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ITS Ribosomal DNA Distinctions and the Genetic Structures of Populations of Two Sympatric Species of *Pavona* (Cnidaria: Scleractinia) from Mauritius

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Kamla Ruby Moothien Pillay, Takashi Asahida, Chaolun Allen Chen, Hiroaki Terashima, and Hitoshi Ida (2006) ITS ribosomal DNA distinctions and the genetic structures of populations of two sympatric species of *Pavona* (Cnidaria: Scleractinia) from Mauritius. *Zoological Studies* **45**(1): 132-144. In this study, we examined the genetic differences between *Pavona cactus* and *P. decussata*, two of the major components of the shallow reef flat coral communities in Mauritius, which not only occur in sympatry but are often intricately associated. Using sequences of ribosomal internal transcribed spacers (ITSs), we conducted phylogenetic, population, and nested clade analyses (NCA) on both species sampled from Bambous Virieux on the southeastern coast and Trou aux Biches on the northwestern coast of the island. The phylogenetic analysis of ITS sequence types supported the distinct species status of *P. cactus* and *P. decussata*. The significant difference detected by the NCA indicated that both *P. cactus* and *P. decussata* in Mauritius constitute statistically distinguishable lineages. No population structure was detected between the two geographic locations. We conclude that *P. cactus* and *P. decussata* remain distinct evolutionary units despite their ecological uniqueness in Mauritius.
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Key words: Species boundaries, Internal transcribed spacer (ITS), Hybridization, Reproductive barriers, Population structure.

In Mauritius, *Pavona cactus* (Forskål, 1775) and *P. decussata* (Dana,1846) constitute two of the major components of the shallow reef flat coral communities (Moothien Pillay et al. 2002a), are ecologically important as they contribute to reef building, and are among the few species that are less susceptible to bleaching (Moothien Pillay et al. 2002b, McClanahan et al. 2005). Both species occur sympatrically and form distinct zones (Montaggioni and Faure 1997, Moothien Pillay et al. 2002a). They may be found as isolated colonies or may form large monospecific stands, especially on channel slopes and in near-surf zones. *P. cactus* dominates the deeper channel slopes and *P. decussata* the shallowest parts of channel slopes and reef flats. *P. decussata* is variable in macro-morphology, having large upright plates in relatively deeper waters and short stunted plates in shallower environments; such morphological variations are most probably related to environmental variations. *P. cactus* usually has thin upright fronds, but tends to develop thicker fronds in shallow, high-energy environments, hence superficially resembling *P. decussata* at the macro-morphological level in such habitats. Although most species of *Pavona* are well defined, they are still known to show wide environmental variations in morphology (Veron 2000). For exam-

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ple, in a study of 3 species of *Pavona* on the Panamanian Pacific, *P. varians* was found to be so morphologically variable as to resemble *P. chiriquiensis* and *P. frondifera* in certain environments, but ecological, genetic, and morphological studies showed distinct species boundaries between the 3 species (Maté 2003). *P. cactus* and *P. decussata* are ecologically unique on the reefs of Mauritius as they are often found intricately associated when they co-occur on reef flats (Fig. 1). Often, *P. cactus* grows amid live colonies of *P. decussata*. Moreover, when both species co-occur in large fields in shallow areas, they merge into one another to the extent that they are confusing at times.

Since *P. cactus* and *P. decussata* are so closely associated on the reef flats of Mauritius, opportunities may exist for natural hybridization, in the absence of mechanisms that limit interspecific breeding in corals, such as temporal reproductive isolation (Szmant et al. 1997, van Oppen et al. 2001, Fukami et al. 2003) and gametic incompatibility (Willis et al. 1997, Hatta et al. 1999). In fact, there are suggestions that closely related coral species in sympatry may hybridize during mass spawning events (Babcock 1995, Willis et al. 1997), although there are intrinsic (genetic) and extrinsic (geographic) reproductive barriers that maintain distinct boundaries between sympatric congeners (Avise and Ball 1990). There is at present little known about the reproductive modes of *P. cactus* and *P. decussata*. Sexual reproduction is reported to be dominant in most of the other studied *Pavona* species (e.g., Marshall and Stephenson 1933, Glynn et al. 1996, Glynn and Ault 2000). On the Great Barrier Reef (GBR), *P. cactus* has been reported to reproduce sexually but most commonly by asexual means of larvae (Willis and Ayre 1985, Ayre and Willis 1988). However, we do not know whether *P. cactus* and *P. decussata* in Mauritius have similar reproductive modes as reported for *P. cactus* on the GBR and the other *Pavona* species from elsewhere, as coral species may show wide geographic variations in their reproductive modes. For example, *Pocillopora damicornis* is known to reproduce by sexually brooded planula larvae along the GBR (Ayre et al. 1997), by asexually brooded planula larvae in Western Australia and Hawaii (Stoddart 1984 1988), and by broadcast spawning (Richmond 1985) or asexual fragmentation of large colonies in the Eastern Pacific (Richmond 1987). However, if *P. cactus* and *P. decussata* are broadcast spawners on the reefs of Mauritius and have similar spawning times, this would be expected to result in cross-species mating, especially considering that they are so closely associated on the reef flat. Hence, examination of their DNA sequences would reveal whether these species are hybridizing or are genetically distinct.

In this study, we used the nuclear ribosomal DNA internal transcribed spacers 1 and 2 (rDNA ITS-1 and ITS-2) to examine genetic differences between *P. cactus* and *P. decussata*. ITS markers have been used in previous studies to clarify phylogenetic relationships at or below the genus level in anthozoans (Beauchamp and Powers 1996, Chen and Miller 1996, Odorico and Miller 1997, Lopez and Knowlton 1997, Medina et al. 1999, van Oppen et al. 2000, Diekmann et al. 2001, Forsman 2003, Lam and Morton 2003, Forsman et al. 2005). Some of those studies concluded that

