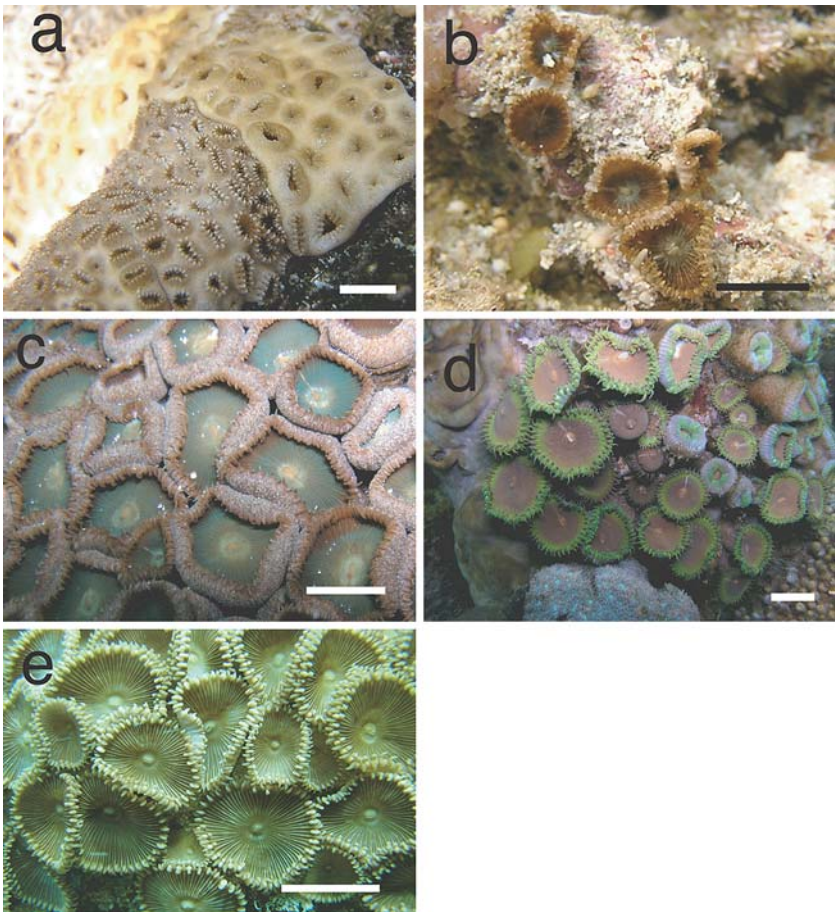

Fig. 2 **a** *Palythoa tuberculosa* at Hoshizuna, Iriomote, Okinawa, depth 0.5 m. Note the two colonies with different coloration. **b** *Palythoa mutuki* 2 at Haemida, Iriomote, Okinawa, depth 0.0 m, **c** *P. mutuki* 1 at Izushita, Miyakejima, Tokyo, depth 0.0 m. Note the differences in oral disk coloration and the slight difference in polyp thickness between *P. mutuki* 1 and *P. mutuki* 2. **d** *Palythoa* sp. sakurajimensis at Hakamagoshi, Sakurajima, Kagoshima, depth 4.0 m, and **e** *Palythoa heliodiscus* at Sumiyoshi, Erabu, Kagoshima, depth 5.0 m. *Scale bars* 1 cm

following procedures outlined in Reimer et al. (2007). Between 2 (sample PtIsO1) and 14 (sample PtIrHo1) clones were sequenced (see Table 1).

Phylogenetic analyses

New sequences obtained in the present study were deposited in DDBJ and GenBank (accession numbers DQ997839–DQ997946). By using CLUSTAL X version 1.8 (Thompson et al. 1997), the nucleotide sequences of mt 16S rDNA from samples were aligned with previously published mtDNA 16S rDNA sequences (Table 1) from *Palythoa* (AB219218-AB219225), and *Zoanthus* spp. sequences (AB219187, AB219191, AB219192) were used as the outgroup. *Zoanthus* ITS-rDNA sequences (particularly ITS-1 and ITS-2 spacers) were highly divergent from *Palythoa* sequences (see Reimer et al. 2007) and thus an ITS-rDNA alignment consisting only of *Palythoa* sequences was generated, using *Palythoa heliodiscus* sequences as the outgroup for the subsequent phylogenetic analyses. The alignments were inspected by eye and manually edited. All ambiguous sites were removed from the dataset before phylogenetic analyses. Consequently, two alignment datasets were generated: (1) 759 alignment positions of 52 sequences (mtDNA 16S rDNA); and (2) 686 alignment positions of 67 sequences (ITS-rDNA). The alignment data are available on request from the corresponding author and also from the European Molecular Biology Lab (EMBL) (alignment numbers: mtDNA 16S rDNA = ALIGN_001072, ITS-rDNA = ALIGN_001073).

