

## Investigations into the Reproductive Patterns, Ecology, and Morphology in the Zoanthid Genus *Palythoa* (Cnidaria: Anthozoa: Hexacorallia) in Okinawa, Japan

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**Eriko Shiroma and James Davis Reimer (2010)** Investigations into the reproductive patterns, ecology, and morphology in the zoanthid genus *Palythoa* (Cnidaria: Anthozoa: Hexacorallia) in Okinawa, Japan. *Zoological Studies* 49(2): 182-194. The zoanthid genus *Palythoa* is found in shallow subtropical and tropical waters worldwide; yet many questions remain regarding the diversity of species and their evolution. Recent progress using molecular techniques has advanced species identifications but also raised new questions. In previous studies, it was hypothesized that *P. sp. yoron* may be the result of interspecific hybridization between the closely related species *P. tuberculosa* and *P. mutuki*. Here, in order to further assess the relationships among these 3 species, their sexual reproductive patterns, distribution, and morphology (tentacle number, colony shape and size, polyp shape, etc.) were investigated in 2008 at Odo Beach, Okinawa, Japan. Results show clear differences in morphology and distribution among all 3 species, with *P. sp. yoron* apparently intermediate between *P. tuberculosa* and *P. mutuki*. From observations of oocyte/egg production, sizes, and frequency, *P. tuberculosa* is believed to spawn within 5 d after the full moon in July. No gamete production was observed in either *P. mutuki* or *P. sp. yoron*, although *P. mutuki* was found to prolifically reproduce asexually. How *P. sp. yoron* propagates remains unknown, but at least it appears that the 3 species do not spawn synchronously. From morphological data collected in this study in conjunction with previous DNA phylogenetic results, it is proposed that *P. sp. yoron* may be the product of a hybridization event in the past between a *P. tuberculosa* egg and *P. mutuki* sperm, followed by occasional introgression with *P. tuberculosa*, although no evidence of sexual reproduction was seen in either *P. mutuki* or *P. sp. yoron*. Some *Palythoa* spp. diversity may be due to reticulate evolution, and this may also contribute to *Palythoa*'s potential for adaptation. <http://zoolstud.sinica.edu.tw/Journals/49.2/182.pdf>

**Key words:** Spawning timing, Zooxanthellae, Zoantharia, Reticulate evolution, Hybridization.

Zoanthids are benthic cnidarians belonging to the order Zoantharia within the subclass Hexacorallia of the class Anthozoa. Most but not all zoanthids incorporate sand and other detritus into their mesoglea to help fortify their structure, and many shallow-water tropical and subtropical

species are in symbiosis with endosymbiotic zooxanthellae (*Symbiodinium* spp.) (Trench 1974, Reimer et al. 2006b).

The zoanthid genus *Palythoa* (family Sphenopidae) is colonial, generally zooxanthellate, and is commonly found in coral reef ecosystems

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worldwide. Traditionally, the identification of *Palythoa* species was based on morphological characters such as polyp shape and size, colony color and shape, as well as locality (see Ryland and Lancaster 2003). According to the most recent estimates, 193 species of *Palythoa* have been formally or informally named in the literature (Fautin 2006). However, due to *Palythoa* species' heavy sand encrustation (up to 45% of total body weight; see Mueller and Haywick 1995), as well as large intraspecific variations in morphology (e.g., polyp shape and size, colony shape, and color), species diversity, identification and taxonomy within this genus remain very difficult and confused (Muirhead and Ryland 1985, Ryland and Muirhead 1993, Burnett et al. 1994), and it is likely that many described zoanthid species are invalid due to inadvertent redescription (Burnett et al. 1997).

In recent years, molecular approaches using allozymes (Burnett et al. 1997) and DNA sequencing and phylogenetics (Sinniger et al. 2005, Reimer et al. 2006a 2007a) have begun to reassess the diversity and species numbers within *Palythoa*. Notably, mitochondrial (mt)DNA sequence analyses have demonstrated that the former genus *Protopalmythoa*, defined as having "liberae" polyps erect and free from the colonial coenenchyme, belongs within *Palythoa* (Reimer et al. 2006a 2007a), which formerly only consisted of species with "immersae" or embedded polyps.

Additionally, recent studies using sequences of the internal transcribed spacer of ribosomal DNA (ITS-rDNA) from *Palythoa* specimens in Japan showed a very close phylogenetic relationship between "liberae" *Palythoa mutuki* and "immersae" *P. tuberculosa* (Figs. 1, 2) (Reimer et al. 2007a). Closer examination of unusual specimens that have an intermediate morphology between the 2 species (hereafter *P. sp. yoron*; Figs. 1B, 2B) demonstrated that they possessed ITS-rDNA with sequence characteristics of both *P. mutuki* and *P. tuberculosa* (Fig. 3), indicating the potential for a reticulate evolutionary past (Reimer et al. 2007a).

Since the initial examination of *P. sp. yoron* from specimens on Yoron I., Japan, populations of this potentially hybrid zoanthid have been noted at a handful of other locations in southwestern Japan (Fig. 4A), usually in areas where large numbers of both *P. mutuki* and *P. tuberculosa* exist. Herein, we examined the morphology, distribution, and in particular the sexual and asexual reproduction patterns of *P. mutuki*, *P. tuberculosa*, and *P. sp. yoron* in order to further explore the possibility that *P. sp. yoron* is the product of past or present



**Fig. 1.** Three species of *Palythoa* in situ at Odo Beach, Okinawa, Japan. (A) *Palythoa tuberculosa*, (B) *P. sp. yoron*, and (C) *P. mutuki*. All scales = 3 cm.

hybridization between *P. mutuki* and *P. tuberculosa*.

## MATERIALS AND METHODS

### Study location

In this study, *P. mutuki*, *P. tuberculosa*, and *P. sp. yoron* (Figs. 1, 2) were investigated at Odo Beach, southern Okinawa I., Japan (26°5'2"N, 127°42'3"E; Fig. 4B). This site is the only known site where large numbers of *P. sp. yoron* are found on Okinawa I.; *P. sp. yoron* has a relatively patchy distribution throughout the Ryukyu Is. compared to *P. tuberculosa* and *P. mutuki*, which are found at almost all coral reef locations.

Odo Beach has a fringing reef with a well-developed backreef moat and reef flat, with the outer edge of the reef dropping quickly to 20-25 m with occasional reef patches, similar to many reefs on Okinawa I. (The Japanese Coral Reef Society and Ministry of the Environment 2004).