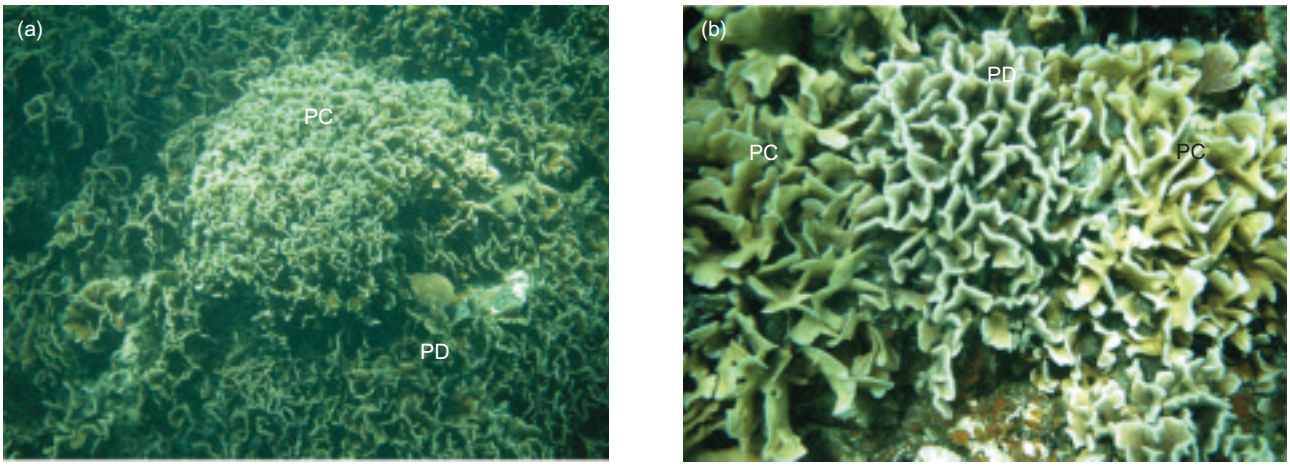

Fig. 1. *Pavona cactus* (PC) and *P. decussata* (PD) on a reef flat in Mauritius. *P. cactus* growing on a large colony of *P. decussata* at TAB (a). *P. decussata* growing in the middle of a *P. cactus* colony at BV (b). Photos by K.R. Moothien Pillay.

potentially hybridizing corals, especially *Acropora* spp., followed a reticulate pattern due to a high degree of intraspecific variation (e.g., Odorico and Miller 1997, van Oppen et al. 2000, 2002, Marquez et al. 2003). However, Vollmer and Palumbi (2004) noted that the ITS might not conclusively resolve coral phylogenetics, as polyphyletic lineages reported in these *Acropora* studies could be due to introgression, slow concerted evolution, or incomplete lineage sorting. On the other hand, it has been suggested that the observed high levels of ITS intragenomic divergence might be specific to *Acropora* spp. and might not reflect reticulate patterns among all corals (Chen et al. 2004), as the ITS marker has successfully resolved relationships at different phylogenetic levels from populations to genera in some non-*Acropora* corals such as *Platygyra* (Lam and Morton 2003), *Siderastrea*, *Porites* (Forsman 2003), and *Siderastrea* (Forsman et al. 2005).

MATERIALS AND METHODS

Study site and specimen collection

The island of Mauritius lies between latitudes 19°58'8"S and 20°31'7"S and longitudes 57°18'0"E and 57°46'5"E (Fig. 2). Its coastline is 200 km long, and the island is surrounded by a fringing