The alignments of mtDNA 16S rDNA and ITS-rDNA were tested for optimal fit of various nucleotide substitution models using MODELTEST version 3.06 (Posada and Crandall 1998). The base frequencies, proportion of invariable sites (and a gamma distribution) were estimated from the datasets. For the mtDNA 16S rDNA and ITS-rDNA datasets, the TN model (Tamura and Nei 1993) incorporating invariable sites (TN + I) and the Hasegawa, Kishino and Yano (HKY) model (Hasegawa et al. 1985) incorporating invariable sites and a discrete gamma distribution (four categories) (HKY + I + Γ) were selected by MODELTEST, respectively. The maximum-likelihood (ML) analyses with PhyML (Guindon and Gascuel 2003) of these datasets were independently performed using an input tree generated by BIONJ (Gascuel 1997) with the models selected by MODELTEST. PhyML bootstrap trees (500 replicates) were constructed using the same parameters as the individual ML trees.

ML distances of the two datasets were calculated under the optimal models described above with PAUP* Version 4.0 (Swofford 1998). Distance trees were constructed using the neighbor-joining (NJ) method (Saitou and Nei 1987). The ML distance bootstrap analyses with 1,000 replicates were also performed.

Bayesian trees were reconstructed by using the program MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003) under the general time reversible (GTR) model (Rodriguez et al. 1990) of nucleotide substitution incorporating invariable sites (GTR + I) for the mtDNA 16S rDNA dataset, and under HKY + I + Γ for the ITS-rDNA dataset [both models selected by MrModeltest (Nylander 2004. MrModeltest v2. Program distributed by the author. Evolutionary Biology Centre, Uppsala University)]. One cold and three heated Markov chain Monte Carlo (MCMC) chains with default-chain temperatures were run for 1,000,000 generations, sampling log-likelihoods (InLs), and trees at 100-generation intervals (10,000 InLs and trees were saved during MCMC). The likelihood plot for mtDNA 16S rDNA and ITS-rDNA datasets suggested that MCMC reached the stationary phase after the first 30,000 and 300,000 generations, respectively. Thus, the remaining 9,700 and 7,000 trees of mtDNA 16S rDNA and ITS-rDNA were used to obtain posterior probabilities and branch-length estimates, respectively.

To examine the levels of sequence variation within each ITS-rDNA species group, all obtained sequences of the entire ITS-rDNA region (ITS-1 + 5.8S rDNA + ITS-2, 18S rDNA and 28S rDNA portions not included) for each species group were aligned and edited, and the total number of variable sites was estimated.

Results

The mtDNA 16S rDNA tree (Fig. 3) showed *P. heliodiscus* forming one highly supported clade (ML bootstrap support = 99%, NJ bootstrap support = 96%, but posterior probability of Bayesian inference [PP] = 0.69), and the *P. mutuki* species group derived from *P. tuberculosa*. *P. mutuki* was divided into two separate clades (*P. mutuki* 1 and 2), with *P. mutuki* 1 differing from *P. tuberculosa* by 1 bp, and *P. mutuki* 2 only by 2 bp. Both *P. mutuki* groups showed generally low bootstrap support values, although the *P. mutuki* 2 clade has a posterior probability of 0.95 in the Bayesian analysis. Included in the *P. tuberculosa* species group were distant samples (from Japan) from the Pacific and Indian Ocean (PMadagascar289, PtIsrael1, PtIsrael2), as well as *P.* cf. *caribaeorum* from Honduras (PcHond1 = sample PcH1 in Reimer et al. 2006c). While bootstrap probability was relatively low for the monophyly of *P. tuberculosa* (ML = 72%, NJ = 61%), posterior probability was moderately high (0.90). Additionally, three unidentified *Palythoa* samples (PErabuWanjoN1, PSakuraHakama1, PWakayamaShira1) formed a separate clade with relatively low support (ML = 82%, NJ = 78%, PP = 0.80), designated *Palythoa* sp. sakurajimensis in this study. Variation between mtDNA 16S rDNA sequences within species groups was very low, i.e., <0.5% (Table 2).