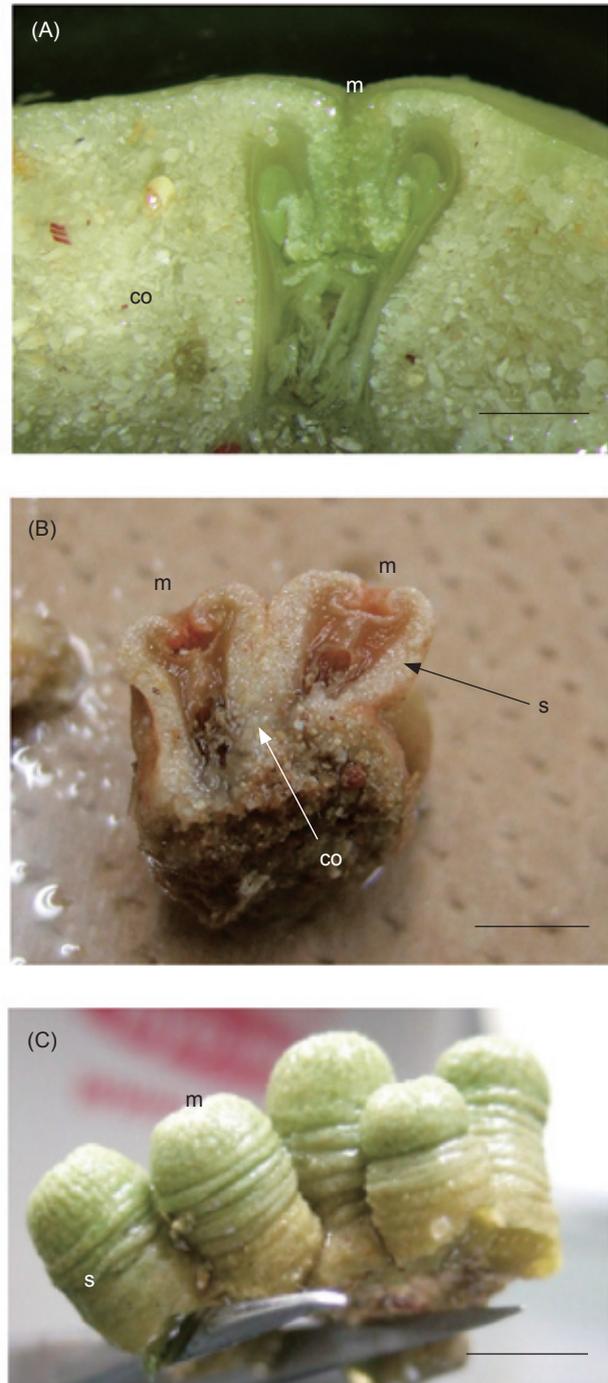
### Distribution of *Palythoa*

Belt transects (10 × 1 m) were conducted by snorkeling and/or scuba diving in 4 different areas within Odo Beach; backreef moat ( $n = 1$ ; at a depth of approximately 1 m), tidal pools in the reef flat ( $n = 3$ ; at depths of approximately 0.5-1 m), reef edge ( $n = 3$ ; at depths of intertidal to 1.0 m in surge channels), and outer reef ( $n = 3$ ; at depths of approximately 7-10 m) (Fig. 4B). Transects were conducted in Feb. and Mar. in both 2008 and 2009. During a transect, the numbers and sizes of colonies of all 3 *Palythoa* species were recorded. Further distributional data were collected during sample collection (see below).