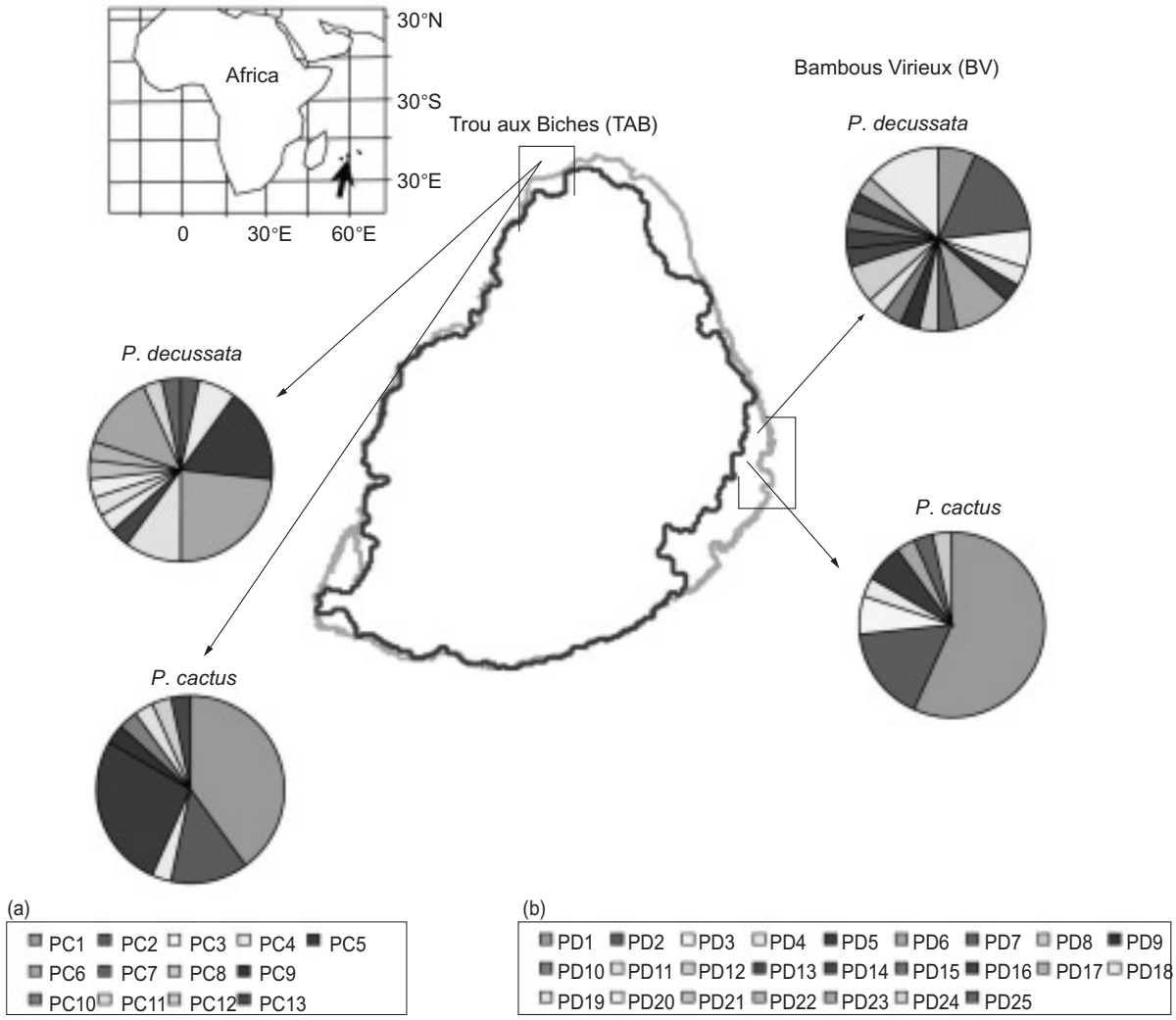

Fig. 2. Map of Mauritius showing sampling locations of *P. cactus* and *P. decussata* (grids showing sampling locations are not to scale). Pie charts show distribution of sequence types of *P. cactus* and *P. decussata* at TAB and BV. Different colors represent sequence types between populations within species. Sequence types are not shared between species. (a) PC1, PC5, and PC2 were the major sequence types shared by populations of *P. cactus*; (b) PD6, PD2 and PD5 were the major sequence types shared by populations of *P. decussata*. These sequences are shown in table 1.

coral reef of over 150 km^2 in areal extent, except for breaks on the southern and western coasts. A short strip of barrier reef is present off the south-eastern coast. The width of the lagoon from the shore to the reef crest greatly varies, ranging from 200-400 m in certain areas on the western coast to nearly 7 km off the eastern coast, with depths averaging 1-6 m. We chose 2 locations for sampling *P. cactus* and *P. decussata*: Trou aux Biches (TAB) on the northwestern (leeward) and Bambous Virieux (BV) on the southeastern (windward) coasts of the island (Fig. 2). These locations are under different hydrodynamic regimes, the lagoon of TAB is sheltered from the Southeast Trade Wind that prevails throughout most of the year, whereas BV is under its direct influence.

The reefs at TAB are approximately 57 km from those at BV along the northern coast and about 125 km along the southern coast. The sampling areas were ~0.115 km^2 at TAB and 2.2 km^2 at BV. The area sampled at BV was larger due to the complexity of the reefs in that region and the distribution patterns of *P. cactus* and *P. decussata*. They were more often found aggregated in large fields in that area. We sampled only one colony from large monospecific stands, which at times extended over areas exceeding 200 m^2.

We sampled 30 colonies of each species within each location. Although *P. cactus* has been reported to be highly clonal over distances of even 93 m (Ayre and Willis 1988), we were unable to separate all sampled colonies by such a large dis-

Table 1. ITS sequence types for *P. cactus* (PC) and *P. decussata* (PD) from BV and TAB with *Leptoseris yabei* as the outgroup

Sequence types	4 4 6	5 5 4	8 6 7	1 6 0	2 4 8	2 7 5	4 0 6	4 8 7	5 5 4	5 6 5	7 6 7	7 7 4	9 4 5	9 5 6	9 6 7	9 7 8	9 8 9	9 9 0	1 0 0 1	1 0 1 2	1 0 2 3	1 0 3 4	1 0 4 5	1 0 5 6	1 0 6 7	1 0 7 3	1 1 4 0	1 4 1	1 5 1 6	1 5 7
PC1	G	A	C	G	T	G	T	C	T	A	C	A	G	T	C	C	G	C	C	G	C	T	T	G	G	C	T	A	C	C	*
PC2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	*
PC3	A	G	T	.	.	.	.	T	C	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	*
PC4	A	G	T	.	.	.	.	T	C	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	*
PC5	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	*
PC6	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	*
PC7	A	G	T	.	C	.	.	T	C	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	*
PC8	A	G	T	.	.	.	.	T	C	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	*
PC9	.	.	.	C	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	*
PC10	A	G	T	C	.	.	T	C	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	*
PC11	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	*
PC12	A	G	T	C	.	.	T	C	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	*
PC13	A	G	T	C	.	.	T	C	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	*
PD1	.	.	.	A	.	.	C	.	C	.	.	T	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	*
PD2	.	.	.	A	.	.	C	.	C	.	.	T	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	*
PD3	.	.	.	A	.	.	C	.	C	.	.	T	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	T	.	.	*
PD4	.	.	.	A	.	.	.	.	C	.	.	T	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	T	.	.	*
PD5	.	.	.	A	.	.	C	.	.	.	.	T	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	*
PD6	.	.	.	A	.	.	C	.	.	T	.	T	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	*
PD7	.	.	.	A	.	.	.	.	C	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	*
PD8	.	.	.	A	.	.	C	.	C	.	.	T	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	*
PD9	.	.	.	A	.	.	.	.	C	.	.	T	*	*	*	*	*	*	*	*	*	*	*	*	*	*	.	.	.	.	*
PD10	.	.	.	A	.	.	.	.	C	.	.	T	*	*	*	*	*	*	*	*	*	*	*	*	*	*	.	.	.	.	*
PD11	.	.	.	A	.	.	.	.	C	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	*
PD12	.	.	.	A	.	.	.	.	C	.	.	T	*	*	*	*	*	*	*	*	*	*	*	*	*	*	.	T	.	.	*
PD13	.	.	.	A	.	.	C	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	*
PD14	.	.	.	A	.	.	.	.	C	.	.	.	*	*	*	*	*	*	*	*	*	*	*	*	*	*	.	.	.	.	*
PD15	.	.	.	A	.	.	.	.	C	.	.	T	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	*
PD16	.	.	.	A	.	.	.	.	C	.	.	T	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	*
PD17	.	.	.	A	.	.	.	.	C	.	.	T	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	*
PD18	.	.	.	A	.	.	.	.	C	.	.	T	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	*
PD19	.	.	.	A	.	.	C	.	C	.	.	T	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	*
PD20	.	.	.	A	.	.	.	.	C	.	.	T	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	*
PD21	.	.	.	A	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	*
PD22	.	.	.	A	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	*
PD23	.	.	.	A	.	.	C	.	.	.	.	T	.	.	.	.	T	.	.	.	.	.	.	.	.	T	.	.	.	.	*
PD24	.	.	.	A	.	.	.	.	C	.	.	T	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	*
PD25	.	.	.	A	.	.	.	.	C	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	*
Leptoseris yabei	.	.	.	A	.	C	.	.	C	.	.	T	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C
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tance due to the relatively small size of each location, our large sample size, and the aggregated distribution pattern of these species. However, we sampled colonies that were separated from each other by at least 10 m. At times, the distance between colonies exceeded 50 m. Sampling was undertaken in the shallower parts of the lagoon where the species co-occur. Tissue samples were collected by snapping off pieces (< 1 cm^2) from the fronds of individual colonies of *P. cactus* and *P. decussata*. Samples were preserved in 96% EtOH and kept refrigerated until they were processed.

Extraction, PCR amplification, sequencing, and sequence alignment

Total DNA was extracted using a DNeasy Tissue kit (Quiagen, USA). One microliter of each eluate was electrophoresed in a 0.7% agarose gel using a Lambda *Hind* III marker to assess the yields. Total DNA was then stored at -20°C. The ITS-1, 5.8S, and ITS-2 regions of the rDNA were amplified from *P. cactus* and *P. decussata* using the primer, ITS4 (5'-CCT CCG CTT ATT GAT ATG C-3'; White et al. 1990) and the coral-specific primer, A18S (5'-GAT CGA ACG GTT TAG TGA GG-3': Takabayashi et al. 1998).

All PCR reactions contained 1 μl of template DNA (12-120 ng/μl), 0.25 μl of each primer, 12.5 μl of premixed *Taq* polymerase (Takara Taq version, Takara, Japan) in a total volume of 25 μl.

Table 1. (Cont.)

Sequence types	165	166	167	168	169	170	171	172	173	180	182	184	185	195	196	199	200	222	227	229	230	231	316	334	347	353	355	358	360	363	377
PC1	A	A	C	C	A	A	T	A	G	A	A	C	G	A	A	C	A	C	T	C	G	G	*	C	T	A	G	G	C	*
PC2	.	.	C	C	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	*	.	.	.	.	.	.	*
PC3	.	.	C	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	*	.	.	.	.	.	.	*
PC4	.	.	C	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	*	.	.	G	.	.	.	*
PC5	.	.	C	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	*	.	.	.	.	.	.	*
PC6	.	.	C	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	*	.	.	.	.	.	.	*
PC7	.	.	C	C	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	*	.	.	.	.	.	.	*
PC8	.	.	C	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	*	.	.	.	.	.	.	*
PC9	.	.	C	C	.	.	.	.	C	.	C	.	.	.	.	.	.	.	.	.	.	.	*	.	.	.	.	.	.	*
PC10	.	.	C	C	.	.	.	.	C	.	C	.	.	.	.	.	.	.	.	.	.	.	*	.	.	.	.	.	.	*
PC11	.	.	C	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	*	.	.	.	.	.	.	*
PC12	.	.	C	C	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	*	.	.	.	.	.	.	*
PC13	.	.	C	C	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	*	.	.	.	.	.	.	*
PD1	.	.	C	C	.	.	.	.	.	.	.	.	A	*	*	.	.	.	.	C	.	.	*	.	C	G	.	.	.	*
PD2	.	.	C	C	.	.	.	.	.	.	.	.	A	*	*	.	.	.	.	T	.	.	*	G	C	G	.	.	.	*
PD3	.	C	C	C	.	.	.	.	.	.	.	.	A	*	*	.	.	.	.	T	.	.	*	G	C	G	.	.	.	*
PD4	.	C	C	C	.	.	.	.	.	.	.	.	A	*	*	.	.	.	.	T	.	.	*	G	C	G	.	.	.	*
PD5	.	.	C	C	.	.	.	.	.	.	.	.	A	*	*	.	.	.	.	T	.	.	*	G	C	G	.	.	.	*
PD6	.	.	C	C	.	.	.	.	.	.	.	.	A	*	*	.	.	.	.	T	.	.	*	G	C	G	.	.	.	*
PD7	.	.	C	C	.	.	.	.	.	.	.	.	A	*	*	.	.	.	.	T	.	.	*	G	C	G	.	.	.	*
PD8	.	C	C	C	.	.	.	.	.	.	.	.	.	*	*	.	.	.	.	T	.	.	*	G	C	G	.	.	.	*
PD9	.	C	C	C	.	.	.	.	.	.	.	.	.	*	*	.	.	.	.	T	.	.	*	G	C	G	.	.	.	*
PD10	.	C	C	C	.	.	.	.	.	.	.	.	A	*	*	.	.	.	.	T	.	.	*	G	C	G	.	.	.	*
PD11	.	C	C	C	.	.	.	.	.	.	.	.	.	*	*	.	.	.	.	C	.	.	*	.	C	G	.	.	.	*
PD12	.	C	C	C	.	.	.	.	.	.	.	.	A	*	*	.	.	.	.	T	.	.	*	G	C	G	.	.	.	*
PD13	.	.	C	C	.	.	.	.	.	.	.	.	A	*	*	.	.	.	.	T	.	.	*	G	C	G	.	.	.	*
PD14	.	C	C	C	.	.	.	.	.	.	.	.	A	*	*	.	.	.	.	T	.	.	*	G	C	G	.	.	.	*
PD15	.	C	C	C	.	.	.	.	.	.	.	.	A	*	*	.	.	.	.	T	.	.	*	G	C	G	.	.	.	*
PD16	.	C	C	C	.	.	.	.	.	.	.	.	A	*	*	.	.	.	.	T	.	.	*	G	C	G	A	A	.	*
PD17	.	C	C	C	.	.	.	.	.	.	.	.	A	*	*	.	.	.	T	T	.	.	*	G	C	G	.	.	.	*
PD18	.	C	C	C	.	.	.	.	A	.	.	.	A	*	*	.	.	.	T	T	.	.	*	G	C	G	A	A	.	*
PD19	.	C	C	C	.	.	.	.	A	.	.	.	A	*	*	.	.	.	T	T	.	.	*	G	C	G	A	A	.	*
PD20	.	C	C	C	.	.	.	.	.	.	.	.	.	*	*	.	.	.	.	T	.	.	*	G	C	G	.	.	.	*
PD21	.	.	C	C	.	.	.	.	.	.	.	.	A	*	*	.	.	.	.	T	.	.	*	G	C	G	.	.	.	*
PD22	.	C	C	C	.	.	.	.	.	.	.	.	.	*	*	.	.	.	.	T	.	.	*	G	C	G	.	.	.	*
PD23	.	.	C	C	.	.	.	.	.	.	.	.	A	*	*	.	.	.	.	T	.	.	*	G	C	G	.	.	.	*
PD24	.	.	C	C	.	.	.	.	.	.	.	.	.	*	*	.	.	.	.	T	.	.	*	G	C	G	.	.	.	*
PD25	.	.	C	C	.	.	.	.	.	.	.	.	A	*	*	.	.	.	.	T	.	.	*	G	C	G	.	.	.	*
Leptoseris yabei	*	*	*	*	*	*	*	*	*	*	*	*	*	G	A	A	T	T	.	.	.	.	C	G	C	A	G	G	T	A
														**	**										**					

Numbers on top refer to positions in the original alignment (excluding the 5.8S region). A full point represents a synonymous nucleotide, an asterisk (*) represents a deletion, and a pair of asterisks (**) represent a fixed genetic difference between the 2 taxa.

Amplifications were performed in a DNA thermal cycler (i Cycler: BIO-RAD, USA) with the following thermal profile: initial denaturation at 90°C for 30 s, 35 cycles at 94°C for 30 s, 55°C for 1 min, and 72°C for 30 s; ending with a thermal extension at 72°C for 1 min. PCR products were electrophoresed in 0.7% agarose gels to assess the yield, and were purified using Exo SAP-IT (Exonuclease I and shrimp alkaline phosphatase in buffer) (USB, USA). The purified PCR products were directly sequenced using a DyEnamic ET Terminator Cycle Sequencing kit (Amersham Biosciences, USA). Both strands of the PCR products were used for sequencing. Sequencing was performed on an ABI Prism 3100-Avant Genetic Analyzer (Hitachi, Japan), PCR primers were used as sequencing primers. Since the ribosomal DNA sequences of these species showed no ambiguous sites in the data or overlapping peaks in the sequences, direct sequencing was used. Sequences were submitted to GenBank under accession numbers AB217876 to AB217913.

Phylogenetic inferences, nested clade analysis, and population genetic analysis

Sequences were evaluated by running them through the Blast program (Altschul et al. 1990) and were manually aligned using the Bioedit sequence alignment editor vers. 7.0.0 (Hall 2004) with the sequence of *Leptoseris yabei* (Pillai and Scheer 1976). Inter- and intra-population genetic diversities were calculated by indices of haplotype diversity (*h*, Nei 1987) and nucleotide diversity (π, Nei 1987) in DnaSp (DNA sequence polymorphism) vers. 4.0.5 (Rozas et al. 2004) for ITS-1+ ITS-2 using the full 120 sequences. We excluded the 5.8S region from the analyses as it was identical in both species.

Phylogenetic trees of the relationships among sequence types were constructed using Maximum parsimony (MP), Neighbor-joining (NJ), and Maximum likelihood (ML) in PAUP*4.0b10 (Swofford 2002). Maximum parsimony (MP) analyses were performed under the heuristic search setting with the random addition of taxa and the tree-bisection-reconnection (TBR) algorithm, with 0-length branches collapsed, and the steepest descent not enforced. Analyses were run with gaps treated as missing data. Bootstrap analyses consisted of 1000 replicates in which the "max" trees were set to 10,000. Multiple bootstrapped MP trees were combined to produce a majority-rule consensus tree.

NJ analysis was performed using the Kimura-2-parameter model of nucleotide substitution. Stability of the NJ phylogeny was assessed by 1000 bootstrap replicates. Maximum likelihood (ML) trees were constructed using the best-fit model of DNA substitution and parameter estimates by performing hierarchical likelihood ratio tests (as reviewed in Huelesenbeck and Crandall 1997, Harris and Crandall 2000) using PAUP* 4.0b10 (Swofford 2002) and Modeltest 3.6 (Posada and Crandall 1998). The best-fit evolutionary model for ITS is the Felsenstein81+G+I (F81+G+I) model, with estimated base frequencies of 0.248 (A), 0.257 (C), 0.233 (G), and 0.263 (T), a rate heterogeneity among sites (G) of 0.0152, and invariable sites (I) of 0.857.

To test whether *P. cactus* and *P. decussata* correspond to phylogenetic lineages, a Nested Clade Analysis (NCA) was performed on the ITS data, using the program, TCS vers. 1.13 (Clement

Table 2. Collection sites, sample size (*n*), nucleotide content (GC%), number of substitutions (Ti, transition; Tv, transversion), intraspecific variability (%), number of sequence types (n_h), haplotypic diversity (*h*), and nucleotide diversity (π) calculated using DnaSp 4.0.5 (Rozas et al. 2004). Standard errors for haplotype and nucleotide diversities are indicated

Species	Localities	ITS-1 + ITS-2					
		n	GC%	Ti/Tv	Intra-specific variability (%)	n_h (*h*)	π
P. cactus	BV	30	49.4	10/6[a]	3.3	8 (0.69 ± 0.08)	0.0069 ± 0.0018
	TAB	30	49.4		3.8	9 (0.77 ± 0.06)	0.0079 ± 0.0021
P. decussata	BV	30	48.8	11/4[a]	3.5	18 (0.94 ± 0.02)	0.0093 ± 0.0009
	TAB	30	49.7		3.9	14 (0.91 ± 0.03)	0.0107 ± 0.0014

BV and TAB, abbreviations for localities are given in figure 2. [a]Average Ti/Tv for each species.

et al. 2000). The program collapses sequences into haplotypes and calculates the frequencies of the haplotypes in the sample. These frequencies are used to estimate haplotype outgroup probabilities, which correlate with haplotype age. An absolute distance matrix is then calculated for all pairwise comparisons of haplotypes. The probability of parsimony (Templeton et al. 1992) is calculated for pairwise differences until the probability exceeds 0.95. The number of mutational differences associated with the probability just before this 95% cut-off is then the maximum number of mutational connections between pairs of sequences justified by the parsimony criterion. Using these connections and the inferred missing intermediates, the program plots a haplotype network. A nested design is then drawn on top of the haplotype tree, using Templeton and Sing's algo-

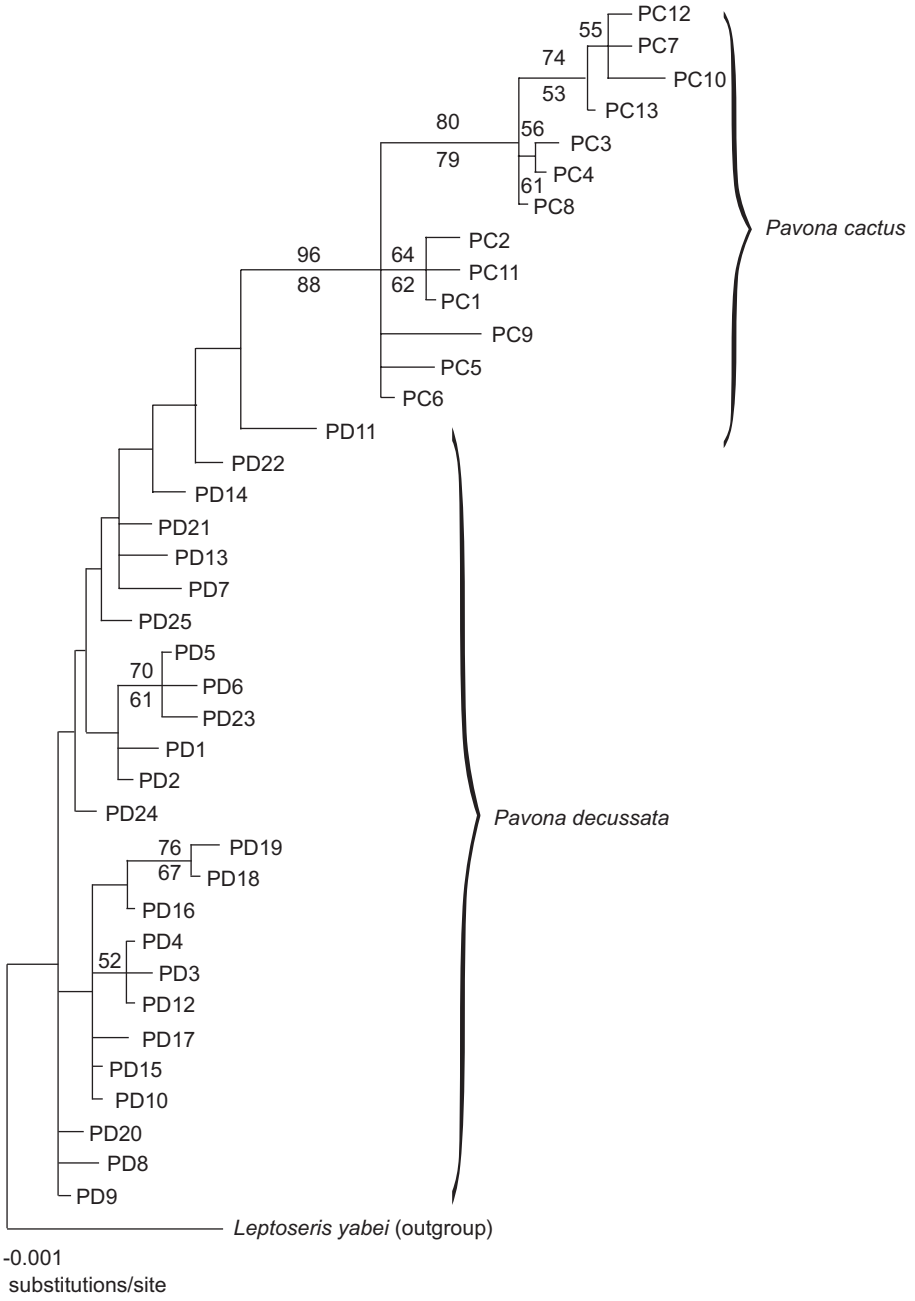

Fig. 3. Rooted Maximum-likelihood tree of *P. cactus* and *P. decussata* using the ITS region. Bootstrap values (1000 replicates) greater than 50% obtained from the NJ and MP trees are shown above the branches for the NJ tree and below the branches for the MP tree.

rithm (Templeton and Sing 1993). For that we need to nest haplotypes (0-step clades) that are in some sense evolutionarily adjacent into 1-step clades (branches of the evolutionary tree), nest adjacent 1-step clades into 2-step clades, and so forth, until finally all data nest into a single clade. To test for associations among clade categories and taxonomic categories, we used contingency table tests within each clade level (Templeton and Sing 1993). Given the small sample sizes, the asymptotic property of a chi-square distribution could not be assumed. Instead, we performed an exact test that uses the random permutation procedure of Roff and Bentzen (1989). In this procedure, a contingency chi-square statistic is calculated, and the probability of observing the exact test statistic or larger is generated using a random permutation procedure that maintains the marginals but simulates the null hypothesis of no association. The random permutation was implemented in Chiperm vers. 1.2 (Chiperm, together with the other programs by D. Posada, Modeltest, and TCS, are freely available at the web site at http://bioag.byu.edu/zool ogy/crandall_lab/programs.htm).

RESULTS

ITS sequence analysis

In total, 60 samples of each species were sequenced. The length of ITS-1 was 200 bp for *P. cactus* and 198 bp for *P. decussata*; the regions of 5.8S and ITS-2 were 158 and 190 bp, respectively, for both species. The length of ITS-1 was 192 bp, that of 5.8S was 158, and that of ITS-2 was 193 bp for the outgroup *Leptoseris yabei* (Pillai and Scheer, 1976). The sequence alignment is available from the senior author upon request.

Out of the ITS-1, 5.8S, ITS-2 region of the 120 sequences, 16 and 15 sites were polymorphic for *P. cactus* and *P. decussata*, respectively (Table 1). Both taxa had fixed differences at the 16th and 353rd positions. Position 356th was fixed in *P. decussata*, and only 1 sequence type of *P. cactus* showed a base substitution. All the mutations observed were substitutions except for 14 nucleotide deletions found in 5 samples of *P. decussata* at TAB and indels found at positions 195 and 196 in *P. decussata* (Table 1). The transition: transversion ratio was 10: 6 for the *P. cactus* population and 11: 4 for the *P. decussata* population. The average GC contents were 49.4% for *P. cactus* and 49.25% for *P. decussata* (Table 2). The levels of intraspecific variation differed between the ITS regions. ITS-1 was more variable than ITS-2 in both species. The average intraspecific variation was 5.8% and 5.25% in ITS-1 as compared to an average of 2.5% and 3.05% in ITS-2 for *P. cactus* and *P. decussata*, respectively. When the datasets were combined, average sequence variabilities were respectively 3.5% and 3.7% for *P. cactus* and *P. decussata* (Table 2). The *p* distance ranged from 0.257% to 2.880% in *P. cactus* and from 0.258% to 2.634% in *P. decussata*. However, the *p* distance between *P. cactus* and *P. decussata* ranged from 1.562% to 5.088%. The sequence divergence was higher between *L. yabei* and *P. cactus* (range, 3.788%-5.190%) than between *L. yabei* and *P. decussata* (range, 1.932%-3.529%). No sequence types were shared between species. *P. decussata* had higher haplotypic diversity (*h*) than *P. cactus*. Nucleotide diversity (π) was higher in *P. decussata* (0.0093 ± 0.0009, 0.0107 ± 0 .0014) than in *P. cactus* (0.0069 ± 0.0018, 0.0079 ± 0.0021) at BV and TAB, respectively.

Table 3. Nested exact contingency analysis of species with clades of the ITS. The nested design is given in figure 4. The standard contingency Chi-square statistic was calculated, and its exact significance was determined by 1000 random permutations that preserve the marginal values. The probability column refers to the frequency with which these randomly generated Chi-square statistics were greater than or equal to the observed Chi-square value

Step	Source clade or haplotype	Chi-square statistic	*p*
3-step clade			
Clade 3.1	Clades 2.1 and clade 2.4	7.00	0.117
5-step clades			
Clade 5.1	Clades 4.1 and Clade 4.2	33.87	< 0.001

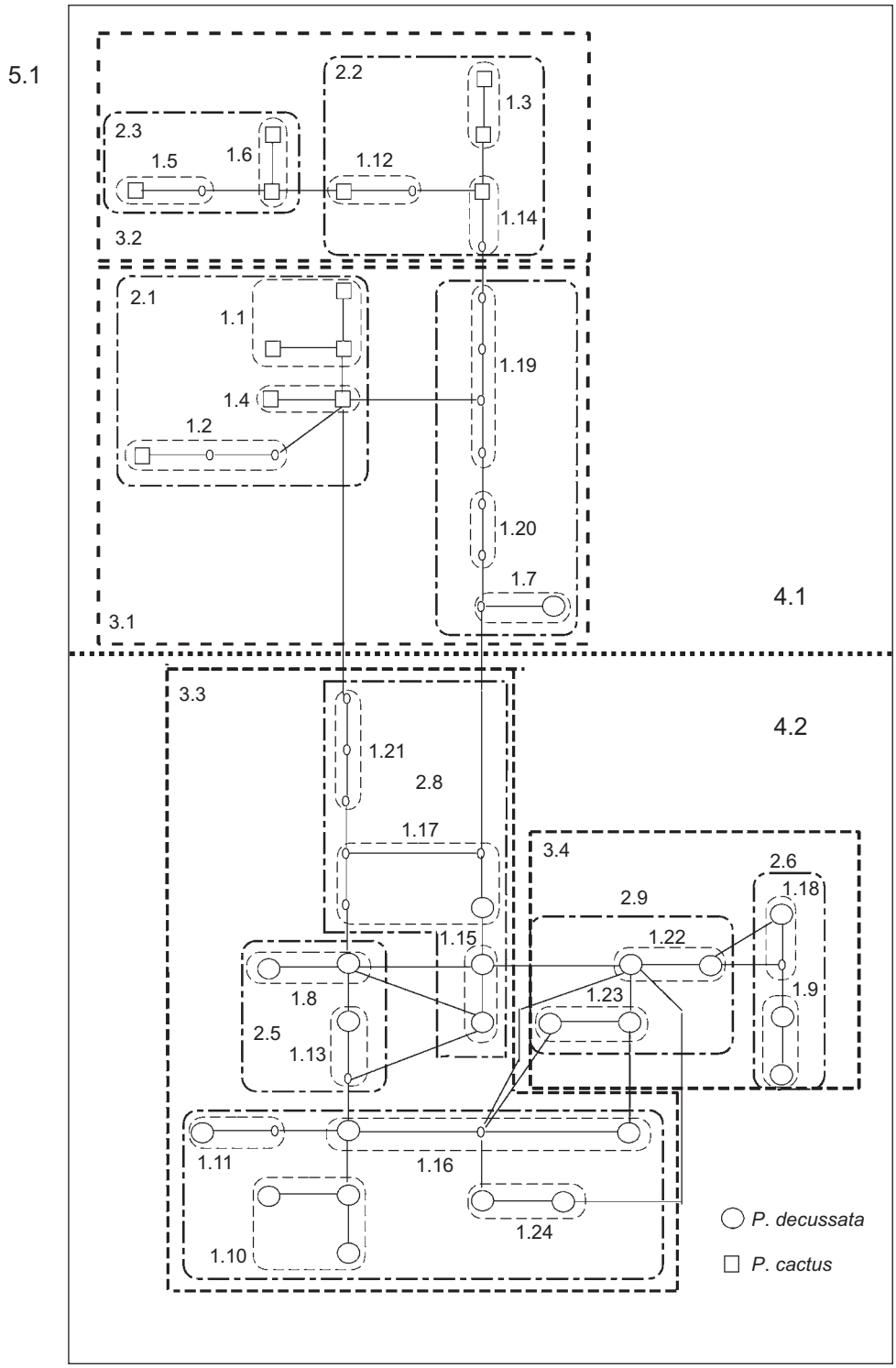

Fig. 4. Haplotype tree and nested clade design for the ITS rDNA of *P. cactus* and *P. decussata*. A zero (0) represents an interior node in the network that was not sampled. Rectangular thin-dashed-lined boxes with rounded corners indicate 1-step clades; thick-dashed-lined boxes with rounded corners indicate 2-step clades; thick-dashed-lined boxes with square-edged corners indicate 3-step clades; solid-bold-lined boxes with dashed lines on the interior represents 4-step clades, and the solid rectangle represents a 5-step clade.

Phylogenetic and nested clade analyses

In total, 13 sequence types of *P. cactus* and 25 of *P. decussata* were used for the phylogenetic analyses. We ran the analyses including and excluding the 5 sequences of *P. decussata* that contained large indels, and their inclusion or exclusion did not affect the topology. The NJ, MP, and ML analyses yielded trees of similar topologies. We present here the ML tree with bootstrap values of the NJ tree shown above the branches and those of the MP tree shown below the branches. Only bootstrap values that were higher than 50% are shown. Relationships between species were resolved by the phylogenetic analyses with *P. decussata* and *P. cactus* clustering into 2 distinct clades, with the *P. decussata* clade basal to the *P. cactus* clade (Fig. 3).