The ITS-rDNA tree (Fig. 4) had four main *Palythoa* groups, three of which corresponded to known species (*P. tuberculosa*, *P. mutuki*, and *P. heliodiscus*) and the *P.* sp. sakurajimensis clade comprising two of the 'unknown *Palythoa* sp.' samples (PSakuraHakama1 and PWakayamaShira1). *P. heliodiscus* formed a well-supported clade (ML, NJ = 100%, PP = 1.00), as did *P.* sp. sakurajimensis with moderate to high support (ML = 99%, NJ = 74%, PP = 0.97). A large clade containing both the separate P. *tuberculosa and P. mutuki* clades was also moderately to highly supported (ML = 98%, NJ = 61%, PP = 0.96), as were both the *P. mutuki* (ML = 96%, NJ = 71%, PP = 0.95) and the *P. tuberculosa* groups (ML = 96%, NJ = 92%, PP = 0.99) within this larger clade. ITS-rDNA sequences from samples distant from Japan (PMadagascar289, PtIsrael1, PtIsrael2, PcHond1) were clearly within the *P. tuberculosa* clade.

However, ITS-rDNA showed larger differences between sequences within each species group. *P. mutuki* formed four separate subclades (two each of *P. mutuki* 1 and *P. mutuki* 2) with moderate to very high support, but the two subclades each of *P. mutuki* 1 and *P. mutuki* 2 mtDNA 16S rDNA did not separately form a monphyly with the other subclade of their putative species group in the ITS-rDNA tree (Fig. 4). However, putative (based on mtDNA 16S rDNA) *P. mutuki* 1 and 2 sequences were not found within the same subclade (Fig. 4). *P. tuberculosa* also showed intraspecific variation, with samples PtYoronShin1 and PtYoronChabana2 forming a highly supported basal group (ML = 98%, NJ = 99%, but PP = 0.54). In some cases, clones from the same *P. tuberculosa* specimens clustered together within the same subclade (PtYakuSango4 and PcHond1). In other cases cloned sequences from individual specimens grouped in more than one subclade (PtIriomoteHoshi1, PtOtsukiFutabae1) (Fig. 4). The *P. mutuki* and *P. tuberculosa* species groups seemed to be very closely related in comparison to the other *Palythoa* species groups.

Intraspecific ITS-rDNA sequence variation ranged between 0.4% (*P.* sp. sakurajimensis) and 23.7% (*P. tuberculosa*) (Table 2). *P. heliodiscus* ($n = 5$) and *P.* sp. sakurajimensis ($n = 2$) groups showed little variation in ITS-rDNA sequences within their own species groups for both length and sequence identity (Table 2), although sample sizes were small. On the other hand, *P. mutuki* and *P. tuberculosa* groups showed much higher levels of ITS-rDNA length and sequence variation within their respective species groups (Table 2). *P. tuberculosa* ITS-rDNA sequences,

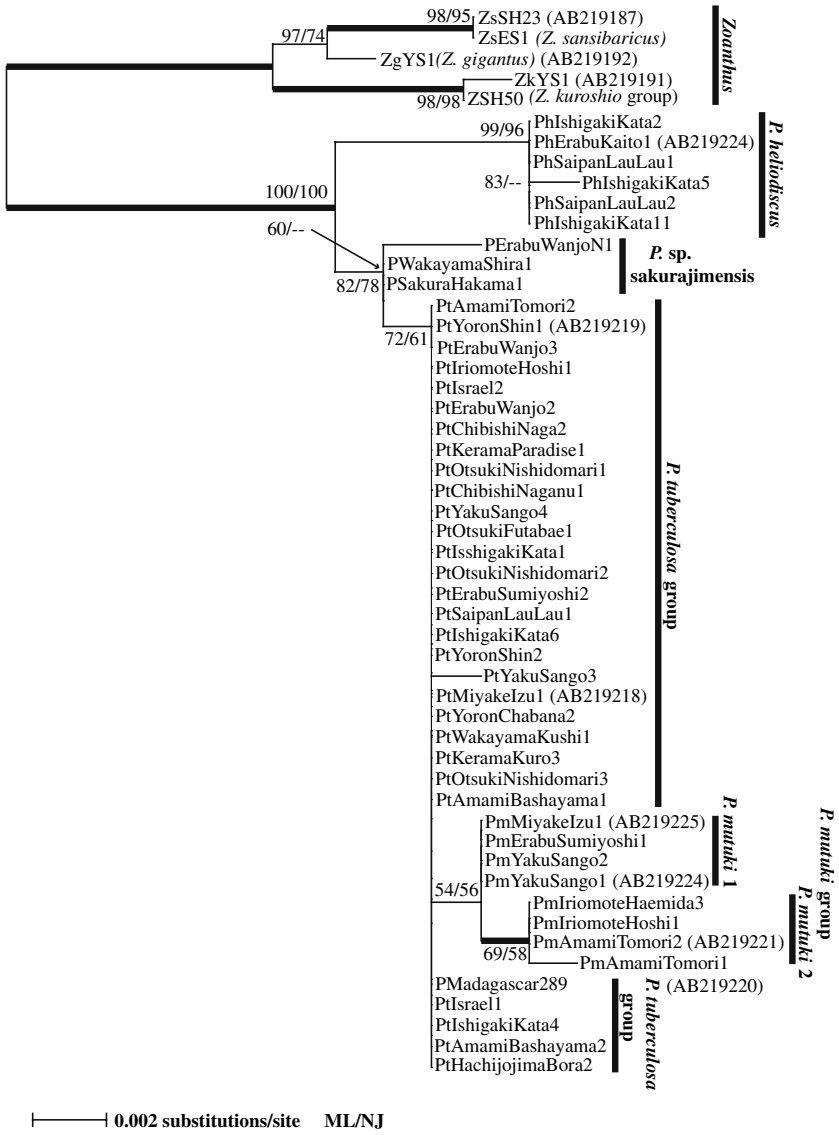

Fig. 3 Maximum likelihood tree of mitochondrial 16S ribosomal DNA (mtDNA 16S rDNA) sequences. *Values at branches* represent ML and NJ bootstrap probabilities, respectively (>50%). Bayesian posterior probabilities of >95% are represented by *thick branches*. For sample name abbreviations see Table 1

while monophyletic and distinct from other *Palythoa* species groups, not only showed variability in length and sequences both between samples (Tables 2, 3) but also between cloned sequences within individuals (maximum variation 11.9% [74/621 bp] between clones [$n = 14$] from sample PtIriomoteHoshi1). For all four species groups, ITS-1 was found to be the most variable region, followed by ITS-2 and then 5.8S rDNA, respectively (Table 2).

Discussion

The utility of ITS-rDNA in species delineation of the genus *Palythoa*

ITS-rDNA has different levels of divergence within different genera of hard corals [i.e., only 2% variation in the *Montastraea annularis* complex (Medina et al. 1999), 4.9% in *Madracis* spp. (Diekmann et al. 2001), 11% in *Porites* spp. (Hunter et al. 1997), and almost 60% reported in ITS-2 rDNA of *Acropora* spp. (Marquez et al. 2003)]. ITS-rDNA in *Palythoa* spp., while sufficient for separating species groups from one another, does not have the huge variability seen previously between *Zoanthus* spp., where differences between species groups of up to approximately 70% of base pairs in the ITS-1 region made alignment impossible (see Reimer et al. 2007). As ITS-rDNA variation levels widely vary among hexacorallian genera, it is somewhat difficult to confidently assign the level of taxonomic relationship(s) seen between *P. tuberculosa, P. mutuki, P. heliodiscus*, and *P.* sp. sakurajimensis based solely on ITS-rDNA sequences. However, with an examination of both ITS-rDNA and mitochondrial marker data (mtDNA 16S rDNA here and COI—see Reimer et al. 2006c) from *Palythoa*, it would appear that these four groups have a congeneric-level relationship. Differences in interspecific variation of ITS-rDNA

Table 2 Intraspecific sequence variation levels of ITS-rDNA and mt 16S rDNA for *Palythoa tuberculosa*, *P. mutuki*, *P.* sp. sakurajimensis and *P. heliodiscus*

DNA region		ITS-1[a]		ITS-2[a]		5.8S rDNA		ITS-1 + 5.8S + ITS-2		mtDNA 16S rDNA	
Species/group	*n*	Differences/total base pairs	% Difference	Differences/total base pairs	% Difference	Differences/total base pairs	% Difference	Differences/total base pairs	% Difference	Differences/total base pairs	% Difference
P. tuberculosa	50	96/299	32.1	51/190	26.8	6/156	3.8	153/646	23.7	2/760	0.3
P. mutuki	7	82/321	25.5	26/174	14.9	0/156	0.0	108/651	16.6	2/760	0.3
P. sp. sakurajimensis	2	3/389	0.8	0/170	0.0	0/156	0.0	3/715	0.4	2/760	0.3
P. heliodiscus	6	20/351	5.7	8/197	4.1	1/156	0.1	29/704	4.1	2/760	0.3

[a] Note that ITS-1 and ITS-2 rDNA sequence lengths are different than in Table 3 as sequences here are aligned while lengths in Table 3 are from unaligned sequences

within a genus (relatively lower in *Palythoa* and higher in *Zoanthus*) may be due to the examined *Palythoa* spp. being more recently diverged than *Zoanthus* spp., or due to much faster ITS-rDNA evolution in *Zoanthus*. How useful ITS-rDNA is in examining congeners from other zoanthid genera besides *Palythoa* and *Zoanthus* remains to be seen, although preliminary data indicate it may be useful in distinguishing between *Parazoanthus* spp. and other genera in the former suborder Brachycnemina (F. Sinniger, personal communication).

5.8S rDNA sequences in the ITS-rDNA region, which clearly resolved the *Zoanthus* congeners *Z. sansibaricus*, *Z. kuroshio*, and *Z. gigantus* (Reimer et al. 2007), showed no variation between *P. tuberculosa* and *P. mutuki*. Similarly, mtDNA COI sequence differences between *P. tuberculosa* and *P. mutuki* were not observed, and while mtDNA 16S rDNA does distinguish between *P. tuberculosa* and *P. mutuki*, this is only by 1–2 bp (Reimer et al. 2006c). For these reasons it is concluded that the best means of specific *Palythoa* identification is the analyses of ITS-rDNA (in particular ITS-1 and/or ITS-2) sequences with confirmatory mtDNA 16S rDNA sequences.

Palythoa phylogeny, and comparison with ecological data

Phylogenetic data based on mitochondrial markers (COI and 16S rDNA) suggest *Protopalythoa* and *Palythoa* are one genus [=*Palythoa* (Reimer et al. 2006c)], and the ITS-rDNA analyses in this study further confirm this hypothesis. ITS-rDNA was observed to be more variable than mtDNA 16S rDNA (Table 2). In the ITS-rDNA phylogenetic analyses, *P. tuberculosa* is sister to *P. heliodiscus* and *P.* sp. sakurajimensis but more closely related to *P. mutuki* (Fig. 4) than to *P. heliodiscus* or *P.* sp. sakurajimensis. In the mtDNA 16S rDNA tree, *P. tuberculosa* is derived from *P.* sp. sakurajimensis, and *P. mutuki* derived from *P. tuberculosa* (Fig. 3). Despite the ITS-rDNA tree having these differences in topology from the mtDNA 16S rDNA tree, ITS-rDNA sequences again clearly show the close relationship between *P. tuberculosa* and *P. mutuki* (Fig. 4). From both the mtDNA 16S rDNA and ITS-rDNA results it is concluded that *P. mutuki* (liberae '*Protopalythoa*' morphology) is more closely related to *P. tuberculosa* (immersae '*Palythoa*' morphology) than to other species with *Protopalythoa* morphology. These results also support the hypothesis posed by Reimer et al. (2006b) that in zoanthids gross morphology (i.e., polyp shape and colony features) often does not reflect relatedness among congeners.

The close relationship inferred between *P. tuberculosa* and *P. mutuki* based on DNA sequence data reflects their similar ecological and distribution data, as they often occur sympatrically in shallow areas of high sunlight, water flow and wave activity. *P. heliodiscus* appears to be somewhat

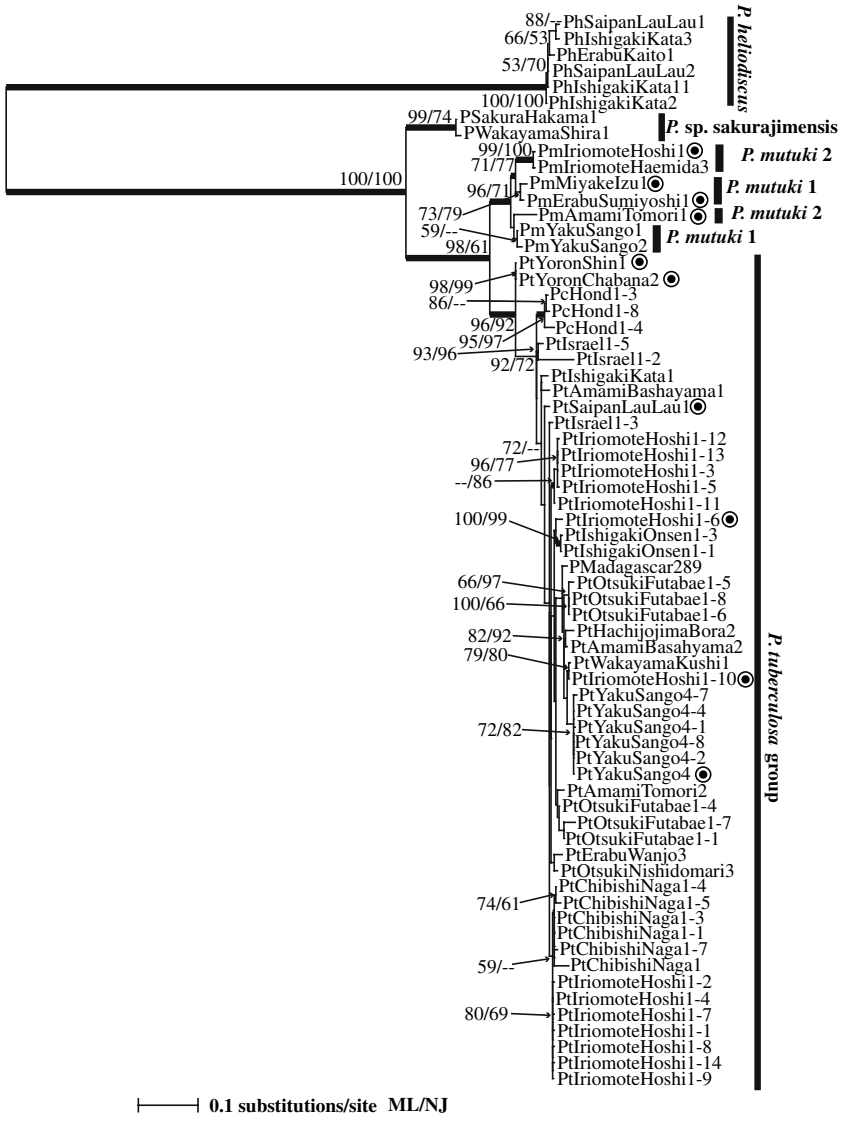

Fig. 4 Maximum likelihood tree of internal transcribed spacer ribosomal DNA (ITS-rDNA) sequences. *Values at branches* represent ML and NJ bootstrap probabilities, respectively (>50%). Bayesian posterior probabilities of >95% are represented by *thick branches*. For sample name abbreviations see Table 1. Sample names with Accession Numbers are from previous studies (see Table 1). Sample names with a *hyphenated number* or *letter ending* represent clones. Samples with *filled in target symbols* are also shown in the alignment in Fig. 5

Table 3 Length variation of different regions of obtained ITS-rDNA sequences for different *Palythoa* spp

Species/group	*n*	ITS-1[a]	5.8S	ITS-2[a]
P. tuberculosa	50	243–271	156	171–192
P. mutuki	7	283–312	156	168–174
P. sp. Sakurajimensis	2	389	156	170
P. heliodiscus	6	339	156	203

[a] Note that ITS-1 and ITS-2 rDNA sequence lengths are different than in Table 2; sequence lengths here are from unaligned sequences, while Table 2 shows aligned sequence lengths

more distantly related to the other three *Palythoa* groups and ecologically this species is also somewhat different from the others, as it is often found in deeper areas with much lower sunlight, and thus appears to be more mixotrophic, obtaining energy from both its symbiotic zooxanthellae and perhaps directly from particles in the water column.

Despite extensive sampling throughout the course of this study, only three samples of *P.* sp. sakurajimensis were found, suggesting that this species may be less common or more cryptic than other *Palythoa* species in the sub-tropical and tropical waters of Japan. More samples are needed before the ecology of this potentially new species group can be discussed, but it is noteworthy that all three samples were found in habitats where other *Palythoa* species were not found [e.g., at the relatively cold locations (winter minimums approximately 15°C) of Sakurajima and Shirahama, and relatively high in the tidal zone at Wanjo North, Okinoerabu Island]. This species may be more adapted to "marginal" or variable conditions than other *Palythoa* species, similar to *Zoanthus sansibaricus* (found at the colder Sakurajima site) compared to other *Zoanthus* species in Japan (Reimer et al. 2006a).

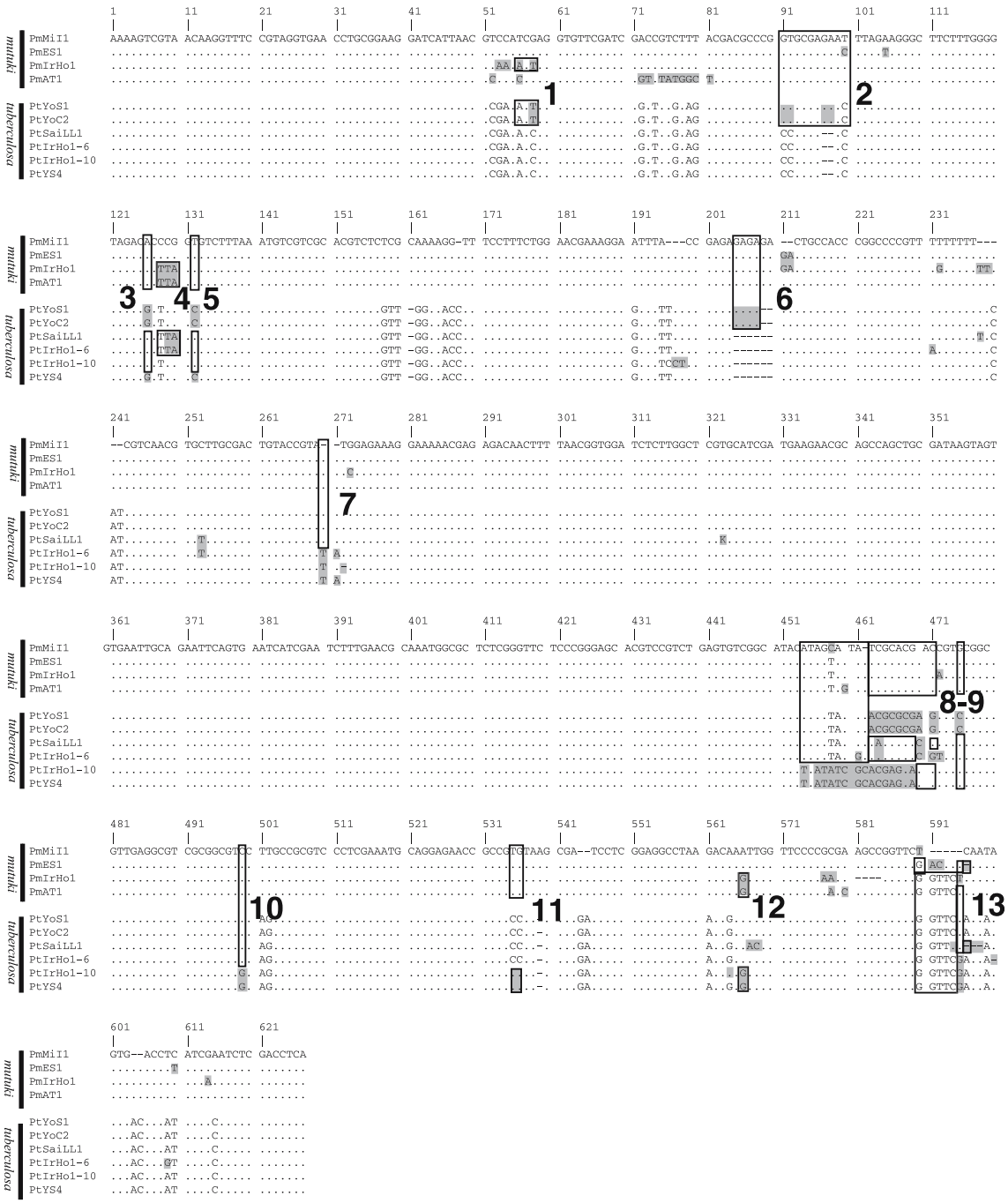

Fig. 5 Alignment of the ITS-rDNA region for various *Palythoa mutuki* and *Palythoa tuberculosa* species groups' sequences. Areas in *open boxes* represent shared common areas of sequence variation between species groups, and *shaded gray areas* represent "minor" areas of sequence variation within a species group (different from the majority of sequences within a species group). Thus, other areas of variation in the *P. tuberculosa* species group *not marked with an open box or a gray area* represent differences between all *P. mutuki* and *P. tuberculosa* sequences shown. Sample names are abbreviated due to limited space in the alignment: *PmMiI1* PmMiyakeIzu1, *PmES1* PmErabuSumiyoshi1, *PmIrHo1* PmIriomoteHoshi1, *PmAT1* PmAmamiTomori1, *PtYoS1* PtYoronShin1, *PtYoC2* PtYoronChabana2, *PtSaiLL1* PtSaipanLau-Lau1, *PtIrHo1-6* and *1–10* PtIriomoteHoshi1 clones 6 and 10, *PtYS4* PtYakuSango4

