



INVITED PAPER

Introgression of the Gamete Recognition Molecule, Bindin, in the Sea Urchin *Diadema*

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Synopsis Hybridization is important in evolution, because it is a necessary (though not sufficient) step in the introgression of potentially adaptive variation between species. Bindin is a gamete recognition protein in echinoids and asteroids, capable of blocking cross-fertilization between species to varying degrees. Four species of the sea urchin genus *Diadema* are broadly sympatric in the Indo-Pacific: *D. paucispinum*, *D. savignyi*, *D. clarki*, and *D. setosum*. Data from three published studies, one of identification of hybrids through allozymes, one of the phylogeography of mitochondrial DNA, and one of the phylogeny of bindin, were combined to assess the degree of bindin introgression between these four species. I analyzed sequences of the ATPase 8 and ATPase 6 mitochondrial genes and of bindin, sampled throughout the species ranges, with an isolation–migration algorithm, IMA3. IMA3 uses a coalescent approach to produce Bayesian estimates of effective population sizes and gene flow between populations. The results showed that bindin alleles coalesce completely within the species bounds of *D. clarki* and of *D. setosum*. The sister species *D. paucispinum* and *D. savignyi*, however, were estimated as having exchanged a bindin allele at an average of every one to two-and-a-half generations since they speciated from each other. As the allozyme study detected nine hybrids between three of these species in Okinawa (most of them between *D. setosum* and *D. savignyi*) in a single sample, hybrids between these species are produced, but bindin does not introgress. Therefore, bindin must not be efficient in blocking heterospecific fertilizations. Complete, or almost complete, reproductive isolation between species of *Diadema* must result from low hybrid fitness.

Introduction

Introgression, the incorporation of genes from one species into the genome of another through hybridization, is important in evolution. Hybridization between closely related species has the potential of transferring adaptive variation from one species to another (Anderson 1949; Arnold 1997; Abbott et al. 2013; Hedrick 2013; Edelman and Mallet 2021), gives rise to new homoploid or polyploid species (Mallet 2007), and reinforces reproductive barriers through selection against low-fitness offspring (Howard 1993). It can also result in the fusion or extinction of species (Rhymer and Simberloff 1996; Wolf et al. 2001). Once thought to be rare in marine animals (e.g., Hubbs 1955; Arnold 1997), hybridization was subsequently found in several marine groups (reviews in Gardner 1997; Willis et al. 2006; Arnold and Fogarty 2009). Among echinoids, hybrids were genetically identified in *Strongylo-*

centrotus (Addison and Hart 2005; Harper et al. 2007; Addison and Pogson 2009; Glasenapp and Pogson 2023), *Echinothrix* (Coppard et al. 2021), *Pseudoboletia* (Zigler et al. 2012), *Lytechinus* (Zigler and Lessios 2004), *Arbacia* (Lessios et al. 2012), *Echinometra* (Geyer and Palumbi 2005), and *Diadema* (Lessios and Pearse 1996). With the notable exception of *Strongylocentrotus*, the importance of introgression in echinoid evolution has remained unexplored. Most of the reports about other genera consist of the identification of a few hybrids in otherwise monophyletic species.

How closely related sympatric species of sea urchins maintain their genetic integrity remains an open question, as there are few potential barriers to heterospecific fertilizations in organisms that shed their gametes into the water column (Lessios 2007). Non-overlapping spawning times are one possible barrier in prezygotic isolation, as is recognition between conspecific

gametes. One of the proteins potentially important in gamete recognition is bindin. Bindin is a gamete recognition protein (GRP) that mediates interaction between egg and sperm. It is expressed on the acrosome of sea urchin sperm, and it is recognized by two receptors of the egg, EBR1 and a 350-kDa protein (Vacquier 2012). Compatibility of bindin with its receptors permits adhesion with the egg vitelline membrane, fusion, and fertilization (Vacquier and Moy 1977; Ulrich et al. 1998; Vacquier 2012). Bindin has been shown experimentally to be capable of blocking heterospecific mating in sea urchins (Metz et al. 1994). Its DNA sequence divergence between species is inversely correlated with compatibility between heterospecific gametes (Zigler et al. 2005). It, thus, has the potential of being a “speciation gene,” as substitutions that alter its affinity to the egg receptors could reproductively isolate populations (Lessios 2011).

GRPs generally evolve at a fast rate under strong positive (diversifying) selection (Swanson and Vacquier 2002; Turner and Hoekstra 2008; Vacquier and Swanson 2011). Sea urchin bindin does not evolve as rapidly as that of some other GRPs (Lessios and Zigler 2012), but its rate of divergence between congeneric species is usually higher when their geographic ranges overlap (Palumbi and Lessios 2005). Positive selection on bindin is present in three sea urchin genera, *Echinometra* (Metz and Palumbi 1996; McCartney and Lessios 2004), *Strongylocentrotus* (Biermann 1998), and *Paracentrotus* (Calderon et al. 2009), but absent in five, *Arbacia* (Metz et al. 1998; Lessios et al. 2012), *Tripneustes* (Zigler and Lessios 2003), *Lytechinus* (Zigler and Lessios 2004), *Pseudoboletia* (Zigler et al. 2012), *Helicoidaris* (Hart et al. 2012, but see Zigler et al. 2003), and *Diadema* (except for one branch) (Geyer et al. 2020). Even in genera in which no positive selection has been found, bindin alleles generally cluster into monophyletic clades. The exception is bindin of *Diadema*.

Species of *Diadema* are abundant in tropical seas around the globe. Four of the eight described species of the genus are sympatric over a large part of their range in the Indo-Pacific (Fig. 1). *Diadema savignyi* ranges from the central Pacific to the east coast of Africa, *D. setosum* is spread from Tonga to Africa (Pearse 1998), *D. paucispinum* was originally thought to be endemic to the central Pacific (Mortensen 1940; Clark 1954), but recent evidence has shown it to be much more widespread (Lessios et al. 2001), and *D. clarki* is only known from the islands of Japan, the Marshall Islands, and Indonesia (Chow et al. 2016; Moore et al. 2019). All species are morphologically very similar (Lessios and Pearse 1996), which has led to misidentifications and erroneous reports of their presence in various regions (Mortensen 1940; Pearse 1998).

John Pearse and I, with the help of Bailey Kessing, studied the mitochondrial phylogeography of *Diadema* (Lessios et al. 2001). We found *D. setosum* to consist of two deeply divided clades, almost certainly different species, *D. setosum*-a, spread widely from the West Pacific to virtually the entire Indian Ocean, and *D. setosum*-b, only occurring around the Arabian Peninsula, where *D. savignyi* is absent and *D. paucispinum* is rare. These two clades were an outgroup to those of all other species in the genus. Mitochondrial DNA (mt-DNA) of *D. paucispinum*, far from being restricted to the central Pacific as it was once thought, was found in the entire Indo-Pacific (Fig. 1). *Diadema setosum* was estimated as having separated from all other species of *Diadema* 6.7–13.9 million years ago (Mya), *D. clarki* from the clade leading to *D. paucispinum* and *D. savignyi* 4–8.5 Mya, while the two sister species split from each other 1.02–1.86 Mya. The study of mt-DNA confirmed the presence of *D. paucispinum* outside the central Pacific, as reported from semi-diagnostic allozymes in Okinawa by Lessios and Pearse (1996). We also discovered a distinct clade of mt-DNA in Kyushu, Honshu, and Majuro, Marshall Islands—more similar to *D. savignyi* than to *D. setosum*—and suggested that it was a distinct species, *D. clarki*, originally described by Ikeda (1939) but synonymized with *D. setosum* by Mortensen (1940). Chow et al. (2014, 2016) subsequently provided morphological and additional mt-DNA evidence that this clade was, indeed, the species originally described by Ikeda. Thus, the distributions of four species of *Diadema* overlap in the West Pacific.

The question of hybridization in the Indo-Pacific species of *Diadema* was first addressed by Lessios and Pearse (1996) at a time when *D. clarki* was still assumed to be a synonym of *D. setosum*, and that *D. paucispinum* was thought to be restricted to the central Pacific. John Pearse collected *Diadema* in Okinawa and classified the specimens according to their morphology as *D. setosum*, *D. savignyi*, or as one of three types of potential hybrids. To determine alleles diagnostic of each species, collections of *D. setosum* were also made at Fantome Island at the Great Barrier Reef in Australia, where it occurs alone, and of *D. paucispinum* at Hawaii, where it is the only species of *Diadema*. Allozymes, diagnostic or semi-diagnostic for each species, indicated that nine of the morphologically intermediate specimens in Okinawa were hybrids between *D. setosum* and *D. savignyi*, or between one (or both) of these species and *D. paucispinum*. The allozyme data established that hybridization between three species was taking place. They could not, however, provide an estimate of the frequency of these hybrids in the population because sampling was deliberately biased in favor of hybrid inclusion by looking for individuals that could not be

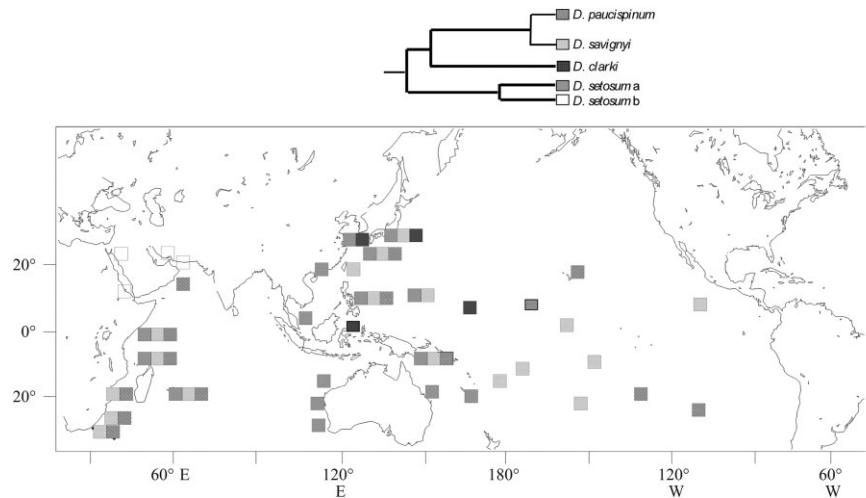


Fig. 1 Localities where each of the species of *Diadema* discussed in the text is known to occur, and a representation of the species relationships as determined by their mitochondrial DNA (after Lessios et al. 2001 with additions from Chow et al. 2016 and Moore et al. 2019).

identified to species by their morphology (Lessios and Pearse 1996). An estimate of historical introgression could, thus, not be obtained, except through weak evidence from linkage disequilibrium and deviations from Hardy–Weinberg equilibrium. The mitochondrial phylogeny of Lessios et al. (2001), based only on maternally inherited loci, could not provide a definitive answer as to whether individuals with *D. paucispinum* haplotypes were pure members of this species, or whether their mt-DNA now existed in a *D. savignyi* nuclear background, having spread outside the central Pacific through introgression.

Sequencing of bindin by Geyer et al. (2020) found that alleles of *D. setosum* were on a separate clade than those formed by alleles of all other species of *Diadema*; the same was true for alleles of *D. clarki*. Alleles of *D. savignyi* and *D. paucispinum*, however, were often in the same clade. Four bindin first exon sequences of these two species were identical. The shared polymorphisms in this genus raise the question of whether they are the result of incomplete sorting (i.e., whether they have been inherited from their common ancestor but did not have enough time to diverge), or introgression that occurred after speciation.

Data from mitochondrial (Lessios et al. 2001) and nuclear (Geyer et al. 2020) DNA provide an opportunity to estimate the degree of historical genetic exchange of bindin between sympatric species of *Diadema* and thus assess the importance of introgression in their evolution. The degree of introgression of bindin also has implications regarding its efficacy in reproductive isolation. Here, I use the data of Lessios and Pearse (1996), Lessios et al. (2001), and Geyer et al. (2020) to ad-

dress this question, quantify rate of bindin introgression, and evaluate the nature of reproductive isolation in this genus.

Materials and methods

Mitochondrial DNA variation in *Diadema* was assessed from sequences of the ATPase8 and ATPase6 genes (642 bp, hereinafter mentioned as “ATPase86”) reported by Lessios et al. (2001). Sequences of eight individuals of *D. savignyi* and one of *D. clarki* from Majuro, the Marshall Islands, were added. Lessios et al. (2001) had found only haplotypes of *D. clarki* (listed as *Diadema* sp.) at this island, but the more recently sequenced haplotypes indicated that *D. savignyi* is also present. The sampled Indo-Pacific species were *D. paucispinum* ($n = 79$, both a and b clades), *D. clarki* ($n = 21$), *D. savignyi* ($n = 107$), and *D. setosum*-a ($n = 87$). *Diadema setosum*-b from the Arabian Peninsula was not included in the analysis of hybridization, because it was not found in the same area with any other species of *Diadema*. Samples came from Clipperton, Easter Island, Pitcairn, Moorea, Kiritimati, and Hawaii in the central Pacific; Samoa, Rarotonga, the Cook Islands, Fiji, New Caledonia, Fantome Island (East Australia), Papua New Guinea, Majuro, Guam, the Philippines, Taiwan, Okinawa, Kyushu, Honshu, Ishigaki (Japan), Hong Kong, and Singapore in the West Pacific; the Lamarck and White Islands, Ningaloo, Geraldton (West Australia), Réunion, South Africa, Zanzibar, Kenya, and Oman in the Indian Ocean. Detailed localities, sample sizes per locality in each species, and methods of DNA extraction, amplification, and sequencing are listed in Lessios et al. (2001), as is the list of many

people to whom J. Pearse and I were indebted for collections.

Sequences of the first exon of mature bindin were from a subsample of individuals used in Lessios et al. (2001): *D. paucispinum* ($n = 16$), *D. savignyi* ($n = 8$), *D. clarki* ($n = 9$), and *D. setosum-a* ($n = 8$) (Geyer et al. 2020). ATPase86 sequences provided species identifications, because morphology in *Diadema* can be misleading. It is, thus, assumed that mitochondrial DNA delineates species of *Diadema*. To determine whether individuals were heterozygotes in bindin, up to five clones were obtained from each. Sequence differences between clones from the same individual were assumed to be indicative of a heterozygote if they differed at more than one site, or if they differed at only one site, but this difference was consistent in more than one clone. The bindin data were from Easter Island, the Cook Islands, Majuro, Okinawa, Kyushu, Ishigaki, the Philippines, Papua New Guinea, Zanzibar, Réunion, and South Africa.

Gene flow between species of *Diadema* since their separation from each other was estimated with the isolation–migration program IMA3 (Hey and Nielsen 2004; Hey et al. 2018), including the two mitochondrial genes as a single (haploid) locus, and the bindin sequences as a diploid locus. The program uses gene genealogies to produce Bayesian estimates, based on coalescence, of effective population size ($2N_e\mu$, where μ is the mutation rate) of ancestral and daughter populations, the time since their initial separation ($T\mu$), and the rate of gene flow in each direction (m/μ). The mitochondrial phylogeny of Lessios et al. (2001) provided a fixed species tree (Fig. 1). I estimated gene exchange and effective population sizes in six pairwise comparisons between the four species. Analyses were implemented with the Hasegawa et al. (1985) (HKY) model for both loci. Preliminary runs were performed to optimize the bounds of the priors, then they were run in 80 chains with geometric heating for 10^7 steps, sampling 10^5 genealogies every 10^2 steps after a burning of 4×10^6 steps. The IMA3 runs were done in the CIPRES portal (Miller et al. 2010). Convergence was determined by comparing estimates resulting from three runs with different random seeds but with the same priors. As the results of the three runs were very similar, estimated parameters of only one are shown here. Because estimates produced by IMA3 are a function of mutation rate, to calculate demographically scaled estimates, I used ATPase86 divergence of 4.24% between *D. antillarum* and *D. mexicanum*, assumed to have been separated by the Isthmus of Panama completion ~ 3 Mya (Lessios et al. 2001). There is no recombination (that confounds coalescence) in the first exon of bindin. Geyer et al. (2020) used four methods of estimating ratios of rate of amino acid replacement

Table 1 Highest probability density points of introgression events between Indo-Pacific species of *Diadema*

sp1	sp2	2NM (95% HPD)	
		sp1→sp2	sp2→sp1
<i>D. setosum</i>	<i>D. clarki</i>	0.002 (0.000–0.256)	0.002 (0.000–0.486)
<i>D. setosum</i>	<i>D. savignyi</i>	0.003(0.000–0.597)	0.003(0.000–0.567)
<i>D. setosum</i>	<i>D. paucispinum</i>	0.003 (0.000–0.571)	0.018 (0.000–0.542)
<i>D. clarki</i>	<i>D. savignyi</i>	0.002(0.000–0.666)	0.012(0.000–0.342)
<i>D. clarki</i>	<i>D. paucispinum</i>	0.002 (0.000–0.339)	0.007 (0.000–0.021)
<i>D. savignyi</i>	<i>D. paucispinum</i>	1.069 (0.046–3.456)	0.412 (0.000–2.791)

Range of 95% Highest Probability Densities (HPDs) are shown in parentheses.

and silent substitutions to assess selection on the mature bindin molecule. They provided no evidence of positive selection in either the first or the second exon of bindin, except for the branch leading to *D. clarki* (Geyer et al. 2020).

Results

Estimated effective population sizes and rate of gene flow between species of *Diadema* are shown in Fig. 2. Hybridization between *D. setosum* or *D. clarki* and the other two species was essentially null; the lowest limits of the range of 95% highest probability densities (HPD) of all migrations in these comparisons were invariably zero (Table 1). Introgression in both directions between the sister species *D. paucispinum* and *D. savignyi*, on the other hand, was more than a hundred times greater than that of any other comparison. From *D. paucispinum*, an average of 0.4 bindin genotypes were transferred into *D. savignyi* every year (or every generation, as *Diadema* probably reaches sexual maturity in 1 year). From *D. savignyi*, 1.1 alleles per year were transferred into *D. paucispinum*. Thus, the species boundary between these two reciprocally monophyletic mitochondrial sister clades has been crossed by bindin every 1–2.5 years since speciation, by hybrids fit enough to backcross and transfer their alleles. The rates of introgression between *D. paucispinum* and *D. savignyi* rival those seen in stony corals, a group in which hybridization is common (Willis et al. 2006), and in which reticulate evolution is thought to be widespread (Veron 1995). $2N_e m$ values of 0.4–2 propagules per generation were estimated by the isolation–migration model as transferred between the scleractinians *Acropora cytherea* and *A. hyacinthus* (Ladner and Palumbi 2012), and 0.29 between *Porites evermanni* and *P. lobata* (Hellberg et al. 2016). The same model provided $2N_e m$ estimates of 0.13–1.05 between sister species of snapping shrimp of the genus *Alpheus* (Hurt et al. 2013).

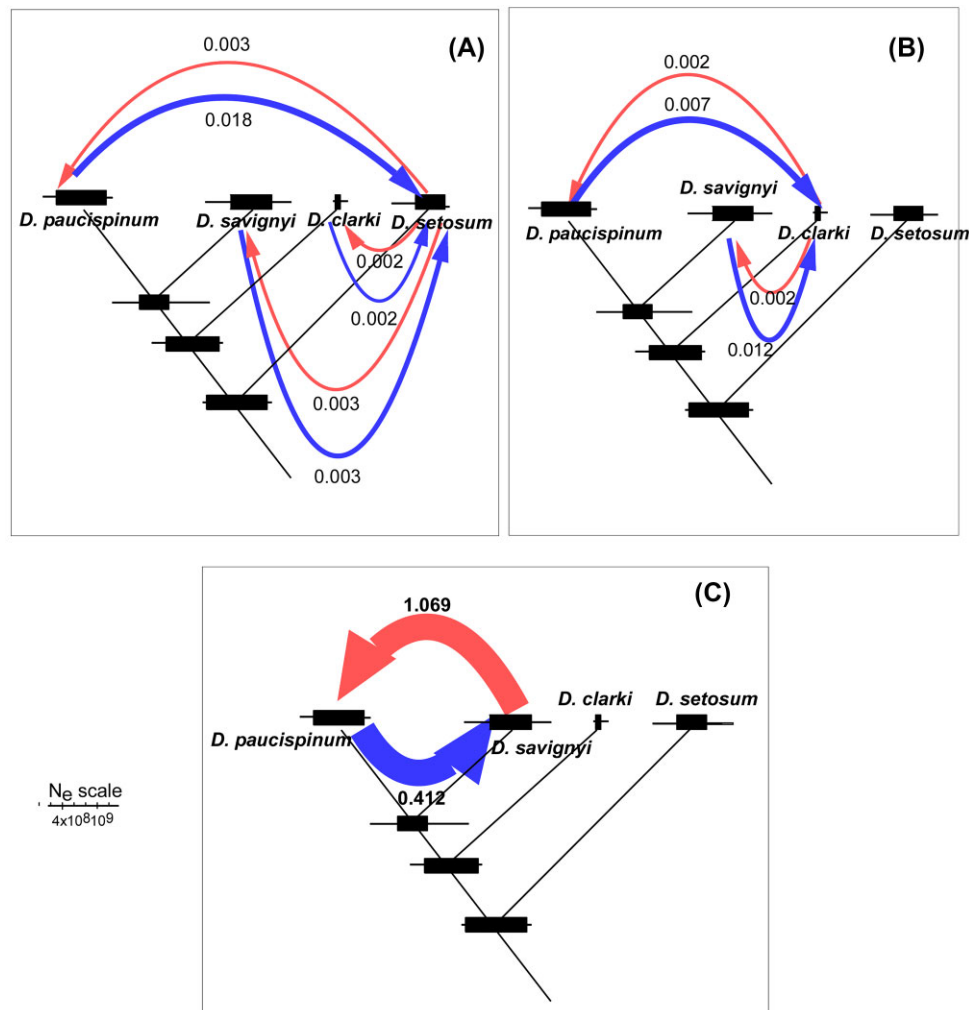


Fig. 2 Estimates of effective population sizes and bindin introgression rates between Indo-Pacific species of *Diadema*, superimposed on the mitochondrial phylogeny of Lessios et al. (2001). Width of rectangles represents the Highest Probability Density (HiPt) of effective population size (N_e), lines represent the 95% HPD intervals. Labels on arrows indicate the HiPt of the number of introgression events ($2N_e m$) per generation between the species in the direction indicated by arrows (forward in time). 95% HPD ranges are shown in Table 1. Estimates of introgression events are shown between *D. setosum* and all other species (panel A), *D. clarki* and *D. savignyi* or *D. paucispinum* (B), and *D. savignyi* and *D. paucispinum* (C).

Effective population sizes of *D. paucispinum* (4.35×10^8 individuals) and *D. savignyi* (5.5×10^8 individuals) were much larger than that of *D. clarki* (9.6×10^7 individuals), while that of *D. setosum* (2.1×10^8 individuals) was intermediate. The relative values of *D. paucispinum* and *D. savignyi* versus that of *D. setosum* may well reflect the expected larger genetic variation in hybridizing species, but the much smaller estimated population in *D. clarki* could be an artifact of smaller sample sizes and of selection on bindin in this species, as coalescence assumes selectively neutral evolution. Nevertheless, population sizes correlate with the known ranges of the species. *Diadema clarki* is only known from Japan (Chow et al. 2016), the Marshall Islands (Lessios et al. 2001), and Indonesia (Moore et al. 2019), whereas the other three species

range over the entire Indo-Pacific. Reports of the distribution of newly rediscovered species are expected to underestimate their range, but out of 29 locations sampled by Lessios et al. (2001) in the Indo-Pacific for ATPase86, *D. clarki* haplotypes were only found in two.

Discussion

The discovery of hybridization in various animal taxa has led to the proposal that species barriers are selectively porous, with some genes introgressing readily between genomes, while others are barred from entering by natural selection (reviews in Abbott et al. 2013; Edelman and Mallet 2021). This view, in turn, favors calls for the abandonment of the biological species

concept (Mallet 2008), as this concept defines species by their reproductive isolation (Mayr 1963). The documentation presented here, that bindin introgression occurs between sister species of *Diadema* but not between more distantly related species, does not directly address the rate of allele transfer in other loci. Bindin, however, is not a random nuclear locus, but a GRP, potentially capable of blocking cross-fertilization between species. Differences between alleles in a locus that affect the efficiency of cross-fertilization directly affect the porosity of the entire genome.

My analysis necessarily assumes that mt-DNA distinguishes between species of *Diadema*, and that bindin, rather than mt-DNA, is being exchanged between *D. paucispinum* and *D. savignyi*. This assumption, made necessary by the unreliability of morphological species identifications, is also based on the monophyly of mt-DNA (Lessios et al. 2001) between the species and the paraphyly of bindin (Geyer et al. 2020). Under this assumption, the sharing of bindin alleles between *D. paucispinum* and *D. savignyi* is not the result of incomplete lineage shorting, but rather of hybridization that has happened since these species were separated from each other. Bindins of *D. clarki* and *D. setosum* coalesce within each of these species.

The allozyme study of Lessios and Pearse (1996) identified one individual that was an F₂ (or later generation) hybrid between *D. savignyi* and *D. paucispinum*. It also discovered seven hybrids between *D. savignyi* and *D. setosum*, and one hybrid carrying alleles from all three species. Thus, hybrids of *D. savignyi* with *D. setosum* can be found on the reef at the same time, but, as the present analysis shows, bindin introgression between them does not occur. Sampling by Chow et al. (2014, 2016) has failed to locate *D. clarki* at Okinawa. Although their exact frequency in the population cannot be calculated, nine natural hybrids in a single generation on two Okinawan reefs, each searched once, indicate that heterospecific fertilizations are far from rare. That eight of these hybrids were F₂ or later generation offspring indicates that hybrids reach sexual maturity and backcross or mate with each other. Yet, the IM analysis indicates that bindin introgression is present only between *D. savignyi* and *D. paucispinum*, and that even between these species bindin allele transfer has occurred only once in a generation in one direction and only every 2.5 years in the other. Frequent hybrids in one generation but low introgression since speciation suggest that prezygotic isolation in *Diadema* is weak. Uehara et al. (1990) reported that gametes of *D. savignyi* and *D. setosum* are capable of fertilizing each other in the laboratory at unspecified sperm concentrations.

In addition to bindin, a potential prezygotic barrier to hybridization between *D. savignyi* and *D. setosum* is

spawning that peaks at different phases of the moon (Coppard and Campbell 2005). It might be thought that the 15-day face shift in spawning has evolved by reinforcement, but a similar difference exists between *D. antillarum* and *D. mexicanum*, separated by the Central American Isthmus (Lessios 1984). Intraspecific synchronization of spawning in *D. setosum* and *D. savignyi* is not tight (Pearse 1968). That hybrids are produced indicates that gametes of the two species are in the water column at the same time, and that neither bindin nor asynchronous spawning cycles are very efficient in blocking interspecific crosses. Introgressed bindin alleles, however, survive over some generations, but in deeply separated species, they are eventually eliminated from the gene pool. Separate species of sympatric *Diadema* are, thus, maintained as independent genetic entities by post-zygotic isolation resulting from low hybrid fitness. Lower hybrid fitness could be caused by alleles that are not fully compatible when introduced into the genome of a sister species. A genomic scan of the species of *Diadema* would help identify such alleles and, thus, begin to shed light into the causes of post-zygotic isolation between species of sea urchins. That a great deal is known about the function of genes in sea urchin development (Davidson et al. 2002) suggests that this type of investigation may be fruitful.

One might expect that marine taxa with external fertilization would be more prone to introgression than taxa with internal fertilization. Animals with behavioral and physiological species recognition should be better insulated against interspecific mating and less prone to show the effects of introgression in their evolutionary histories. Yet, there is a host of documented cases of introgression in arthropods, amphibians, birds, or mammals, but a paucity of examples of interspecific allele transfer in sponges, anthozoans (except corals), bivalves, or echinoderms. That many cases of introgression have been discovered in groups such as arthropods may be simply the result of the probability that large, diverse groups are more likely to illustrate any given phenomenon. The trend also undoubtedly reflects the intensity of effort devoted to evolutionary studies in each group. As the study of introgression has implications regarding the porosity of species genomes, the utility of species concepts, and ultimately the manner in which variation in nature is generated and maintained, its occurrence in taxa with external fertilization deserves attention.

Acknowledgments

As the citations in this paper make clear, this study was made possible by John Pearse, an ideally amicable, hard-working, and knowledgeable collaborator. I also thank

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Conflict of interest

The author declares no conflict of interest.

Data availability

Sequences used in this study have been deposited in GenBank under accession numbers AY012956–AY013079, AY013081, AY013082, AY013084, AY013085, AY013087, AY013092–AY013101, AY013104–AY0131235, PP481646–PP481653 and PP858888 for ATPase 8 and ATPase 6; and MT365802–MT365868, MT375187, and MT375188 for bindin.

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