Marine Biology (1996) 126: 715–723

H. A. Lessios · J. S. Pearse

Hybridization and introgression between Indo-Pacific species of *Diadema*

Received: 18 January 1996 / Accepted: 22 February 1996

Abstract We present the first case of hybridization between echinoid species evaluated through genetic markers and morphology. We examined the three tropical Indo-Pacific species of the genus *Diadema*: *D. paucispinum* A. Agassiz, *D. savignyi* (Audouin) Michelin and *D. setosum* (Leske). Specimens morphologically intermediate between two of these species, *D. savignyi* and *D. setosum* have previously been noted. Fertile hybrids have also been produced in the laboratory. To determine extent of hybridization, we first assayed the allozyme products of 22 loci in individuals which, on the basis of morphology and collection locality, could be unambiguously assigned to one of the three species. We found four loci that were either diagnostic or semi-diagnostic between *D. setosum* and the other two species, and one locus semi-diagnostic between *D. savignyi* and *D. paucispinum*. We then assayed individuals of intermediate morphology to find out whether they had hybrid genotypes. In the Ryukyu Islands, where *D. setosum* and *D. savignyi* coexist, we found one specimen which on the basis of all four diagnostic loci was an F_1 hybrid, and several individuals that could be either F_2 (or later-generation) hybrids or progeny of backcrosses. We also found one individual that on both genetic and morphological grounds appeared to belong to *D. paucispinum* (even though this species has only been reported from Hawaii and Kiribati) and three other individuals that carried alleles characteristic of *D. paucispinum*. Thus, previous reports of hybridization between *D. setosum* and *D. savignyi* were correct; it is also possible that larvae of *D. paucispinum* occasionally arrive at localities outside Hawaii, reach sexual maturity, and hybridize with the other two species. Counts of pure and hybrid morphotypes in other populations across the western tropical Pacific revealed a low but widespread incidence of apparent F_1 hybrids and backcrosses of *D. savignyi* and *D. setosum*. However, the existence of diagnostic or semi-diagnostic loci, low interspecific gene-flow estimates based on F_{ST} statistics, and the lack of Hardy–Weinberg or linkage disequilibria among individuals of pure morphology all suggest that gene introgression between the three species is limited.

Introduction

It is still not known whether interspecific hybridization is an important evolutionary process in the animal kingdom. Anderson (1949) viewed it as a significant means of introducing new gene combinations into species genomes, whereas Mayr (1963, pp 130–133) held that "... the total weight of the available evidence contradicts the assumption that hybridization plays a major evolutionary role among higher animals.". More recently, Heiser (1973) speculated that hybridization has the potential of being an important process, but admitted that "... there is no strong evidence to support such a claim.". Arnold (1992) concluded that molecular and ecological analyses "... suggest a prominent role for natural hybridization in numerous species complexes.". Studies of the dynamics of hybrid zones (e.g. Bigelow 1965; Moore 1977; Shaw 1981; Barton and Hewitt 1985, 1989; Howard 1986; Harrison et al. 1987; Bert and Harrison 1988; Dillon and Manzi 1989; Rand and Harrison 1989; Harrison 1990, 1993; Howard and Waring 1991; Howard et al. 1993) have demonstrated gene introgression in a number of species capable of hybridization. Such studies, however, encompassing a relatively small number of taxa, beg the question of how widespread hybridization is in

Communicated by N. H. Marcus, Tallassee

H. A. Lessios (✉)
Smithsonian Tropical Research Institute, Box 2072, Balboa, Panamá

J. S. Pearse
Institute of Marine Science, University of California, Santa Cruz, California 95064, USA

different animal groups from a variety of habitats. To answer this question, it is necessary to accumulate data from many phyla. The possibility of hybridization in marine species in particular is often raised, but rarely documented.

Echinoids are a group of marine animals in which the evolutionary role of hybridization has never been assessed. There are reported cases of artificial crosses between echinoid species that produced offspring which were subsequently raised to metamorphosis and sexual maturity in the laboratory (e.g. Shearer et al. 1914; Strathmann 1981; Pearse and Cameron 1991). Natural hybridization between species of sea urchins has also been postulated on the basis of observations of morphologically intermediate specimens (e.g. Mortensen 1940, 1943; Vasseur 1952; Swan 1953; Mayr 1954; Hagström and Lönning 1961; Kelso 1970; Strathmann 1981; Palumbi and Metz 1991). Hagström and Lönning, based on the appearance of laboratory-reared hybrids, concluded that 10 to 20% of a Norwegian population of *Echinus* spp. were hybrids. However, we are aware of no genetic evidence that has directly shown echinoids to hybridize in nature, or that has been used to assess the extent of introgression.

Two echinoid species long suspected of forming hybrids are *Diadema setosum* and *D. savignyi* (Mortensen 1940; Mayr 1954). They occur sympatrically throughout the western tropical Pacific and Indian Ocean from the coast of Africa to Polynesia (Mayr 1954), often in mixed aggregations (Pearse and Arch 1969). There appears to be ample opportunity for hybridization between these two species. Both their annual gametogenic cycles (Uehara et al. 1990) and their lunar spawning cycles (Pearse unpublished data) can overlap, so there are times during which their gametes are in the water column at the same time. In the laboratory, sperm of each species can fertilize eggs of the other at rates equal to those of intraspecific crosses (Uehara et al. 1990). The resultant hybrids have been reared in aquaria to sexual maturity, producing motile sperm (T. Uehara, personal communication to JSP). A third Indo-Pacific species, *D. paucispinum*, is mainly known from Hawaii (Mortensen 1940) (where the other two species are absent), although it has also been reported from Kiribati (0°; 174°W) (Clark 1954). There is no information regarding the ability of *D. paucispinum* to cross with either of the other two species.

To find out whether natural hybridization between *Diadema setosum*, *D. savignyi* and *D. paucispinum* occurs, and to assess the degree to which such hybridization may be responsible for genetic introgression across species borders, we collected morphological and electrophoretic evidence from the three Indo-west Pacific species of *Diadema*. We first asked whether there are allozymes that distinguish individuals of pure morphology characteristic of each species, and then we assessed the genetic makeup of individuals with mixtures of morphological features suggesting a hybrid origin. We also assessed the frequencies of apparent hybrid morphotypes in five regions of the western tropical Pacific. Our data indicate that although hybridization occurs, it results in little interspecific gene flow.

Materials and methods

Collections of *Diadema setosum* (Leske), *D. savignyi* (Audouin) Michelin and morphologically intermediate specimens were made in the harbor of Motobu, Okinawa-Jima and at the islet of Sesoko-Jima, immediately offshore, (26° 38′N; 127° 52′E) in the Ryukyu Islands, Japan, between 15 May and 2 September, 1989. Each specimen was classified by one of us (JSP) on the basis of color pattern and the morphology of its pedicellariae into either of the two species, or into one of three categories of suspected hybrids. *D. savignyi* was distinguished from *D. setosum* by three color dichotomies (Pearse and Arch 1969): (1) There is no orange ring around the tip of the anal cone in *D. savignyi*, but there is a conspicuous orange ring in *D. setosum*; (2) spots on interambulacra above the ambitus are faint or absent during the day in *D. savignyi*, but they are noticeably white and always present in *D. setosum*; (3) there are nearly continuous iridescent blue lines around the periproct and down the interambulacra in *D. savignyi*, whereas there is a discontinuous pattern of iridescent blue spots in *D. setosum*. In addition, we examined the tridentate pedicellariae, which are wide and spoon-shaped in *D. savignyi*, but narrow and needle-like in *D. setosum*. The characters of each class of suspected hybrid morphotype were as follows:

Type 1. With five yellow-golden spots and a thin anal ring of the same color, and elongate tridentate pedicellariae, that are wide at the tip similar to those of *Diadema savignyi*, this type resembles hybrids produced by H. Asakura and T. Uehara from eggs of *D. savignyi* and sperm of *D. setosum* (JSP personal *observation*).

Type 2. With five conspicuous white spots, but no orange ring on the anal cone, conspicuous blue spots and dashes but not lines, and long tridentate pedicellariae that are, however, not as long and needle-like as those of *Diadema setosum*, this is the morphological class most similar to *D. setosum*.

Type 3. This type has no white spots or anal ring, very little or no blue color anywhere, and tridentate pedicellariae that resemble those of Type 2. It resembles *Diadema savignyi* more than it does *D. setosum*, but it also fits the description of *D. paucispinum* A. Agassiz.

A special effort was made to locate these suspected hybrids, so their frequencies in the electrophoretic sample are severely biased.

Tissue samples of *Diadema setosum* were also collected at Fantome Island (18° 30′S; 147° 10′E) near the Marine Station of James Cook University at Orpheus Island, Great Barrier Reef, Australia, on 13 December 1988. Other species of *Diadema* were not found in this area, and no suspected hybrids were seen. *D. paucispinum* was collected for electrophoretic analysis off Honolulu, Hawaii (21° 19′N; 157° 52′W), between 1 and 11 March 1989.

Methods of sample preparation and electrophoresis were similar to those previously described (Lessios 1981). Enzymes that produced scorable results are shown in Table 1. The Mendelian interpretation of zymograms is consistent with the existence of a total of 22 loci. Loci diagnostic between the species were identified according to the method of Ayala and Powell (1972). To analyze the data for possible gametic disequilibria between loci resulting from hybridization, we used the program LINKADES of Garnier-Gere and Dillmann (1992). For this analysis, alleles with frequencies of < 0.01 were ignored, because pooling of rare alleles can produce spurious disequilibria (Weir and Cockerham 1978; Zapata and Alvarez 1992). F_{ST} statistics (Wright 1965) were calculated using the BIOSYS program (Swofford and Selander 1989).

Table 1 *Diadema paucispinum*, *D. savignyi*, and *D. setosum*. Enzymes assayed (*abbreviations in parentheses*), tissues and buffers used, and number of putative loci scored (Tissues: *M* muscle, *G* gut; Buffers: *A* discontinuous 0.076 *M* Tris-citrate, pH 8.65; *B* 0.087 *M* Tris-versene-borate, pH 9.10; *C* 0.009 *M* Tris-versene-citrate, pH 7.00; *D* 0.023 *M* Tris-citrate, pH 8.00; *E* discontinuous 0.046 *M* Tris-citrate-borate-LiOH, pH 8.30; *F* 0.214 M phosphate-citrate, pH 7.00)

Enzyme		Tissue	Buffer	No. of loci
Acid phosphatase	(ACPH)	M	A	1
α-glucosidase	(aGLU)	G	F	1
β-N-acetyl-galactosaminidase	(bGALA)	G	E	1
Creatine kinase	(CK)	M	D	1
Esterases	(EST)	G	A	1
Fumarate hydratase	(FUM)	M	D	1
Glucose-6-phosphate-dehydrogenase	(G6PDH)	M	F	1
Aspartate aminotransferase	(GOT)	M	D	1
Isocitrate dehydrogenase	(IDH)	M	D	1
Leucine amino peptidase	(LAP)	G	A	1
Mannose-6-phosphate isomerase	(M6PI)	M	A	1
Malate dehydrogenase	(MDH)	M	D	2
Octanol dehydrogenase	(ODH)	G	B	1
L-leucyl-L-tyrosine peptidase	(PEPLT)	G	E	2
Phosphoglucose isomerase	(PGI)	M	A	1
Phosphoglucomutase	(PGM)	M	C	2
Superoxide dismutatse	(TO)	G	B	1
Triosephosphate isomerase	(TPI)	M	C	1
Xanthine dehydrogenase	(XDH)	G	B	1

The frequencies of *Diadema setosum*, *D. savignyi* and of suspected hybrid morphotypes were determined at 18 localities in five regions spread across the western tropical Pacific on the following dates: (1) Shirahama, Honshu Island, Japan (33° 40′N; 135′ 20′E) in June to July 1989; (2) Okinawa, Ryukyu Islands, Japan (26° 30′N; 128° 00′E) in April, June and August 1989; (3) Iriomote Island, Japan (24° 20′N; 123° 40′E) in May 1989; (4) NE Sulawesi Island and southern Sangihe Islands, