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NATURAL HYBRIDIZATION IN THE SEA URCHIN GENUS *PSEUDOBOLETIA* BETWEEN SPECIES WITHOUT APPARENT BARRIERS TO GAMETE RECOGNITION

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Marine species with high dispersal potential often have huge ranges andminimal population structure. Combined with the paucity of geographic barriers in the oceans, this pattern raises the question as to how speciation occurs in the sea. Over the past 20 years, evidence has accumulated that marine speciation is often linked to the evolution of gamete recognition proteins. Rapid evolution of gamete recognition proteins in gastropods, bivalves, and sea urchins is correlated with gamete incompatibility and contributes to the maintenance of species boundaries between sympatric congeners. Here, we present a counterexample to this general pattern. The sea urchins *Pseudoboletia indiana* and *P. maculata* have broad ranges that overlap in the Indian and Pacific oceans. Cytochrome oxidase I sequences indicated that these species are distinct, and their 7.3% divergence suggests that they diverged at least 2 mya. Despite this, we suspected hybridization between them based on the presence of morphologically intermediate individuals in sympatric populations at Sydney, Australia. We assessed the opportunity for hybridization between the two species and found that (1) individuals of the two species occur within a meter of each other in nature, (2) they have overlapping annual reproductive cycles, and (3) their gametes cross-fertilize readily in the laboratory and in the field. We genotyped individuals with intermediate morphology and confirmed that many were hybrids. Hybrids were fertile, and some female hybrids had egg sizes intermediate between the two parental species. Consistent with their high level of gamete compatibility, there is minimal divergence between *P. indiana* and *P. maculata* in the gamete recognition protein bindin, with a single fixed amino acid difference between the two species. *Pseudoboletia* thus provides a well-characterized exception to the idea that broadcast spawning marine species living in sympatry develop and maintain species boundaries through the divergence of gamete recognition proteins and the associated evolution of gamete incompatibility.

KEY WORDS: **Bindin, echinoid, gamete compatibility, hybridization, *Pseudoboletia*, speciation.**

Despite inhabiting a significantly smaller portion of the planet, species of macroscopic terrestrial animals outnumber their marine counterparts by an order of magnitude (Vermeij and Grosberg 2010). A variety of factors contribute to this pattern, one of which is the remarkable connectivity of marine environments on a global scale. A general lack of geographic barriers in the oceans allows marine species with high dispersal potential to have huge ranges and minimal population structure. This decreases the likelihood that populations become isolated and diverge from one another, a first step toward speciation. How, then, does speciation occur in the sea? Addressing this question, Palumbi (1992, 1994) suggested that transient isolation of populations, combined with the rapid evolution of gamete recognition proteins, could explain the evolution of reproductive isolation and speciation in marine species.

In the past 20 years, examples of rapid evolution of gamete recognition proteins and the associated evolution of reproductive isolation between species have been discovered. The best studied are lysin in abalones (reviewed by Kresge et al. 2001) and bindin in sea urchins (reviewed by Zigler 2008), although other examples are known in oysters (Moy et al. 2008; Springer et al. 2008), mussels (Riginos and MacDonald 2003; Riginos et al. 2006; Springer and Crespi 2007), and teguline snails (Hellberg and Vacquier 1999; Hellberg et al. 2000). The mechanisms causing rapid evolution of these molecules are not always clear, and may vary across molecules and organisms (Zigler 2008; Lessios 2012). As divergence of these molecules generally correlates with reproductive isolation between taxa (e.g., Zigler et al. 2005), it is likely that changes in these molecules played a role in the evolution of reproductive isolation and the formation of new species (reviewed in Lessios 2012). Nonetheless, reproductive isolation between sympatric taxa is often incomplete; in these cases, hybridization may occur.

Hybridization between species may result in the production of unfit hybrids (potentially leading to reinforcement of isolating reproductive barriers), the exchange of genetic material between species (introgression), the fusion of species, or the formation of a new species (through polyploid or homoploid speciation) (reviewed in Mallet 2005, 2007; Baack and Rieseberg 2007). Recent work has shown that hybridization is more common in animals than was previously appreciated (Mallet 2005, 2007). The increased appreciation for the evolutionary role of hybridization in animals rests largely on data from well-studied terrestrial groups (specifically, in Mallet 2005: butterflies, mammals, *Drosophila*, and birds); much less is known about hybridization in marine organisms. A review of hybridization in marine environments (Gardner, 1997) listed 95 cases of hybridization between marine animals, half of which involved vertebrates. Only three phyla of marine invertebrates were included: arthropods, mollusks, and echinoderms, although cnidarians were also discussed. Nearly all cases relied on morphological evidence alone; fewer than a quarter were confirmed by molecular data. Gardner (1997) called for a multidisciplinary approach to studies of hybridization in the sea, incorporating molecular information, reproductive ecology, and gamete compatibility.

Lessios (2007) emphasized the rarity of hybrids in echinoids (sea urchins and sand dollars) as evidence of the efficacy of reproductive isolation barriers between echinoid species. He noted that examples of hybridization that relied on morphology alone were unconvincing, and discussed two genera of echinoids where hybridization, at low frequencies, has been confirmed using genetic markers (Lessios and Pearse 1996; Geyer and Palumbi 2005). Recent population genetic studies in two other genera of echinoids found evidence of hybridization (Addison and Pogson 2009; Lessios et al. 2012), as have studies of two genera of sea stars (Kwast et al. 1990; Harper and Hart 2007), one genus of sea cucumbers (Uthicke et al. 2005), and one genus of brittle stars (Muths et al. 2010). Confirmed cases of natural hybridization in echinoderms are currently limited to these eight genera. In the present study, we examined the possibility of hybridization in the sea urchin genus *Pseudoboletia*.

Pseudoboletia is a genus of sea urchins with broad distribution. Two species, *P. indiana* and *P. maculata*, are found in the Indo-Pacific, and two subspecies are found in the Atlantic: *P. m. maculata*, and *P. m. atlantica* (Pawson, 1978). The genus is poorly known, particularly in the Atlantic, where it is generally found at greater than 20 m depth (Turner and Graham 2003). The adults of these species can only be distinguished by their color pattern, as the morphology of tests of *P. indiana* and *P. maculata* is virtually identical (Schultz 2005). *Pseudoboletia indiana* is white with white or pink, purple, or green tipped spines, whereas the test and spines of *P. maculata* are prominently patterned with brown. Shigei (1986), finding it impossible to separate the two species morphologically, synonymized them. In spite of this, recent taxonomic treatments (e.g., Rowe and Gates 1995; Turner and Graham 2003) list them as separate species. The two species have broadly overlapping distributions. *Pseudoboletia indiana* occurs from Madagascar to Hawaii and Easter Island and from Japan to Australia, and *P. maculata* occurs from Sri Lanka to the Philippines and Australia (Turner and Graham 2003). The affinities of the Atlantic taxa are uncertain; the tests of *P. m. maculata* are virtually identical to those of *P. indiana*. Turner and Graham (2003) suggested a need for a molecular evaluation of the genus.

We suspected hybridization between *P. indiana* and *P. maculata* based on the presence of individuals with intermediate coloration in sympatric populations at Sydney, Australia (Fig. 1). We first sequenced part of the mitochondrial cytochrome oxidase I (COI) gene to confirm the distinctiveness of the two taxa and to estimate their time since divergence from a common ancestor. We then examined the potential for hybridization by

Figure 1. Representative *Pseudoboletia maculata* (top), *P. indiana* (right), and intermediate (bottom and left) individuals from Green Point, Sydney, Australia.

characterizing their distribution in the field, annual reproductive cycles, cross-fertilization efficiencies in the laboratory and the field, and molecular divergence at the gamete recognition protein bindin. We confirmed the hybrid character of intermediate individuals by examining their COI and bindin genes, and, when possible, egg size. We show here that *P. maculata* and *P. indiana* are an exception to the general pattern that broadcast spawning marine species living in sympatry develop and maintain species boundaries through the evolution of gamete recognition proteins and the associated evolution of gamete incompatibility.

Materials and Methods

SAMPLES

Pseudoboletia maculata, *P. indiana*, and individuals with intermediate color patterns were collected at depths of 2–10 m in the boulder habitat at Green Point, Camp Cove, Sydney Harbour, Australia (33.8419°S, 151.2767°E) and transferred to the laboratory, where they were maintained in aquaria for as long as one week. Animals were collected in December 2002, 2003, and 2009 for fertilization experiments and genotyping, and every 2–3 months from January 2004 to November 2005 for study of the annual reproductive cycle. Additional specimens of *P. indiana* were collected at Easter Island on 1 January 1998. We also undertook reconnaissance of sites near Green Point, including the rubble/sand habitat at Camp Cove Beach (33.8429°S, 151.2807°E) to 15 m depth and the boulder habitat at the adjacent headland (33.8483°S, 151.2758°E) to 8 m depth to assess the local distribution of the *Pseudoboletia* species in SCUBA surveys taken over several years (2003–2008).

We placed each individual into one of five categories based on their test and spine coloration while alive (Fig. 1). Typical *P. maculata* individuals had five bands of brown on the spines and the underlying test that extend from the oral to the anal surface. Typical *P. indiana* individuals did not have any brown spots on their test and had spines that were white, pink, green, or some combination thereof (Miskelly 2002). The "*indiana*-like" individuals were similar to *P. indiana* but had small patches of brown test and/or spines. "Hybrid-like" individuals had extensive brown patterns and spines, but not as much or in as regular an array as in *P. maculata*. The "*maculata*-like" individuals were similar to *P. maculata* but the spine and test coloration was not complete.

Pseudoboletia individuals collected in December 1997 from Sao Tome in the eastern Atlantic were tentatively identified by D. Pawson (National Museum of Natural History) as *P. m. maculata*. Our molecular results (see below) indicate that the individuals from Sao Tome are distinct from both Pacific *Pseudoboletia* species and are more closely related to *P. indiana* than to *P. maculata*. Referring to the Sao Tome individuals as "*P. m. maculata*" would be confusing and phylogenetically misleading, so from this point forward we refer to the individuals from Sao Tome as "*P.* sp. Sao Tome."

MITOCHONDRIAL DNA SEQUENCING AND PHYLOGENETIC ANALYSIS

A fragment of the mitochondrial COI gene was amplified and sequenced using combinations of the forward primers COIa (5′-AGTATAAGCGTCTGGGTAGTC-3′) or CO1aPI (5′-CGAGTACCGTCGAGGCATTCC-3′), and reverse primers COIf (5′-CCTGCAGGAGGAGGAGAYCC-3′), CO1p (5′-GGTCACCCAGAAGTGTACAT-3′), or CO1fPI (5′-TGATTCTTTGGCCACCCAGAAG-3′), as described in Lessios et al. (1999). These primers amplified approximately 600 bp of the COI gene. Sequences were obtained from 10 *P. indiana* individuals (eight from Sydney and two from Easter Island), seven *P. maculata* individuals (all from Sydney), one individual with intermediate morphology (from Sydney), and five *P.* sp. Sao Tome individuals. No indels or stop codons were present and sequences were aligned by eye. The sequences have been deposited in GenBank (accession numbers JQ048553-JQ048575).

After excluding identical haplotypes (also excluding those that differed only by ambiguous bases or length), we used Modeltest (version 3.7; Posada and Crandall 1998) to identify the model that best described the evolution of the sequences (HKY [Hasegawa et al. 1985] with a Γ distribution of rates, as selected by the Akaike Information Criterion). This model was used for both Bayesian and distance-based phylogenetic analyses. We used MrBayes (version 3.1.2, Ronquist and Huelsenbeck 2003) to conduct Bayesian phylogenetic analyses. We calculated clade credibility values from 4000 trees by sampling every 1000th tree from two runs of 5,000,000 trees after discarding the first 3001 sampled

trees of each run. We used AWTY (Nylander et al. 2008) to confirm stationarity and convergence of the Bayesian analyses. We rooted our phylogenetic trees with sequences from *Lytechinus variegatus* (GenBank accession AY183277) and *L. euerces* (AY183196) (Zigler and Lessios 2004). *Lytechinus* and *Pseudoboletia* are members of the echinoid family Toxopneustidae.

We also conducted a distance-based neighbor-joining bootstrap analysis (1000 replicates) in PAUP* (version 4.0; Swofford 2001) using the parameters identified in Modeltest. We also used PAUP* to calculate the mean Kimura 2-parameter (K2P; Kimura 1980) genetic distance between haplotypes of the three taxa we sampled (*P. indiana*, *P. maculata*, and *P.* sp. Sao Tome).

FIELD OBSERVATIONS

We quantified the distribution of *Pseudoboletia* sea urchins at Green Point in two ways. First, the distance between individuals was determined using surveyor's tape while diving on SCUBA in December 2003 and January 2004. Around 30 specimens of each species were selected haphazardly and the distance and identity of the nearest neighbor (e.g., *P. indiana* or *P. maculata*) were recorded. Second, we ran four 50 × 1 m transects at 2–11 m depth while diving on SCUBA in December 2005 and recorded morphology (*P. indiana*, *P. maculata*, or intermediate) and the depth at which each individual was encountered. We compared the distribution of *P. maculata* and *P. indiana* individuals across the 2–11 m depth range by Mann–Whitney *U*-test.

ANNUAL REPRODUCTIVE CYCLE

Pseudoboletia indiana (test diameter 53–74 mm) and *P. maculata* (test diameter 53–80 mm) were collected from January 2004 to November 2005 in five samples taken over each year. On each occasion, 10 specimens of each species were collected and the gonads were fixed in Bouin's fixative and processed by routine methods for histological examination of gametogenesis. The gonads were embedded in paraffin wax, sectioned (7-μm thick), stained (haematoxylin and eosin) and viewed with a light microscope. Scoring of gonad gametogenic stages was based on the following stages: recovering/growing, mature, partly spawned, and spent. These stages are typical of those used to describe sea urchin gonad development (e.g., Byrne 1990) with merging of the recovering/growing stages. Briefly, recovering/growing gonads have proliferating gonia, developing gametes and may have a few advanced gametes; mature gonads are filled with fully formed eggs or sperm; partly spawned gonads have initiated spawning as indicated by reduced packing of gametes in the lumen, and spaces vacated by gametes; spent gonads are reduced in size, often lacking gametes or have relict-degenerating gametes. Due to our interest in the potential for synchrony of mature gametes of *P. indiana* and *P. maculata*, we focused on identification of the temporal pattern of maturation and spawning.

The spawning response of the two species was also monitored in spawning trials in specimens collected on 10 occasions between December 2003 and December 2005. On each occasion, 10–20 specimens were injected with about 0.5 mL 0.5M KCl, which induces spawning in gravid sea urchins.

EGG SIZE MEASUREMENTS

Spawned eggs were washed several times in filtered sea water (FSW). An aliquot of eggs was transferred to a microscope slide and covered with a cover slip supported by modeling clay at the corners to prevent compression of the eggs. Mean egg sizes were determined for 17 females (three *P. indiana*, nine with intermediate morphology, and five *P. maculata*) from a sample of 10 or 20 eggs per female. Only round eggs were considered, and the same calibrated eyepiece micrometer was used for all measurements. Egg size measurements showed little variation per female (mean standard deviation [SD] of all measurements per female was 2.6 μm).

FERTILIZATION EXPERIMENTS—LABORATORY

Five experimental crosses within and between *P. maculata* and *P. indiana* were performed. Individuals were used in a single cross, and each was crossed with both hetero- and conspecific animals at a range of sperm concentrations. Eggs were collected from the gonopores of females and subsequently washed several times in FSW. The eggs were then resuspended at a concentration of approximately 1000 eggs/mL. A total of 800 μl of this egg suspension were placed in each of 12 wells of a 24-well cell culture plate.

Sperm were collected "dry" from the gonopores of males. A series of five fivefold sperm dilutions in FSW was prepared, beginning with a 1:100 dilution of dry sperm. A total of 200 μl of each sperm dilution were added to the appropriate con- and heterospecific egg suspensions, and the culture plate was briefly swirled to mix the sperm and eggs. After 5 min, 3 mL of FSW were added to each well. After 10 min, 3 mL of FSW were removed from the top of each cell of the culture plate, leaving the settled eggs undisturbed, and then replaced with 3 mL of new FSW. The fertilized eggs were then allowed to sit at room temperature for at least 2 h, by which time cleavage had begun. One hundred eggs per well were then examined to determine if they had cleaved. Immature oocytes (evidenced by a large germinal vesicle) were not scored. Fertilization percentages from a total of 132 different combinations of egg and sperm were determined.

A 1:1000 dilution of dry sperm was preserved by the addition of paraformaldehyde. Fixed sperm samples were briefly mixed using a vortex mixer, and a 10-μl aliquot was transferred onto a hemacytometer. The number of sperm in each sample was counted twice in two separate aliquots, and the mean of the two counts was recorded.

FERTILIZATION KINETICS CALCULATIONS

To quantify levels of gametic compatibility within and between *P. maculata* and *P. indiana*, we calculated the linear regression of logit-transformed fertilization percentages against the log sperm concentration (after McCartney and Lessios 2002), as in Zigler et al. (2008). To arrive at a single F_{50} (the number of sperm/μl required to fertilize 50% of the eggs) and F_{90} (the number of sperm/μl required to fertilize 90% of the eggs) value for each of the four possible crosses (female × male: *P. maculata* × *P. maculata*, *P. maculata* × *P. indiana*, *P. indiana* × *P. maculata*, and *P. indiana* × *P. indiana*), we calculated a linear regression between sperm concentration and percent fertilization for all values tested for a particular class of crosses. We used the F_{90} values from these linear regressions to calculate the ratio of hetero-/conspecific fertilization percentages at the sperm concentration necessary to fertilize 90% of conspecific eggs, as in Zigler et al. (2005).

FERTILIZATION EXPERIMENTS IN THE FIELD

We examined fertilization success within and between *P. indiana* and *P. maculata* under natural conditions at Green Point in December 2005. Trials were conducted using SCUBA in 1.5–2 m of water depth on a sandy bottom during high tide. Adult sea urchins were collected from a shallow rocky area and held until needed away from the experimental area. Trials ($n = 4$ for each species) consisted of a single male sea urchin (*P. indiana* or *P. maculata*) paired with two females, one of each species. Animals were spawned at the surface by injection with 0.5M KCl to identify their sex. Each female was immediately placed in an individual plastic container with 70-μm mesh panels on each end of the container, allowing sperm to enter but retaining the eggs. Spawning males were placed approximately 0.25 m upstream (as determined by the release of a small amount of fluorescein dye) of the containers with the two spawning females. The pairs of spawning females were exposed to a spawning male for 10 min, after which females and shed eggs in their mesh-sided boxes were placed in closed plastic boxes and brought to the surface. After 10 min, eggs were collected from each container into 50-mL conical plastic tubes. Eggs were fixed at a final volume of 1% paraformaldehyde/SW, and counts of the number of eggs with a fertilization envelope/total number of eggs were done in the laboratory. Experimental males were removed from the sea after each experiment, and no other spawning males were located within 10 m of an experimental trial.

BINDIN DNA SEQUENCING AND MOLECULAR EVOLUTION ANALYSIS

Bindin sequences from testis mRNA of *P. maculata* were amplified, cloned, and sequenced using a RACE protocol as described in Zigler and Lessios (2003a). From those sequences, we developed primers PsF1 (5′-TTTTCGGACGATTCAGAAAGAGG-3′) and PsR6 (5′-GTACTGATAGTCGTTTCGCCCTC-3′), which amplified the complete mature bindin molecule, including the intron, from genomic DNA of *P. maculata* individuals. We were unable to amplify through the intron of *P. indiana* or *P.* sp. Sao Tome individuals, so for these taxa we amplified bindin in two parts: the 5′ exon (using primers PsF1 and PSGETTISTrev, 5′-GGTACTGATAGTCGTTTCGCC-3′), and approximately 500 bp at the 3′ end of the intron and the 3′ exon (using primers PSseqf1 5′-CATTTCGGTGGCTAAATTGCCC-3′ and PSRX 5′-TTAGCCTTGAAAATAACCCTGATT-3′). We cloned the DNA and sequenced 3–5 colonies (mean = 4.7) per amplification from each individual. Individual clones were considered to represent the same allele if they differed by no more than one nucleotide. Clones that differed by two or more nucleotides were considered to represent distinct alleles. In this manner, we identified nine unique full-length bindin alleles from five *P. maculata* individuals, as well as a single full-length bindin allele from an individual with intermediate morphology. We identified 12 5′ bindin exon alleles and 11 3′ exon alleles from eight *P. indiana* individuals. We also identified four 5′ bindin exon alleles from three *P.* sp. Sao Tome individuals and four 3′ bindin exon alleles from four *P.* sp. Sao Tome individuals. The sequences have been deposited in GenBank (accession numbers JQ236576-JQ236616).