Figure 4 shows the nested clade design on top of the haplotype network from the NCA. At level 3.1, clade 1.7 consisting of 1 haplotype of *P. decussata* (haplotype PD11) is linked to PC6 of clade 1.4 by 5 intermediate haplotypes. The Chi-square statistics show clades 4.1 and 4.2 to significantly differ (χ^2 = 33.87, $p < 0.001$) (Table 3). Overall, the results indicate that *P. cactus* and *P. decussata* constitute statistically distinguishable lineages.

Population differentiation

Distribution and sequence type frequencies of both species at BV and TAB are shown in figure 2. *P. cactus* populations contained 8 sequence types at BV and 9 sequence types at TAB, 4 of which were shared between populations (sequence types 1, 2, 4, and 5). Sequence type 1 was most common. Populations at BV and TAB had 56.6% and 40% of sampled individuals with sequence type 1. *P. decussata* populations had 18 sequence types at BV and 14 sequence types at TAB, seven of which were shared between populations (sequence types 2, 4, 5, 6, 11, 13 and 18). Sequence type 6 was most common. At BV and TAB, 10% and 23.3% of the sampled individuals had sequence type 6. Haplotypic diversity did not significantly differ among populations, being 0.69 ± 0.08 and 0.77 ± 0.05 ($p > 0.05$) for *P. cactus* and 0.94 ± 0.02 and 0.90 ± 0.03 for *P. decussata* ($p > 0.05$).

DISCUSSION

Our results showed the ITS sequences of *P. cactus* and *P. decussata* to be homogenous with signature sequences that are species-specific. *P. cactus* and *P. decussata* had levels of intraspecific variation (3.3%-3.9%) in the ITS regions comparable to those reported for *Madracis* corals (3.3%-3.5%, Diekmann et al. 2001) and *Balanophyllia elegans* (2.7%, Beauchamp and Powers 1996). Other studies have reported either very low levels of intraspecific variability, for example, 0.39% in *Siderastrea* corals (Forsman et al. 2005), 1% in the closely related species *Montastrea annularis*, *M. franksi*, and *M. faveolata* (Lopez and Knowlton 1997), and 2% in *Heliofungia actiniformis* (Takabayashi et al. 1998), or very high levels of intraspecific divergence, for example, 29% in *Acropora valida* (Odorico and Miller 1997), 31% in *Stylophora pistillata* (Takabayashi et al. 1998), and about 59% in *A. aspera* (van Oppen et al. 2001). The high intra- and interspecific levels of sequence heterogeneity in *Acropora* have been suggested to result from introgressive hybridization (Hatta et al. 1999, van Oppen et al. 2000 2001) and more recently to the presence of pseudogenes (Marquez et al. 2003). Our phylogenetic analyses clearly separated *P. cactus* and *P. decussata* into 2 clades in the NJ, MP, and ML trees. In our NCA, within level 3.1, haplotype PD11 from clade 2.4 linked to PC6 of clade 2.1 via 5 missing haplotypes. The Chi-square statistics found no significant association between clades 2.1 and 2.4. However, at level 5.1, the Chi-square statistics showed clades 4.1 and 4.2 to significantly differ, suggesting *P. cactus* and *P. decussata* to be statistically distinct lineages.

It has been suggested that sympatric taxa maintain distinct species boundaries due to accumulation of some fixed genetic differences through evolutionary time as a result of intrinsic (genetic) or extrinsic (geographic) reproductive barriers (Avise and Ball 1990). However, in the absence of reproductive barriers, species may hybridize resulting in shared DNA sequences among species (Odorico and Miller 1997, Hatta et al. 1999, van Oppen et al. 2000 2001 2002, Diekmann et al. 2001, Fukami et al. 2003). The existence of fixed differences between *P. cactus* and *P. decussata* suggests that there are some intrinsic mechanisms that help keep these species distinct. Without sufficient biological data at the moment, it is hard to tell the determinants of their genetic isolation, although differential spawning times (e.g., Knowlton and Weigt 1997, Szmant et al. 1997, van Oppen et al. 2001, Fukami et al. 2003, Wolstenholme 2004), gamete incompatibility (e.g., Willis et al. 1997,

Hatta et al. 1999, Wolstenholme 2004), the presence of sperm attractants in eggs of conspecifics (Coll et al. 1994), or different modes of reproduction between the 2 species could be one or more of the factors that maintain the distinctness of these species despite being so intricately associated in the field.

This study also revealed that populations of *P. cactus* and *P. decussata* from BV and TAB may be genetically connected populations. Four of 13 sequence types were shared between populations of *P. cactus*, and seven of 25 sequence types were shared between populations of *P. decussata*. Moreover, haplotypic diversities did not significantly differ between populations of *P. cactus* (0.69 ± 0.08 and 0.77 ± 0.05, $p > 0.05$) and *P. decussata* (0.94 ± 0.02 and 0.90 ± 0.03, $p > 0.05$) at BV and TAB, respectively. Genetic connectivity between reefs in relatively close proximity was seen in previous studies using allozyme electrophoresis (e.g., Ayre and Hughes 2000, Ridgeway et al. 2001). For instance, reefs along the Maputaland coastline in Northern KwaZulu-Natal, South Africa, separated by less than 3 km and up to 70 km, were found to be genetically connected (Ridgeway et al. 2001).

In conclusion, *P. cactus* and *P. decussata* are not hybridizing, and their lineage sorting is nearly complete. The existence of fixed differences between these sympatric closely related taxa suggests that there are intrinsic factors which help maintain them as discrete species in the field. However, the determinants of their genetic isolation are still unknown. Clearly, there is a need to look into the reproductive biology of these taxa in detail; in addition to providing greater insights into their evolutionary trajectory, such information is necessary to better understand these species in light of their importance as reef builders and bleaching-resistant taxa in Mauritius. The preliminary results obtained from the present study suggest that there might be larval connectivity between the studied reefs, although there is a need to locally extend the geographic sampling before we can draw any conclusion as to whether all reefs are genetically interconnected. Such information will be important for reef management as current management plans are based on the assumption that the reefs are interconnected. Results from this study also showed the utility of the ITS regions in resolving species boundaries among sympatric species.

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