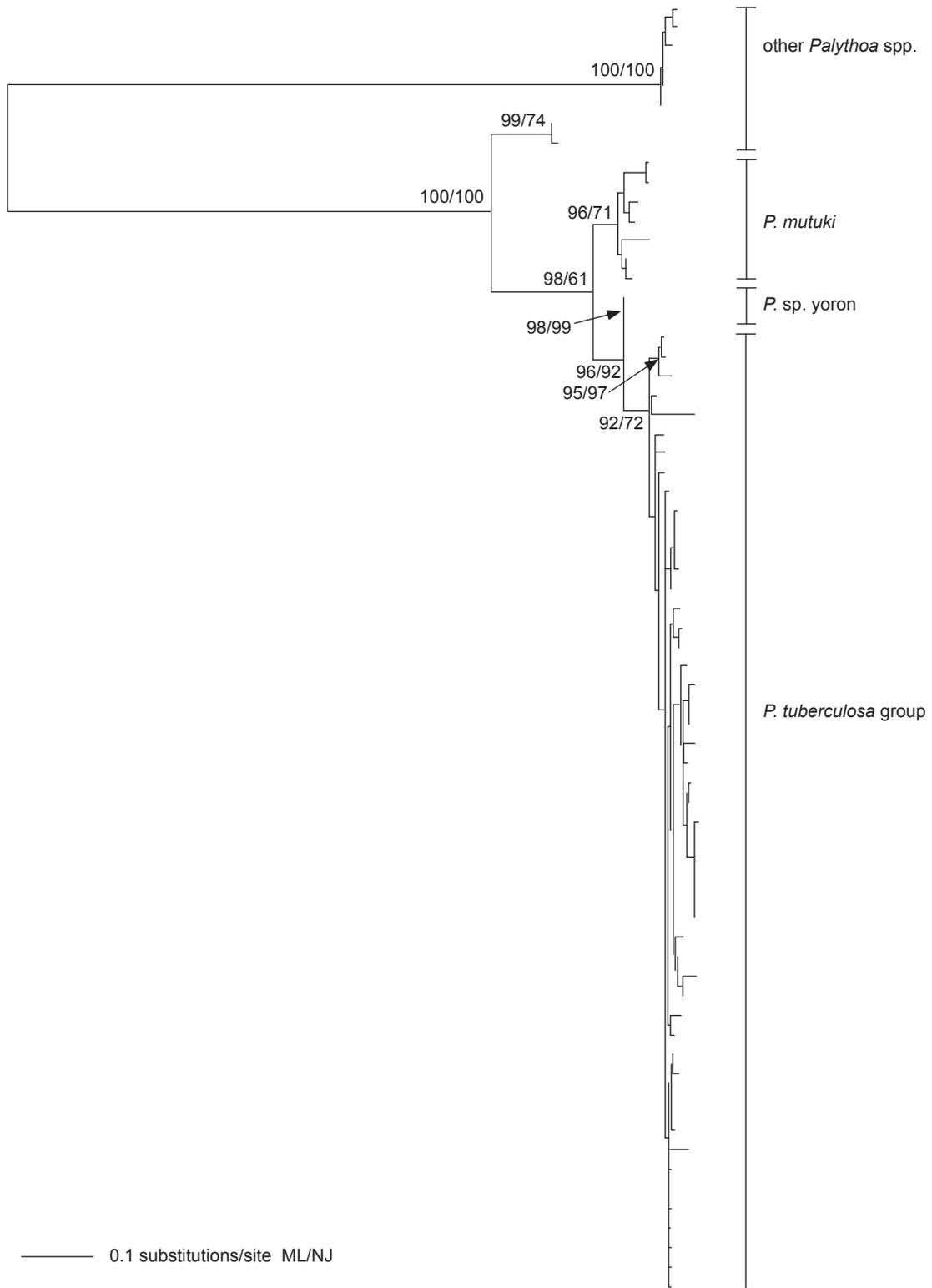
### Morphological analyses

To compare the morphology of the 3 *Palythoa* species, morphological analyses were conducted. Four or more colonies from the 3 *Palythoa* species were analyzed; only polyps from the center of colonies were examined as polyps along the edge of colonies may be immature and not fully grown (Karlson 1981). Morphological data collected included the number of tentacles, oral disk diameter and color, polyp diameter, and colony and polyp shape (following Pax 1910, see also Fig. 2). Most data were collected from in situ images as sampled specimens shrink upon fixation (Ryland and Babcock 1991, Ryland and

Lancaster 2004). Images were taken with a Fuji FinePixF11 digital camera on macro mode with a



**Fig. 2.** Cross-sections of 3 species of *Palythoa* from Odo Beach, Okinawa, Japan. (A) "immersae" *P. tuberculosa* with polyps immersed within the coenenchyme (co), (B) "intermediae" *P. sp. yoron* with polyps connected by some coenenchyme, and (C) "liberae" *P. mutuki* with polyps free and clear of the coenenchyme. Note the sand encrustations in all 3 images. m, mouth; s, scapus. All scales = 1 cm.



**Fig. 3.** Maximum-likelihood (ML) tree of internal transcribed spacer ribosomal DNA (ITS-rDNA) sequences from *Palythoa* specimens (adapted from Reimer et al. 2007a) showing the intermediate position of *P. sp. yoron* between *P. tuberculosa* and *P. mutuki*. Values at the branches represent ML and Neighbor-joining (NJ) bootstrap probabilities (> 50%). Bayesian posterior probabilities of > 95% are represented by thick branches.

scale, and sizes calculated using Adobe Illustrator (San Jose, CA, USA) imagery software. Polyp lengths (heights) were calculable but not recorded as the microenvironment (exposure to waves and overhangs) is thought to influence this parameter (Koehl 1977).

### Sexual reproduction timing analyses

Approximately 3-7 specimens (= 5 colonies) of each *Palythoa* species were collected 10 times ( $n \geq 30$  for each species) between Apr. and Oct. 2008. Sampling dates were 30 Apr., 15 and 29 May, 14 and 28 June, 21 July, 2 and 16 Aug., 30 Sept., and 16 Oct. Samples were collected with a diving knife and placed in separate containers filled with seawater (SW) immediately upon collection. Sampling dates in July and Aug. were chosen to coincide with large tides, as many coral reef hexacorallians in Okinawa spawn in this period (Heyward et al. 1987), and eggs in *P. tuberculosa* had first been noted during sampling in late June.

Specimens collected for this purpose contained 5-20 polyps. Samples from *P. tuberculosa* and *P. mutuki* were only collected from the center of large marked colonies ( $> 35 \times 35$  cm) as these areas produce more gametes compared to the edges of colonies as in other zoanthids (Boscolo et al. 2005, Ono et al. 2005). Thus, the same colonies were sampled every time for these 2 species. In contrast, samples of *P. sp. yoron* included the entire colony, as this *Palythoa* species only forms small ( $< 10$  polyp) colonies. Digital images of each colony were taken as mentioned above.

Collected specimens were kept in ambient SW until being taken to the Univ. of the Ryukyus within 1 h. Specimens were then individually fixed in 10% SW, transferred to 70% ethanol, and examined under a dissecting microscope (Santa2, Microscope Network, Kawaguchi, Saitama, Japan). Examinations consisted of the complete dissection of at least 10 polyps/

specimen (except for *P. sp. yoron* for which entire colonies were dissected). As a dissecting microscope was used throughout this experiment, only eggs with diameters  $> 100 \mu\text{m}$  were clearly visible and therefore recorded. Digital images of all observed eggs and ovaries were taken, and egg numbers and their maximum diameters were calculated using digital imagery software as described above. Eggs were then grouped into  $100 \mu\text{m}$  diameter categories ( $100 \mu\text{m}$  class,  $100\text{-}199 \mu\text{m}$ ;  $200$ ,  $200\text{-}299 \mu\text{m}$ ;  $300$ ,  $300\text{-}399 \mu\text{m}$ ; and  $400$ ,  $400\text{-}499 \mu\text{m}$ ). The presence or absence of *Symbiodinium* spp. (zooxanthellae) on the eggs was also noted.

## RESULTS

### Transect and sampling results

Transect data are summarized in table 1. From both transects and observations made during sampling, *P. tuberculosa* was found in the backreef moat, on the reef flat, and at the outer reef at Odo Beach. Although *P. tuberculosa* was found in most areas, only 1 colony was noted in the backreef moat during transect surveys (with an average occurrence of 0.3 colonies/transect,  $n = 3$  transects), and none were noted in surge channels near the reef edge. *Palythoa tuberculosa* was more common on the reef flat (ranging 1-11 colonies per transect, with an average of 5 colonies/transect,  $n = 3$ ) and was most common on the outer reef (ranging 9-38 colonies per transect, with an average of 21.3 colonies/transect,  $n = 3$ ).

*Palythoa sp. yoron* only appeared on the reef flat, albeit in large numbers (ranging 1-23 colonies per transect, with an average of 14.8 colonies/transect,  $n = 3$ ).

*Palythoa mutuki* was confirmed only from intertidal areas and surge channels along the reef edge slightly to the west of the other transect locations. Although limited to a small area, many

**Table 1.** Number of colonies of three *Palythoa* spp. from transects at Odo Beach, Okinawa, Japan

Species	Backreef moat ( $n = 1$ ) <sup>1</sup>	Reef flat ( $n = 5$ ) <sup>1</sup>		Reef edge ( $n = 3$ ) <sup>1</sup>		Out reef ( $n = 3$ ) <sup>1</sup>	
		Total Avg.	transect	Total Avg.	transect	Total Avg.	transect
<i>P. tuberculosa</i>	1	25	5	0	0	64	21.3
<i>P. sp. yoron</i>	0	74	14.8	0	0	0	0
<i>P. mutuki</i>	0	0	0	9	3	0	0

<sup>1</sup>Numbers in parentheses equal total number of transects for each area (transects  $10 \times 1$  m - see Materials and Methods).

very large colonies (up to 2 × 5 m) appeared in this area. Similarly, although *P. sp. yoron* was present in the reef flat transects, it was not observed anywhere else at Odo Beach, and has only been reported from a few scattered locations in Okinawa and Kagoshima (Fig. 4A).

Overall, *P. tuberculosa* was the only *Palythoa* species present in both the backreef moat and outer reef, while *P. sp. yoron* dominated the reef flat (74.8% of total colonies in this area), and *P. mutuki* dominated the reef edge and surge channels (100% of colonies).

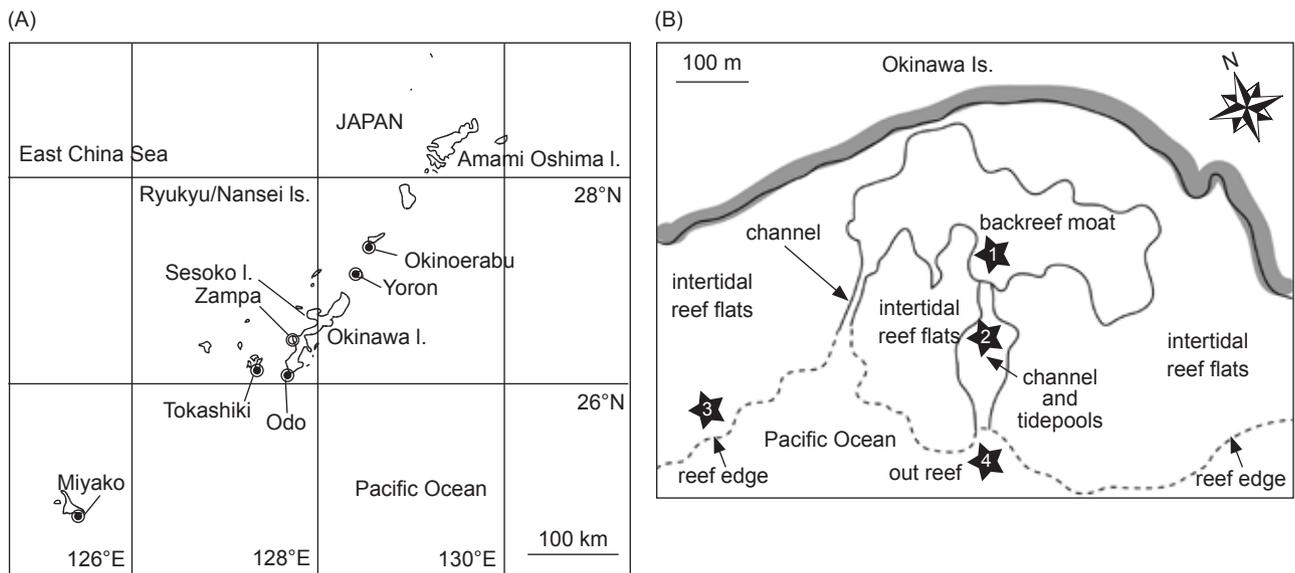
**Morphological comparisons among *Palythoa* spp.**

A summary of the observed morphological

characteristics of *P. tuberculosa*, *P. mutuki*, and *P. sp. yoron* is given in table 2.

Both *P. tuberculosa* and *P. mutuki* were often found in large colonies with diameters of > 50 cm at Odo Beach. These colonies numbered more than 100 polyps each, and often had several hundred. In contrast, *P. sp. yoron* was predominantly found in small colonies of 2-6 polyps, and never formed large colonies consisting of more than 10 polyps (E. Shiroma, pers. obs.).

*Palythoa tuberculosa* polyps were embedded within a well-developed coenenchyme (= immersae; Fig. 2A), while *P. mutuki* polyps were free and clear of the coenenchyme (= liberae; Fig. 2C), although the coenenchyme was still much-more developed than the very liberae and stoloniferous *P. heliodiscus* (see Reimer et



**Fig. 4.** Maps of (A) known locations of *Palythoa sp. yoron* in southern Japan, and (B) Odo Beach, Okinawa, Japan. In A, locations with large (> 100) colonies of *P. sp. yoron* populations are represented by filled circles, while the open circle at Zampa represents a small population (< 10 confirmed colonies). In B, stars represent transect locations and dashed lines the reef edge. Star 1, backreef moat (see Table 1); 2, reef flat; 3, reef edge; 4, outer reef.

**Table 2.** Comparative morphological data of three *Palythoa* species from Odo Beach, Okinawa, Japan

	<i>P. tuberculosa</i>	<i>P. sp. yoron</i>	<i>P. mutuki</i>
Environment with most common in <b>bold</b>	<b>Backreef moat to out reef</b>	<b>Reef flat, tide pools</b>	<b>Reef flat, reef edge, surge channels</b>
Depth (m)	Intertidal to >20	Intertidal to 1.5	Intertidal to 0.5
Coenenchyme development	Well-developed	Moderately developed	Not well developed; or stoloniferous
Polyp structure type <sup>1</sup>	immersae	intermediae	liberae
Avg. # of tentacles	33 ± 2 (n = 10)	40 ± 4 (n = 10)	54 ± 7 (n = 16)
Avg. oral disk diameter (cm)	0.66 ± 0.12 (n = 12)	0.60 ± 0.07 (n = 4)	1.24 ± 0.26 (n = 10)
Oral disk diameter/scapus diameter ratio	1	1	> 1

<sup>1</sup>Polyp structure based on Pax (1910); see also figure 2.

al. 2006a 2007a). *Palythoa* sp. yoron polyps were seen to be intermediate between those of *P. tuberculosa* and *P. mutuki* in form, with polyps somewhat free and clear of a rather well-developed coenenchyme (intermediae; Fig. 2B).

*Palythoa tuberculosa* had an average of  $33 \pm 2$  tentacles (range, 30-37,  $n = 10$ ) on fully grown polyps; *P. mutuki* had an average of  $54 \pm 7$  tentacles (range, 42-66,  $n = 16$ ); and *P. sp. yoron* had an average of  $40 \pm 4$  tentacles (range, 30-44,  $n = 10$ ) (Table 2). Both *P. tuberculosa* and *P. mutuki* were observed to occasionally open their oral disks during the daytime, while *P. sp. yoron* was only observed with open polyps at night, and even then very rarely.