Implications of ITS-rDNA variation within *P. tuberculosa* and *P. mutuki*

Veron (1995) described a theory of reticulate evolution in hard corals. Under this scenario, species groups undergo repeated sexual reproductive isolation, differentiation, and secondary contact based on continuously changing patterns of distribution, perhaps due to changing ocean currents and/or ocean levels. Many hard corals and other Anthozoa (including at least some zooxanthellate Zoantharia—see

Ono et al. 2005) undergo mass spawning events (Levitan et al. 2004; Penland et al. 2004), providing an opportunity for hybridization and reticulate evolution. Many studies have detailed molecular and/or reproductive evidence of potential reticulate patterns in zooxanthellate hard corals (e.g., Odorico and Miller 1997; Hatta et al. 1999; Medina et al. 1999; van Oppen et al. 2000, 2002; Diekmann et al. 2001).

In this study there were moderate amounts of intraspecific variation in ITS-rDNA sequences (see Table 3) from *P. mutuki* and both intraspecific and intragenomic variation from *P. tuberculosa*. An examination of the ITS-rDNA sequence alignment reveals potential "reticulate evolution" patterns, such as additive polymorphic sites (APS).

Additive polymorphic sites can potentially indicate hybridization and reticulate evolution, particularly in ITS-rDNA (Sang et al. 1995; Campbell et al. 1997; Whittall et al. 2000; Aguilar and Feliner 2003; Feliner et al. 2004). One kind of APS that hints at hybridization is when some sequences share portions of their sequences with sequences normally found only within another clade (Aguilar and Feliner 2003). In the case of *P. tuberculosa*, samples PtYoronShin1 and PtYoronChabana2 show such a pattern. Both ITS-rDNA sequences are clearly in the *P. tuberculosa* clade, yet both have two regions that are found in *P. mutuki*, and not in any other *P. tuberculosa* sequences (Fig. 5, marked regions 2 and 6). Figure 5 shows 13 main potential ITS-rDNA regions shared between *P. mutuki* and *P. tuberculosa* that have this type of APS (numbered areas). The question of whether PtYoronShin1 and PtYoronChabana2 are *P. tuberculosa–P. mutuki* hybrids or not remains to be investigated, as although PtYoronShin1 displayed "intermediae" polyps more clear and free of the coenenchyme than most *P. tuberculosa* specimens and reminiscent of *P. mutuki*, PtYoronChabana2 had "immersae" polyps common to *P. tuberculosa*.

Another kind of APS that hints at a reticulate history is when observed intragenomic variation patterns (such as an indel, etc.) are also found in other individuals (Sang et al. 1995; Campbell et al. 1997; Whittall et al. 2000; Aguilar and Feliner 2003). This APS type can also be seen in the ITS-rDNA alignment (Fig. 5, grey regions). For example, *P. tuberculosa* PtIriomoteHoshi1 clones (PtIriomoteHoshi1-6, PtIriomoteHoshi1-10, i.e., distinct intragenomic sequences) have many variable regions different from each other that are also found in *P. tuberculosa* sequences from other samples (Fig. 5). It is possible that the results are due to slow concerted evolution with the presence of multiple ITS-rDNA copies (ancestral polymorphism). However, variable ITS-rDNA regions of PtIriomoteHoshi1 match with regions found in other individuals (not only with just other *P. tuberculosa* specimens but even with *P. mutuki* sequences—see region 4—Fig. 5), supporting the hybridization hypothesis. The data suggest that *P. tuberculosa* and *P. mutuki* potentially have a history of reticulate evolution.

The data further show that morphology between even closely related zoanthid congeners can vary drastically (as between *P. tuberculosa* and *P. mutuki*) and highlight the need for additional genetic investigation of zoanthid diversity. To investigate more fully the possibility of a potential reticulate evolutionary history in *Palythoa*, studies of sexual reproduction timing and patterns (as conducted in *Zoanthus*, Ono et al. 2005) need to be conducted. The molecular techniques used in this study to distinguish between congeners and identify a potential reticulate evolutionary history may provide a reliable method for analyses of other Zoantharia groups.

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