BINDIN MOLECULAR ANALYSIS

Bindin sequences were aligned by eye. We tallied the number of fixed nonsynonymous differences between the three taxa (*P. indiana*, *P. maculata*, and *P.* sp. Sao Tome). We calculated bindin divergence at nonsynonymous and synonymous sites (d_N and d_S) as in Zigler et al. (2005), with the following modification: because we amplified *P. indiana* and *P.* sp. Sao Tome bindin in two pieces, we calculated d_N and d_S for the 5′ and 3′ bindin exons separately, and then calculated a weighted average (based on the size of each region) from those values.

IDENTIFYING COI AND BINDIN GENOTYPES BY POLYMERASE CHAIN REACTION AND RESTRICTION ANALYSIS

To determine whether individuals had *P. maculata* or *P. indiana* mtDNA without sequencing, we amplified 458 bp of the COI gene using the primers COIfPseudo (5′-CATGTTATTGCACACTACTCAGG-3′) and COIaPseudo (5′-AATGGGTGTAGGTTGTATCCTG-3′) and then digested the product with the restriction enzyme ApaI (New England BioLabs Inc., #R0114). A single restriction site for this enzyme that cleaved the product into fragments of 101 and 357 bp was present in all of the previously sequenced *P. maculata* individuals and in none of the *P. indiana* individuals. After digestion, the fragments were resolved and sized on a 1.2% agarose gel.

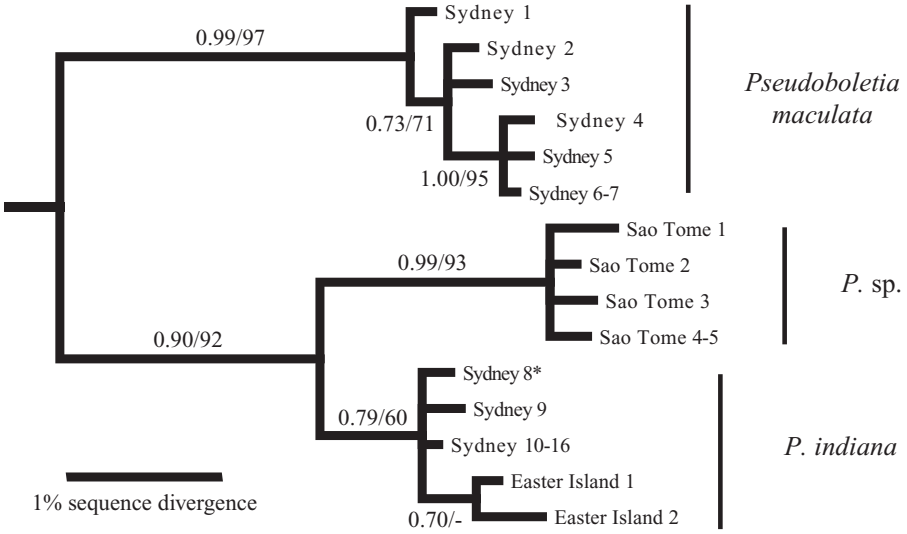

Figure 2. Bayesian majority rule consensus tree based on cytochrome oxidase I sequences. The tree is rooted with sequences of *Lytechinus* (not shown). Bayesian clade credibility values (from 4000 trees) are indicated before neighbor-joining bootstrap percentages (from 1000 replicates) on branches. One individual with intermediate morphology is indicated with an asterisk.

To determine the bindin genotype of individuals, we used the primers PsF2 (5′-GCATTTTCACACAGAAGTTGGGC-3′) and PsR2 (5′-CCTGAATGAGGCAGACAACGTG-3′), which flank a 9 bp indel in the 3′ region of the bindin intron. Relative to the outgroup and *P. indiana*, there was a deletion in all of the alleles from *P. maculata* we sequenced. These primers amplified a 207 bp fragment from *P. indiana* alleles and a 198 bp fragment in *P. maculata* alleles. This size difference was resolved on 3% MetaPhor agarose (Lonza, no. 50181) gels. Individuals genotyped by this method were scored as either homozygous or heterozygous for the *P. maculata* or *P. indiana* bindin alleles.

Results

PHYLOGENETIC ANALYSES

We recovered individuals with *P. maculata* and *P. indiana* morphology from Australia, as well as *P.* sp. from Sao Tome, as monophyletic clades in the COI genealogy (Fig. 2). We found support for a sister-group relationship between *P. indiana* and *P.* sp. Sao Tome; members of these two groups differed by a mean K2P genetic distance of 3.2%. *Pseudoboletia maculata* was the sister taxon to the *P. indiana*/*P.* sp. Sao Tome clade. *Pseudoboletia maculata* haplotypes differed by a mean K2P distance of 7.3% from those of the *P. indiana*/*P.* sp. Sao Tome clade. *Pseudoboletia indiana* from Easter Island grouped with *P. indiana* from Sydney. The bindin gene genealogy was similar, with strong support for *P. maculata* and *P. indiana*/*P.* sp. Sao Tome clades (not shown). Bindin alleles from *P. indiana* and *P.* sp. Sao Tome did not form distinct clades as there were no fixed differences between the two taxa.

DISTRIBUTION OF *PSEUDOBOLETIA* IN THE FIELD

Pseudoboletia maculata, *P. indiana*, and individuals with intermediate spine and test coloration were examined at Green Point, Sydney. Across our transects, we encountered *P. maculata* (33%), *P. indiana* (52%), and intermediate individuals (15%). The distribution of *P. indiana* individuals (6.0 ± 2.4 m, mean $\pm$ SD, $N = 48$) was slightly shallower than that of *P. maculata* individuals (7.4 ± 1.4 m, $N = 31$; Mann–Whitney U-test, $P = 0.002$). *Pseudoboletia* individuals were closest to conspecific individuals 73% of the time (46 of 63 cases) at a mean distance of 0.83 ± 0.93 m (mean $\pm$ SD). *Pseudoboletia* individuals were closest to heterospecific individuals 27% of the time (17 of 63 cases) at a mean distance of 1.04 ± 0.65 m. Heterospecific nearest neighbors were as close as 0.35 m away from each other.

Observations of the benthos at Camp Cove Beach revealed the presence of abundant *P. indiana* down to 15 m in the rubble/sand habitat, but *P. maculata* was not present. Both species were present in the boulder habitat at the adjacent headland (to 8 m depth), but *P. maculata* was not abundant.

ANNUAL REPRODUCTIVE CYCLE

For both *P. maculata* and *P. indiana*, examination of gonad histology revealed the gonad stages were not sharply defined in time due to the prolonged pattern of development of the gonial layer and maturation of gametes. For instance, partly spawned animals often showed signs of recovery of the germinal layer without going through a spent stage.

Pseudoboletia maculata had mature or partly spawned gonads with abundant fertile gametes from January to May. This indicated that this species has a prolonged, potentially six-month

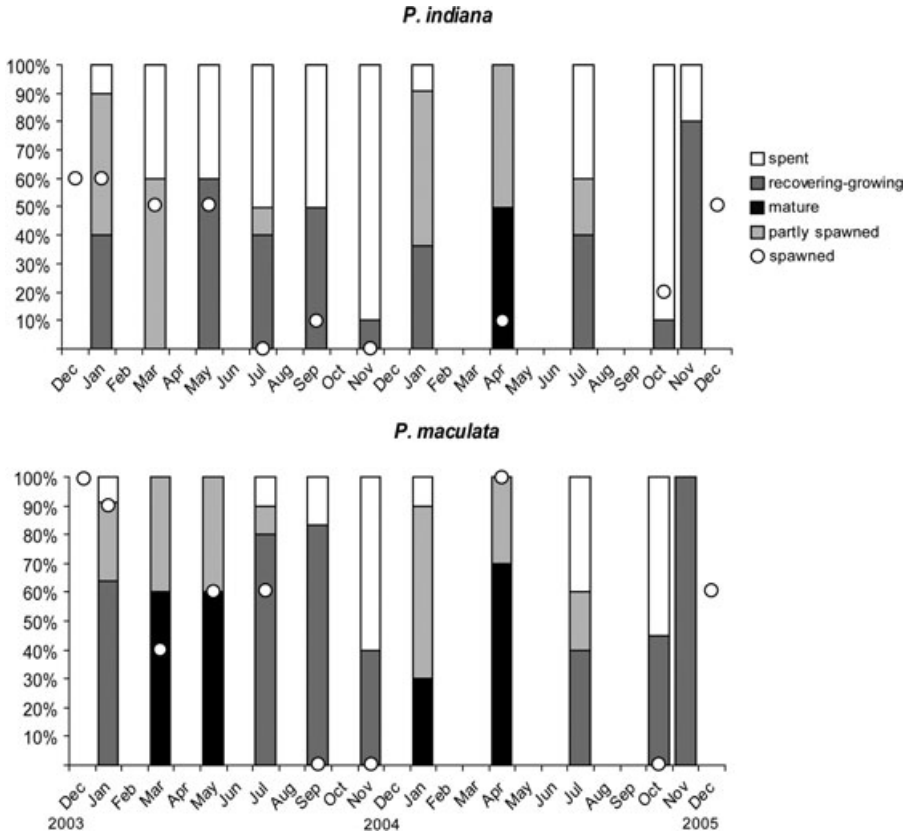

Figure 3. **Frequency of four categories of reproductive condition in gonads of *Pseudoboletia indiana* and *P. maculata* over a two-year period. Gonads were classified into one of four categories by histological methods. On 10 occasions, spawning frequency was determined from a sample of 10–20 individuals after KCl injection (indicated by empty circles).**

spawning season from December/January to May/June (Fig. 3). Consistent with this, spawning was induced in specimens injected with 0.5M KCl solution in samples collected December to July (Fig. 3). The highest incidence of mature and partly spawned *P. maculata* was from March to May indicating that this is the peak breeding period for this species. This may be when spawning in the field is most intense, as also indicated by the presence of spent individuals with reduced gonads in July. By July, some *P. maculata* had renewed gametogenesis in recovering/growing stage gonads.

For *P. indiana*, mature and partly spawned gonads were present from January to April (Fig. 3). This species has a three- to four-month spawning period December/January to March/April. By May, the gonads were spent or in the recovering/growing condition. Spawning was induced in injected specimens in samples collected December to May.

Gonad histology and the spawning response indicated that mature gametes of *P. indiana* and *P. maculata* are both present in the summer months from December to May. Thus, it appears that the spawning activity of the two species potentially overlaps for six months (Fig. 3).

Table 1. **Summary of fertilization results for *Pseudoboletia indiana* and *P. maculata*.**

Female	Male	N	R^2	$F_{(reg)}$	F_{50}	F_{90}
P. indiana	*P. indiana*	21	0.714	47.57*	59	676
P. indiana	*P. maculata*	26	0.776	83.06*	23	357
P. maculata	*P. indiana*	27	0.737	70.10*	104	1138
P. maculata	*P. maculata*	30	0.848	155.82*	48	578

n = number of sperm concentrations tested, R^2 = the coefficient of determination between log sperm concentration and logit-transformed percent fertilization, F(reg) = significance value of the regression between log sperm concentration and logit-transformed percent fertilization: *$P < 0.000001$. F_{50} and F_{90} indicate the number of sperm/μl required to fertilize 50% and 90% of eggs, respectively.

FERTILIZATION EXPERIMENTS

Intraspecific F_{50} values for *P. indiana* and *P. maculata* were similar, at 59 and 48 sperm/μl, respectively (Table 1). The two species cross-fertilized readily (Fig. 4). Indeed, eggs of *P. indiana* required less sperm of *P. maculata* for fertilization than sperm of their own species. The F_{50} value for crosses between *P. indiana*

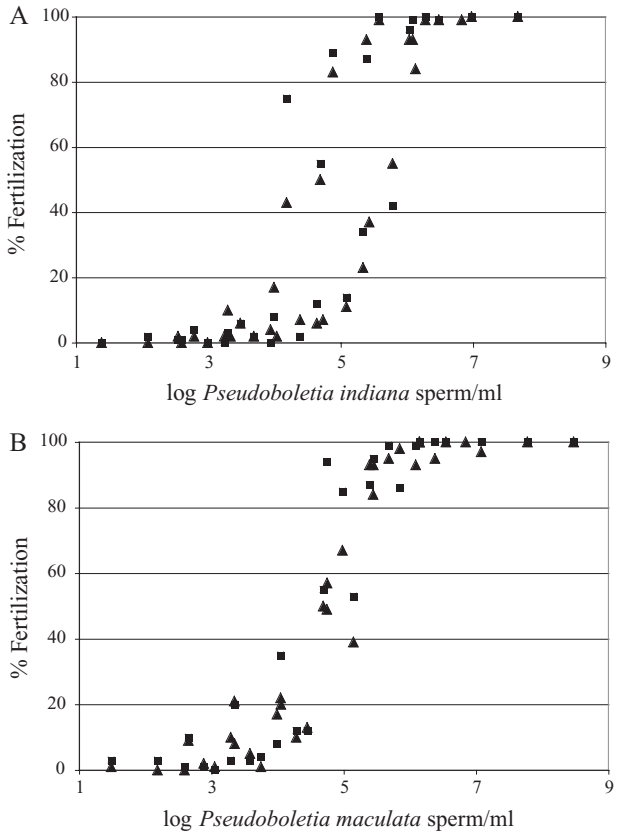

Figure 4. Results of fertilization experiments with *Pseudoboletia indiana* and *P. maculata*. Percent fertilization of *P. indiana* and *P. maculata* eggs by *P. indiana* sperm (top panel). Percent fertilization of *P. indiana* and *P. maculata* eggs by *P. maculata* sperm (bottom panel). In both panels, *P. maculata* eggs are indicated by triangles and *P. indiana* eggs are indicated by squares.

sperm and *P. maculata* eggs was 104 sperm/μl, and that for the reciprocal cross was 23 sperm/μl (Table 1). F_{50} values for con- and heterospecific crosses of *P. maculata* and *P. indiana* gametes were similar to those reported for conspecific crosses of other echinoderms (Harper and Hart 2005; Zigler et al. 2008).

We performed a fertilization test in the sea to confirm cross-species fertilization under more natural conditions than in the laboratory. Fertilization in our field experiment was low (3.7% of 2882 eggs examined). However, we found that heterospecific fertilization took place, showing that hybridization is possible not only in the laboratory, but also in the waters where the natural hybrids occur (in conspecific crosses, 10 of 1435 eggs were fertilized; in heterospecific crosses, 97 of 1447 eggs were fertilized).

EVOLUTION OF BINDIN

As in other sea urchins, *Pseudoboletia* bindin is translated from two exons. The second exon begins with a conserved region of approximately 50 amino acids that is involved in sperm-egg fusion. Regions 5′ and 3′ of the conserved region mediate sperm-egg attachment (reviewed in Zigler 2008). In *Pseudoboletia*, as in other sea urchins of the family Toxopneustidae, glycine-rich repeats are present both 5′ and 3′ of the conserved region (Zigler and Lessios 2003b, 2004; Fig. 5).

Bindin alleles were distinct between *P. maculata* and *P. indiana*, but there was minimal divergence of the amino acid sequence of bindin between the two species. Between *P. indiana* and *P. maculata*, we found nine fixed nucleotide differences. Seven of these differences did not influence the protein sequence; there were six fixed differences (including a 9 bp indel) in the intron and one silent difference in the second exon. There were two fixed differences that affected the protein sequence: a single amino acid difference in the first exon, and a small indel in the second exon (Fig. 5). The *P. indiana* consensus sequence also differs from the *P. maculata* consensus sequence by three unfixed amino acid differences (Fig. 5). There was intraspecific variation in the glycine-rich regions of *Pseudoboletia* as large as eight residues in the 5′ repeat region and three residues in the 3′ repeat region. d_N between *P. indiana* and *P. maculata* was 0.006, whereas d_S was 0.024. There were no fixed amino acid or indel differences between *P. indiana* and *P.* sp. Sao Tome (Fig. 5).

EVIDENCE FOR HYBRIDIZATION IN *PSEUDOBOLETIA*

We characterized 53 *Pseudoboletia* individuals from Green Point, Sydney, in terms of test and spine coloration, egg size (when possible), COI haplotype, and bindin genotype (Table 2). Among 25 individuals with *P. indiana* morphology, 24 had *P. indiana* COI haplotypes and bindin alleles. One individual with *P. indiana* morphology had *P. maculata* mtDNA and *P. indiana* bindin alleles (Table 2). Given this genotype, this individual is an F_2 or later generation hybrid. *Pseudoboletia indiana* females had an egg diameter of 91 ± 1 μm (mean ± SD). Among 10 individuals with *P. maculata* morphology, we identified only *P. maculata* COI haplotypes and bindin alleles. *Pseudoboletia maculata* females had an egg diameter of 105 ± 2 μm. Egg size data from individuals with *P. indiana* and *P. maculata* morphologies were compared with a general linear model in which size of eggs from each sea urchin were nested within each morph. Egg size of these two morphs was marginally significantly different ($F = 2.180$, df = 6, 83, $P = 0.053$).

Of 18 individuals with intermediate spine and test coloration ("*indiana*-like," "hybrid-like," or "*maculata*-like"; Table 2), eight were identified as hybrids, showing a mix of *P. indiana* and *P. maculata* COI haplotypes and bindin alleles. Three of these eight individuals were fertile females, with a range of egg sizes (94, 97, and 104 μm; Table 2). Two additional individuals with intermediate phenotypes had COI and bindin markers that corresponded to one species, but had egg sizes that were intermediate for the two species (95 and 100 μm; Table 2). Individuals with "*indiana*-like" morphology were genetically and gametically more similar

```
                1                                                                                80
P. maculata     YGNMNYPPPMNQQMGGGGYPGQQPQQNYAPQAMGGPVGGGGSMAGPIGGPFGGPVGGPVSGPVGGPVGGPIGGGGGGAAG
P. indiana      ???????.....................................................G...................
P. sp. Sao Tome ???????.....................................................G...................

               81                                                                              165
P. m.           GGGLQDYGEDSEVSDDDLSVDINDYSAAEVSEGETTISTKVMHDIKAVLGATKIDLPVDINDPYDLGLLLRHLRHHSNLLANIGD
P. i.           ............................I..????????.....................................................
P. sp.          ............................I..????????.....................................................

               166                                                                              251
P. m.           PEVREQVLTAMQEEEEEEEEDAANGVLNNLNANSPGQGGFGSTQGGMFGGAGGGGGGGGGGGGMMNNQAMGGQPAGNAYNQGYFQG
P. i.           ............................M.................................--..S.............???????
P. sp.          ............................M.................................--..S.............???????
```

Figure 5. Consensus mature bindin amino acid sequences from *Pseudoboletia*. Sequences begin with the first amino acid after the presumed cleavage site from preprobindin. The conserved core region is underlined and the glycine-rich repeat regions 5′ and 3′ of the conserved region are in bold. Amino acids identical to the first sequence are indicated by a period; gaps are marked with dashes; and missing data indicated with a question mark.

Table 2. Mitochondrial genotype, bindin genotype, and egg diameter of *Pseudoboletia* individuals with various color patterns as described in Methods. When only one bindin allele is indicated, we identified only one allele after cloning and sequencing. All individuals collected at Green Point, Sydney, Australia.

Color pattern	Mean egg diameter (μm)	mtDNA	Bindin allele 1	Bindin allele 2
Typical color pattern and genotype				
indiana (*n*=24)	91±1 (mean±SD, *n*=6)	*indiana*	*indiana*	*indiana*
maculata (*n*=10)	105±2 (mean±SD, *n*=5)	*maculata*	*maculata*	*maculata*
Typical color pattern and hybrid genotype				
indiana		*maculata*	*indiana*	*indiana*
Intermediate color pattern				
indiana-like	90	*indiana*	*indiana*	*indiana*
indiana-like	90	*indiana*	*indiana*	*indiana*
indiana-like		*indiana*	*indiana*	*indiana*
indiana-like	91	*indiana*	*indiana*	*indiana*
indiana-like	91	*indiana*	*indiana*	*indiana*
indiana-like		*indiana*	*indiana*	*indiana*
indiana-like	95	*indiana*	*indiana*	*indiana*
indiana-like		*indiana*	*indiana*	*maculata*
indiana-like	94	*indiana*	*indiana*	*maculata*
indiana-like		*indiana*	*indiana*	*maculata*
indiana-like		*indiana*	*indiana*	*maculata*
Hybrid-like		*indiana*		*maculata*
Hybrid-like	97	*maculata*	*indiana*	*maculata*
Hybrid-like	104	*indiana*	*indiana*	*maculata*
Hybrid-like		*indiana*	*indiana*	*maculata*
maculata-like	100	*maculata*	*maculata*	*maculata*
maculata-like		*maculata*	*maculata*	*maculata*
maculata-like		*maculata*	*maculata*	*maculata*

to *P. indiana*, whereas those with "hybrid-like" or "*maculata*-like" morphologies were genetically and gametically more similar to *P. maculata* (Table 2).

Discussion

Pseudoboletia maculata and *P. indiana* have broadly overlapping ranges in the Pacific and Indian oceans. They can be found side-by-side in nature and their annual reproductive periods overlap. They have nearly identical bindin genes and compatible gametes. Hybrids between the two species are viable and fertile. Despite all of this, *P. maculata* and *P. indiana* remain distinct species, showing consistent differences in color pattern, egg size, mtDNA, and nuclear DNA. It is unclear what allowed these species to form in the first place, and what has kept them from merging.

Contributing to the mystery is the fact that *P. maculata* and *P. indiana* are not young species. The mean rate of K2P COI divergence in eight genera of echinoids separated by the Isthmus

of Panama is 3.5%/my (Lessios 2008). Assuming that *Pseudoboletia* COI diverged at this rate, we estimate that *P. indiana* and *P. maculata* diverged around 2 mya. The actual divergence time between the two species could be greater if mtDNA introgression occurred at some point in the past. Thus, these species are 2 my old at a minimum.

The minimal bindin divergence between these species is consistent with the high level of gametic compatibility observed. With only one fixed amino acid difference and an overall d_N between species of 0.006, we predicted and observed highly compatible gametes, as has been found in other comparisons between sea urchin species with interspecific bindin $d_N < 0.010$ (Zigler et al. 2005). Similarly low bindin divergence (and high levels of gamete compatibility between species) has been observed in the sea urchin genus *Arbacia*, which consists of six species that are almost completely allopatrically distributed from one another (Metz et al. 1998; Lessios et al. 2012). Similar to our observations here, Lessios et al. (2012) recently found molecular evidence of hybridization between *A. spatuligera* and *A. dufresnei* on the coast of Chile, one of the few places where more than one species of *Arbacia* can be found. Our results from *Pseudoboletia* contrast with various comparisons from the sea urchin genera *Echinometra* and *Strongylocentrotus*, where species with comparable levels of COI divergence had greater bindin divergence and lower gamete compatibility (Zigler et al. 2005; Lessios 2007).

Some morphologically intermediate specimens of *Pseudoboletia* turned out to be hybrids. Of 35 individuals with spine and test coloration typical of one species or the other, 34 had COI haplotypes, bindin alleles, and egg sizes consistent with their morphology (Table 2). Of 18 individuals with intermediate morphologies, 10 had hybrid genotypes and/or intermediate egg sizes (Table 2). With only a mitochondrial marker and a single nuclear marker our power to identify hybrid individuals was limited, and it is likely that some individuals we surveyed were hybrids that we did not detect. Nonetheless, hybridization seems to be widespread in this population. The presence of mtDNA from both species in individuals that had bindin alleles from both species indicated that these species are hybridizing and/or backcrossing in both directions. Our identification of an individual with *P. indiana* morphology and *P. indiana* bindin alleles but *P. maculata* COI indicates that backcrossing (or the formation of F2 hybrids) has occurred. Further, the morphological diversity observed in individuals with intermediate morphology suggests that backcrossing is common, but without analyzing other nuclear markers we cannot confirm this possibility. However, the presence of distinct morphologies in *P. maculata* and *P. indiana* individuals and a general lack of heterospecific bindin alleles in these individuals indicates that hybridization has not significantly homogenized the genomes of these species.

Given that the two species of *Pseudoboletia* cross-fertilize easily and form fertile hybrids, what reproductive barriers keep them from merging? Following the example of Ramsey et al. (2003), we can consider the components of reproductive isolation between the two species. The species are ecogeographically isolated on two scales. First, the ranges of the two species are only partially overlapping, with the range of *P. indiana* extending both further west (to Madagascar) and further east (to Hawaii and Easter Island) than that of *P. maculata* (Miskelly 2002). Second, at Green Point where both species were present, they exhibited partial habitat isolation. Across our shallow transects *P. maculata* were significantly deeper, but the difference in mean depths (*P. indiana* 6.0 m, *P. maculata* 7.4 m) was not large. Although the species were at slightly different depths at Green Point, this may not be a general feature. At the adjacent beach, *P. indiana* is abundant down to 15 m depth, but *P. maculata* was not present. Further sampling at more sites and at a greater range of depths is required to clarify the degree of habitat isolation between these species.

Although individuals of the two species can be found within 1 m of each other, they are generally found closer to conspecifics, which may contribute to increased conspecific fertilization. The annual spawning periods of the two species overlap substantially (Fig. 3), but we do not know anything about their spawning behavior or spawning cues. Even with overlapping annual spawning periods, sympatric marine species can be reproductively isolated by spawning at different times of the day (e.g., Levitan et al. 2004). Postfertilization, we know nothing about the fitness of hybrid larvae, juveniles, or adults, other than that at least some hybrid individuals grow into fertile adults. Acknowledging our lack of information about spawning behavior and larval ecology, we have not identified any single barrier to hybridization between these species, so it may be that multiple components of reproductive isolation contribute to keeping these species distinct.

A limitation of our study is that we examined the distribution and annual reproductive cycle of these species at a single location on the southern edge of the broad range of both species. There are regional differences in the frequency of hybridization in some widespread marine taxa (e.g., Fukami et al. 2004; Combosch et al. 2008), so our observations at Sydney may not apply to other areas. We have no information about habitat preferences of *Pseudoboletia* species in other regions, nor about how their annual reproductive cycles vary across the Pacific and Indian oceans. Nonetheless, the low levels of bindin divergence observed across *Pseudoboletia* suggest that hybrids could form wherever the two species are found together, and there is the potential for hybrid larvae to disperse widely in the plankton. There are no previous studies suggesting hybridization in *Pseudoboletia*. However, it is interesting that Shigei (1986) reported "dark grayish patches" on the tests of *P. indiana* specimens from Japan, raising the possibility

Table 3. Examples of natural hybridization in echinoderms.

Family and genus	Species A	Species B	COI divergence[1]	Hybrid frequency[3]	Evidence?[4]	Range overlap	Gamete compatibility[5]		Hybrid fertility?	Backcrosses?	References[7]
							A × B	B × A			
Asteroidea											
Asterias	*forbesii*	*rubens*	11.4%	9.4%	m, mt	Northwest Atlantic	0.66	0.92	Yes	Yes	7, 8, 9, 24
Leptasterias	*aequalis*	*hexactis*	3.4%	2.3%	a, mt	Northeast Pacific					4, 5, 10
Echinoidea											
Arbacia	*spatuligera*	*dufresni*	6.9%		m, mt, n	Southeast Pacific			Yes	Yes	14
Diadema	*savignyi*	*setosum*	16.0%	<1.0%	m, a	Indian and west Pacific			Yes	Yes	12, 13
	paucispinum	*savignyi*	3.8%		m, a	Okinawa					12, 13
Echinometra	*oblonga*	sp. C	3.9%	1.4%	mt, n	West Pacific	0.96	0.88	Yes		6, 11, 22
	mathaei	*oblonga*	2.6%	1.0%	m, mt, n	West and central Pacific	0.01	0.34	Yes		11, 18, 19, 22
	mathaei	sp. C	3.7%	1.0%	m, mt	West Pacific	0.00	0.07			11, 18, 19, 22
Pseudoboletia	*indiana*	*maculata*	6.7%	8.3%	m, mt, n	Indian and Pacific	1.00	0.92	Yes	Yes	26
Strongylocentrotus	*droebachiensis*	*pallidus*	3.8%	3.0%	m, mt, n	Holarctic	0.82	0.07	Yes	Yes	1, 2, 3, 21
	droebachiensis	*purpuratus*	7.2%	1.0%	m, n	Northeast Pacific	0.75	0.00	Yes	Yes	2, 3, 15
Holothuroidea											
Holothuria	*scabra*	*lessoni*	1.9%[2]	5.8%	m, a, mt	Indian and west Pacific			Yes[6]	No	23, 25
Ophiuroidea											
Acronida	*brachiata*	*spatulispina*	19.6%	2.6%	m, a, mt	Northeast Atlantic					16, 17, 20

[1]Mitochondrial cytochrome oxidase I (COI) Kimura 2-parameter divergence (Kimura 1980).

[2]16S divergence (Uthicke et al. 2005).

[3]In studied population.

[4]m = morphology, a = allozymes, mt = mitochondrial DNA, n = nuclear DNA.

[5]Female listed first, after Zigler et al. (2005) and Lessios (2007).

[6]Gonads develop (Uthicke et al. 2005).

[7]References: 1 = Addison and Hart (2005); 2 = Addison and Pogson (2009); 3 = Biermann et al. (2003); 4 = Foltz (1997); 5 = Foltz et al. (2008); 6 = Geyer and Palumbi (2005); 7 = Harper et al. (2007); 8 = Harper and Hart (2005); 9 = Harper and Hart (2007); 10 = Hrincevich et al. (2000); 11 = Landry et al. (2003); 12 = Lessios et al. (2001), 13 = Lessios and Pearse (1996); 14 = Lessios et al. (2012); 15 = Levitan (2002); 16 = Muths et al. (2006); 17 = Muths et al. (2010); 18 = Palumbi (2009); 19 = Palumbi et al. (1997); 20 = Stohr and Muths (2010); 21 = Strathmann (1981); 22 = Uehara et al. 1990; 23 = Uthicke et al. (2005); 24 = Wares (2001); 25 = Massin et al. (2009); 26 = this study.

of hybridization at the northern edge of the range of these two species.

To place our observations of *Pseudoboletia* in a comparative context, we searched the literature for other examples of natural hybridization in echinoderms, considering only cases that were confirmed by genetic evidence. We identified 13 cases (including *Pseudoboletia*) involving 21 species in nine genera and four classes of echinoderms (Table 3). All hybridization occurred between congeners. In three genera, a single species has been shown to hybridize with more than one congener (Table 3). The frequency of hybrid individuals in the studied populations was generally < 3% (Table 3). The estimates of hybrid frequency are limited in their precision for several reasons. First, as in our study, these estimates are for one or a few studied populations. Second, in some cases, researchers sought individuals with intermediate morphology (e.g., Lessios and Pearse 1996), whereas in other cases individuals with intermediate morphology were intentionally excluded from sampling (e.g., Addison and Pogson 2009). Third, the evidence of hybridization varies from study to study, typically mtDNA and morphology, but in several cases nuclear DNA or allozymes are used, which provide more accurate information about hybridization than mtDNA. In cases where F_1 individuals are not observed (or in studies where the markers used are unable to confirm F1 hybrids), it is unclear whether hybridization is ongoing, or whether the genetic signal observed is left from hybridization at some time in the past.

In most cases, hybrids are fertile, and backcrossing has been confirmed in five cases (Table 3). In *Holothuria*, all morphologically intermediate individuals appear to be F1 hybrids, suggesting that backcrossing is not occurring, although the hybrids develop gonads (Uthicke et al. 2005). COI sequences were available for 12 of 13 cases and percent divergence between the hybridizing species varied from 2.6% to 19.6% (Table 3); in several cases, the hybridizing species were not sister taxa. All of these species have planktonic larvae with the exception of *Leptasterias aequalis* and *L. hexactis*, which brood their embryos (Foltz et al. 2008). Gamete compatibility between hybridizing species ranged from high to low and was often asymmetric. *Pseudoboletia* is notable for exhibiting the highest level of gamete compatibility in any case of natural hybridization in echinoderms, and for having a relatively high percentage of hybrid individuals in the population studied (Table 3). Overall, there are relatively few examples of hybridization in echinoderms, and most of the known cases have not been studied in detail.

Conclusion

The idea that speciation and reproductive isolation in free-spawning marine invertebrates is strongly influenced by the evolution of gamete recognition proteins has become the dominant hypothesis over the past 20 years. Compelling examples of selection-driven gamete recognition protein evolution and gamete incompatibility in gastropods, bivalves and sea urchins lend strong support to this idea. *Pseudoboletia maculata* and *P. indiana* are an exception to the typical pattern of sympatric congeners having divergent gamete recognition proteins and incompatible gametes. Despite their broadly overlapping ranges, their gamete compatibility and minimal bindin divergence resembles that of sea urchin species that evolved in allopatry. How these species formed and how they remain distinct is not yet understood.

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