The oral disk diameter of expanded *P. tuberculosa* polyps was found to average  $0.66 \pm 0.12$  cm (range, 0.5-0.9 cm,  $n = 12$ ), while *P. mutuki*'s average oral disk diameter was larger at  $1.24 \pm 0.26$  cm (range, 0.8-1.8 cm,  $n = 10$ ), and *P. sp. yoron*'s average oral disk diameter was smallest, at  $0.60 \pm 0.07$  cm (range, 0.5-0.7 cm,  $n = 4$ ) (Table 2). Additionally, *P. mutuki* polyps' scapus (polyp column) was observed to be smaller in diameter than the oral disk, while the scapi in both *P. tuberculosa* and *P. sp. yoron* was approximately the same diameter as their oral disks (Table 2).

### Sexual reproduction – *P. tuberculosa* egg production

Egg numbers and sizes from *P. tuberculosa* samples are shown and summarized in figures 5 and 6 and table 3.

*Palythoa* samples collected on 30 Apr., and 15 and 29 May 2008 were observed to contain no noticeable gametes  $> 100 \mu\text{m}$  in diameter ( $n$  of

total colonies collected = 1, 5, and 3, respectively) (Fig. 6, Table 3). However, 1 *P. tuberculosa* sample collected on 14 June was observed to contain 1 egg (of 4 total colonies collected). The egg was  $100 \mu\text{m}$  in diameter and whitish. No *Symbiodinium* zooxanthellae were observed on the outer surface of the egg (Fig. 6, Table 3).

One *P. tuberculosa* colony collected on 28 June also contained eggs, of which the largest was  $450 \mu\text{m}$  (of 5 total colonies collected). Again, no *Symbiodinium* was observed on the outer surface of the egg (Fig. 5A). From 10 polyps of this colony, 330 eggs were observed, with an average size of  $267 \pm 112 \mu\text{m}$ . Eggs in the  $300 \mu\text{m}$  size class were most numerous, comprising 43.9% of the total number (Fig. 6, Table 3).

Two colonies collected on 21 July had eggs (of 5 total colonies collected). Eggs were numerous inside the polyps of these colonies, and most had *Symbiodinium* on their outer surface (Figs. 5B, C). From a total of 20 polyps (10 from each colony), 594 eggs were collected. The average egg size was  $368 \pm 100 \mu\text{m}$ , and 46.1% of eggs were in the  $400 \mu\text{m}$  class (Fig. 6, Table 3). Eggs were orange before fixation. After fixation in 70% ethanol, eggs consistently turned greenish.

Three colonies collected on 2 Aug. were found to have eggs (of 7 total colonies collected). Eggs of  $> 400 \mu\text{m}$  had *Symbiodinium* on their surfaces. The total number of eggs from 10 polyps was 151 (with an average diameter of  $261 \pm 85 \mu\text{m}$ ), and 37.8% of eggs were in the  $300 \mu\text{m}$  class (Fig. 6, Table 3).

No eggs were observed from colonies collected on 16 Aug. (of 4 total colonies collected) (Fig. 6, Table 3). On 30 Sept., no eggs were observed (of 4 total colonies collected) (Table 3).

Eggs were seen in 1 colony (of 5 total colonies

**Table 3.** Numbers of eggs ( $>100 \mu\text{m}$  in diameter) observed in *Palythoa tuberculosa* colonies (Apr.- Oct. 2008) from Odo Beach, Okinawa, Japan by month and class sizes, and their maturity

Collection month (2008)	Egg size class ( $\mu\text{m}$ )					Total eggs	Total mature ( $> 300 \mu\text{m}$ ) eggs	Percentage of mature eggs
	100 <sup>1</sup>	200 <sup>1</sup>	300 <sup>1</sup>	400 <sup>1</sup>	500 <sup>1</sup>			
Apr.	0	0	0	0	0	0	0	0
May	0	0	0	0	0	0	0	0
June	1	160	158	12	0	331	170	51.4
July	0	12	245	292	45	594	582	98.0
Aug.	25	67	64	5	0	161	69	45.7
Sept.	0	0	0	0	0	0	0	0
Oct.	0	3	0	0	0	3	0	0

<sup>1</sup>For size class definitions - see Materials and Methods.

collected) collected on 16 Oct. Three eggs in total were seen from 10 polyps, and they had an average diameter of approximately 200  $\mu\text{m}$ , with no *Symbiodinium* on the surface (Fig. 6, Table 3).

Additionally, throughout the study, there were several cases of copepods found inside *P. tuberculosa* polyps, and these data will be reported elsewhere.

### Sexual reproduction – *P. mutuki* and *P. sp. yoron* egg production

During the period between 30 Apr. and 16 Oct., no sampled colonies of either *P. mutuki* ( $n = 32$ ) or *P. sp. yoron* ( $n = 37$ ) were found to contain any eggs, and no copepods were found within any polyps.

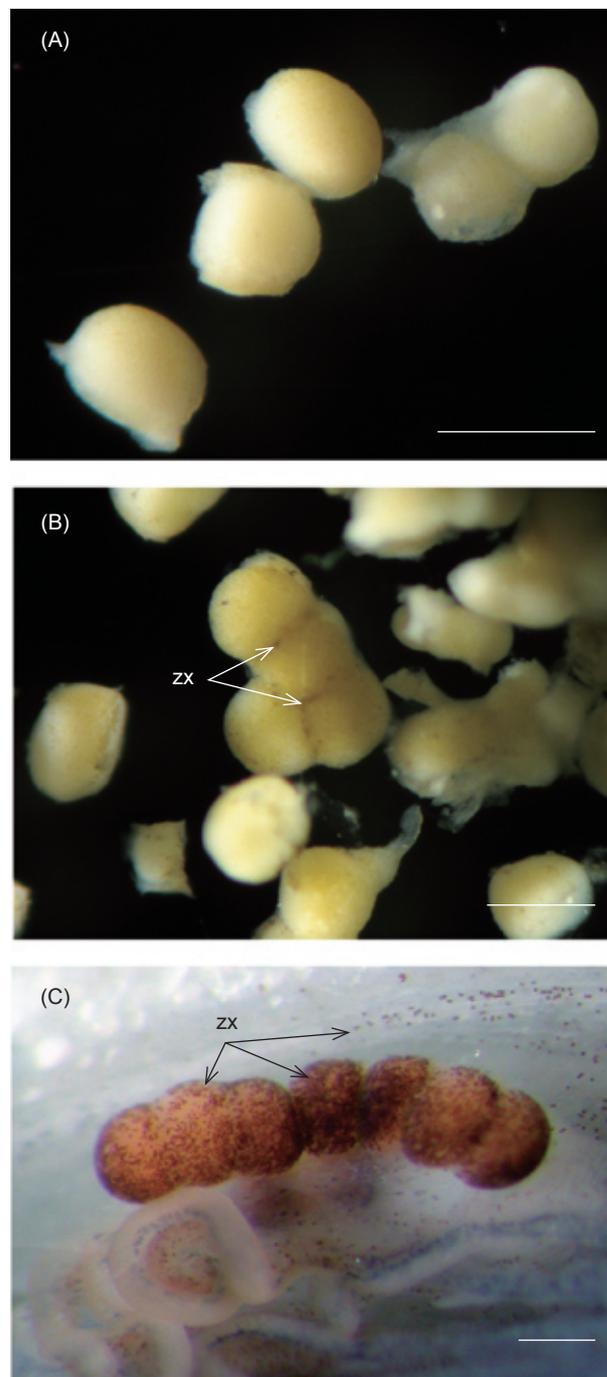
## DISCUSSION

### Distribution and ecology of *Palythoa* spp.

In general, zooxanthellate zoanthids including *Palythoa* show distribution patterns in Japan congruent with sea surface temperatures (Reimer et al. 2008), similar to those seen with corals in Taiwan (Chen 1999).

At Odo Beach, *P. tuberculosa* showed a wide distribution from the shallow waters of the backreef moat and reef flat to > 10 m in depth at the outer reef. This is similar to that previously reported for *P. tuberculosa* in Japan (Reimer et al. 2006b), and further demonstrates that this species is a “generalist” that lives in a variety of coral reef environments. With its relatively massive (for a zoanthid) immersae morphology and heavy sand encrustation, *P. tuberculosa* can live in environments with strong currents or waves. Additionally, *P. tuberculosa* has been shown to be a very efficient planktonivore (Fabricius and Metzner 2004), and is mixotrophic (Tanner 2002), fulfilling its nutritional needs by both symbiosis with *Symbiodinium* (Reimer et al. 2006b) and plankton feeding. This flexibility may allow *P. tuberculosa* to exist in a wide variety of environments from shallow to deep (> 35 m) (Reimer et al. 2006b) and even in very shaded locations under rocks (Reimer unpubl. data).

In contrast, *P. mutuki* found at Odo Beach was limited to very shallow waters no more than 1 m deep, including the reef edge where it often formed large colonies. On the exposed reef flat, however, *P. mutuki* did not form large colonies, and



**Fig. 5.** Eggs from *Palythoa tuberculosa* colonies at Odo Beach. (A) Eggs collected from inside polyps on 14 June 2008 and fixed in 10% seawater (SW) formalin and then 70% ethanol with no visible zooxanthellae (zx) on their surfaces; (B) eggs collected from inside polyps on 21 July 2008 and fixed in 10% SW formalin and then 70% ethanol with visible zooxanthellae (zx) on their surfaces; and (C) eggs collected from inside polyps on 21 July 2008 not yet fixed with many visible zooxanthellae (zx) on their surfaces. All scales = 300  $\mu\text{m}$ .

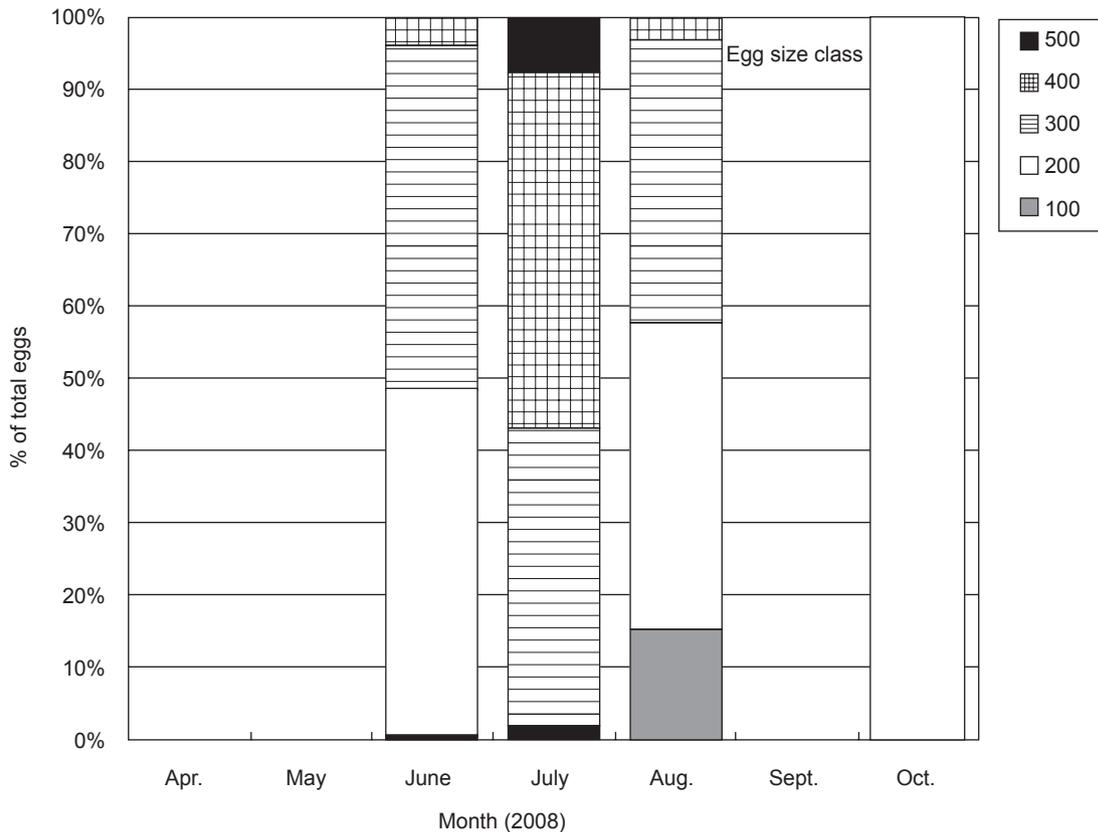
was found only in a patchy distribution of small colonies of no more than 40-50 polyps. The reef flat environment experiences both strong waves and desiccation during extreme tides. *Palythoa mutuki* may escape some of the harsh influences of light, heat, and waves by growing in small cracks that provide some shelter and shade, similar to what was reported for some *Zoanthus* species (Reimer et al. 2007b), but *P. mutuki* colonies are often found in completely exposed locations. Compared to the other 2 *Palythoa* types in this study, *P. mutuki* seems to prefer this harsher environment, and is generally found in this shallow zone (intertidal and shallow subtidal) throughout southwestern Japan.

Although also found in the low intertidal zone, *P. sp. yoron* seems to prefer a slightly different environment than *P. mutuki* or *P. tuberculosa*. This species was found slightly more inshore than the other 2 species, in small holes or divots on the reef flat, and often with sand or other loose

detritus covering the hard dead coral substrate to a few centimeters in depth. Although attached to the substrate, *P. sp. yoron* was not found to be as tightly attached as the other 2 *Palythoa* species and could readily be removed from the substrate by hand without exerting any special effort. *Palythoa sp. yoron* is found in a similar microenvironment at locations on Yoron and Okinoerabu Is., Japan, but not in other locations. From these observations it appears that *P. sp. yoron* strongly prefers low intertidal locations that do not experience strong waves or currents, but are not completely sheltered.

**Sexual reproduction of *P. tuberculosa***

*Palythoa tuberculosa* polyps possessed the largest number of eggs on 21 July ( $n = 582$ ), and these eggs were largest in size, with approximately 98% > 300  $\mu\text{m}$ , which is considered to be the size of mature eggs in *Palythoa* (Yamazato et al. 1973).



**Fig. 6.** Monthly summary of percentages of eggs of 5 different size classes found in polyps of *Palythoa tuberculosa* collected from Odo Beach, Okinawa, Japan in May-Oct. 2008. Size classes: 100, 100-199  $\mu\text{m}$  in maximum diameter; 200, 200-299  $\mu\text{m}$  in maximum diameter; 300, 300-399  $\mu\text{m}$  in maximum diameter; 400, 400-499  $\mu\text{m}$  in maximum diameter; and 500,  $\geq 499 \mu\text{m}$  in maximum diameter. For details see "Materials and methods" and table 3. "No data" indicates that no eggs were found in *P. tuberculosa* samples collected for that month.

By 2 Aug., both the number of eggs ( $n = 69$ ) and the mature proportion (45.7%) had drastically declined, suggesting that spawning in *P. tuberculosa* at Odo Beach occurred between these 2 dates. Additionally, the amount of time taken for *P. tuberculosa* to produce mature eggs (from the 1st observation to estimated spawning of approximately 5 wk) appears to be in line with time scales previously observed in other zoanthids (e.g., Ono et al. 2005). The diameter of *P. tuberculosa* eggs in this study also correlated well with previous size observations by Kimura et al. (1972), who reported mature *P. tuberculosa* eggs of 300-500  $\mu\text{m}$  in diameter.

An examination of previous research into *Palythoa* spp. spawning timing on the Great Barrier Reef showed that 1 species of *Protopalythoa* (= *Palythoa*) mass spawned 3 nights after a full moon (Babcock and Ryland 1990), and another species of *Palythoa* mass spawned 3-5 d after a full moon (Burnett et al. 1994). Very similarly, in the closely related genus, *Zoanthus*, spawning was theorized to occur in *Z. sansibaricus* in Kagoshima, Japan in summer (July) 3-5 d after a full moon (Ono et al. 2005). From these observations it appears that at least some coral reef zoanthids spawn at night close to the full summer moon when tides are large. Thus, based on data in this study and previous research, we surmise that *P. tuberculosa* at Odo Beach most likely mass-spawned at night within 5 days after a full moon which occurred on 21 July 2008.

Both Babcock and Ryland (1990) and Burnett et al. (1994) observed that *Palythoa* mass-spawned at approximately the same time as the Scleractinia, something that we did not conclude from our results here. In particular, acroporids on Okinawa I. are believed to spawn 3-7 d after a full moon in May or June (Hayashibara et al. 1993). Similarly, *Goniastrea* spawns in June and early July, and 2 spawning peaks may exist (Sakai 1997). The only research thus far on zoanthid spawning at Okinawa was conducted by Kimura et al. (1972) and Yamazato et al. (1973), who investigated *P. tuberculosa* at Sesoko I. just off northwestern Okinawa I. (Fig. 4A). Unlike our studies, they observed spawning from May to Nov. with a peak in June. The discrepancy in observations may have been due to the different geographical positions of the 2 sites (separated by > 60 km), and this is something that needs to be investigated in the future. While it is possible that the few ( $n = 3$ ) eggs observed in Oct. could have developed and additional spawning had occurred in *P. tuberculosa*, based on the very small number

of eggs observed and their small size of < 200  $\mu\text{m}$ , we feel this is unlikely. Another possibility is that eggs observed in Oct. were reabsorbed by *P. tuberculosa*, as seen in *Z. sansibaricus* with eggs that remained after a spawning event (Ono et al. 2005).

One more unexpected finding during the course of this study was the presence of *Symbiodinium* on the outer surface of eggs. All *Palythoa* species except one (see data in Ryland and Babcock 1991, Burnett et al. 1997, Ryland 1997, Ryland et al. 2000) are currently thought to acquire their *Symbiodinium* from the environment after larvae settle on a substrate. While the presence of *Symbiodinium* on spawned eggs (e.g., in the water column; outside of polyps) was not confirmed, it is likely that eggs are spawned with *Symbiodinium* on their surfaces for 2 reasons. First, no *Symbiodinium* were observed on eggs on 28 June (Fig. 5A), despite 51.4% of eggs ( $n = 170$ ) being of a mature size (> 300  $\mu\text{m}$  in diameter). On the other hand, *Symbiodinium* was observed on large eggs on both 21 July and 2 Aug. (Figs. 5B, C). Thus, it appears as if *P. tuberculosa* eggs specifically acquire *Symbiodinium* on their surface just prior to spawning. Second, unpublished digital images of *Zoanthus* egg bundles during a spawning event at Otsuki, Kochi, Japan in summer 2008 clearly also show the presence of *Symbiodinium* on the surfaces of eggs (T. Mezaki, Biological Institute on Kuroshio, pers. comm.). Similar to most *Palythoa* species, *Zoanthus* species are thought to acquire their symbionts horizontally (from the environment), but our *P. tuberculosa* data as well as images of *Zoanthus* show this might not always be the case, and many zooxanthellate zoanthids may acquire their *Symbiodinium* vertically (from parent colonies). If so, this could help explain the high levels of specificity and stability for *Zoanthus* and *Palythoa* species to particular types of *Symbiodinium* (Reimer et al. 2006b c).

No evidence of sexual reproduction in *P. mutuki* or *P. sp. yoron* was seen during the course of this study, and the spawning timing of these 2 species could not be elucidated. However, at least at Odo Beach the 3 *Palythoa* species evidently did not spawn synchronously in summer 2008. It may be that *P. mutuki* and *P. sp. yoron* only spawn under certain “just-right” conditions, as oocyte production is a costly undertaking for hexacorals (e.g., Loya and Sakai 2008). However, summer 2008 at Odo Beach was a rather “usual” summer with regards to water temperature, and very little

bleaching was seen in zoanths (only 1 bleached *P. tuberculosa* colony of all *Palythoa* colonies examined;  $n \geq 100$ ).

Another possibility is that *P. mutuki* and *P. sp. yoron* spawn in winter. As the experiment here examined polyps in both Apr. and Oct. that had no or few gametes, and given that oocyte production takes at least a month or 2 from data of other zoanthid species, it would appear that the most likely time for a potential winter spawn would be in the period between late Dec. and early Apr. Year-round examination of *Palythoa* species' polyps will clarify this issue, but to date only a very few species of hexacorals (e.g., Caribbean *Mycetophyllia ferox* - see Szmant 1986, some Taiwanese Scleractinia, see Fan and Dai 1995 1998) were found to be winter spawners, and we deem this explanation for the lack of summer spawning in *P. mutuki* and *P. sp. yoron* to be unlikely.

One more possibility is asynchronous spawning between *P. mutuki* and *P. sp. yoron* colonies, as theorized to occur in Caribbean *Z. sociatus* (Karlson 1981) and in the actinarian, *Aipstasia pulchella* (Chen et al. 2008). However, this theory also seems unlikely as not a single oocyte/egg in either species was observed during the 6 mo period of observation, counter to what would be expected if asynchronous spawning was occurring.

Thus, it appears that *P. mutuki* and *P. sp. yoron* may rely more on asexual reproduction than sexual spawning. Data from *P. mutuki* collected from Odo Beach and kept in tanks at Sesoko Marine Station support this idea, as 5 colonies under observation in May-Oct. 2008 (approximately 6 mo) increased an average of more than 200% in polyp number (Shiroma and Reimer unpubl. data). However, *P. sp. yoron* and *P. tuberculosa* did not increase in size in the tanks, and were not observed to increase in size or number at Odo Beach during the course of the study. How *P. sp. yoron* increases its numbers remains an enigma.

Somewhat similar results of differences in reproductive patterns were observed by Karlson (1981) in 3 Caribbean *Zoanthus* species. It was found that 1 species, *Z. pulchellus*, was far more fecund than 2 other species despite having smaller polyps, producing many more eggs, and having a higher percentage of fertile polyps, and thus directing a larger proportion of its energy budget towards sexual reproduction. Of the other 2 species, one was found to direct a very high proportion of energy towards asexual growth,

particularly when colonies were small (Karlson 1988). Such differences in reproductive energy allotments in zoanthid species may be due to differing strategies and mechanisms of dealing with competition for space, escape from predators, or differing mortality rates (Karlson 1988). It appears that the 3 *Palythoa* species in this study also have differing reproductive patterns and strategies.

### Intermediate morphology of *P. sp. yoron*

Morphological data show that *P. sp. yoron* is generally intermediate in morphology between *P. tuberculosa* and *P. mutuki*. This trend was seen with both polyp shape and tentacle number (Table 2), although *P. sp. yoron* had a smaller oral disk diameter than the other 2 species which was much closer in size to *P. tuberculosa*. Similarly, although intermediate, the tentacle number was closer to the number of tentacles of *P. tuberculosa*. Overall, these data reflect that although *P. sp. yoron* is somewhat intermediate in morphology between *P. tuberculosa* and *P. mutuki*, it is morphologically closer to *P. tuberculosa*.

It should be noted, however, that Burnett et al. (1997) found that 3 different *Palythoa* morphotypes on the Great Barrier Reef belonged to a monospecific group (*P. caesia*), and thus identification of *Palythoa* species based solely on morphology does involve an inherent chance of misidentification. Similarly, there are at least 3 different *P. tuberculosa* (which may be synonymous with *P. caesia*) morphotypes present at Okinawa, although over the course of this study at Odo Beach, we only investigated 1 morphotype.

### Evolutionary history and taxonomic position of *P. sp. yoron*

In this study, however, morphological data are reflected in the genetic data. ITS-rDNA from *P. sp. yoron* has some *P. mutuki* elements, but was closer to *P. tuberculosa* (Reimer et al. 2007a) (Fig. 3), and clearly showed additive polymorphic sites (APSSs), an indication of potential reticulate evolution (Aguilar and Feliner 2003), while mtDNA sequences (cytochrome oxidase I (COI), mt 16S rDNA) from *P. sp. yoron* were identical to those of *P. tuberculosa* (Reimer et al. 2006a 2007a). Finally, even if spawning timing between these species proves to be similarly timed, at least the reproductive effort and strategy of each group appear to differ, with *P. tuberculosa* prolifically spawning, *P. mutuki* prolifically asexual

reproducing, and *P. sp. yoron*'s reproductive patterns are as yet undetermined.

From these combined results, we propose that *P. sp. yoron* is the product of an initial 1-way hybridization event in the past between *P. mutuki* (sperm) and *P. tuberculosa* (egg), followed by subsequent introgression between this hybrid and *P. tuberculosa*. Such events would explain both the presence of mtDNA identical to *P. tuberculosa* in *P. sp. yoron*, and ITS-rDNA sequences of *P. sp. yoron* being phylogenetically more closely related to *P. tuberculosa* than to *P. mutuki*.

It is yet unconfirmed if *P. sp. yoron* sexually reproduces, and until this is confirmed, we cannot formally designate this group a new species. It may be that *P. sp. yoron* is simply some form of a subpopulation of *P. tuberculosa*, as we could not confirm any sexual reproductive characteristics in either *P. sp. yoron* or *P. mutuki*. Likewise, the possibility that *Palythoa* ITS-rDNA has a relatively high rate of evolution resulting in the ITS-rDNA patterns observed by Reimer et al. (2007a) cannot be discounted, and may be responsible for the ITS-rDNA patterns observed, as hypothesized to explain high intraspecific ITS-rDNA sequence variation in the coral genus *Acropora* (Wei et al. 2006). However, from the presence of APSs in *P. sp. yoron* ITS-rDNA, it appears that at least in the past, *P. sp. yoron* did very likely sexually reproduce and then introgressed (backcrossed) with *P. tuberculosa*. It may be that *P. sp. yoron* experienced sexual reproduction breakdown, a phenomenon that can occur in interspecific hybrids (e.g., Vollmer and Palumbi 2002). Regardless, if any current sexual reproduction can be confirmed in this putative species, it will be demonstrated that reticulate evolution in zoanthids has contributed to their evolution and diversity, and may contribute to their adaptive potential, as speculated for some scleractinian genera (Richards et al. 2008).

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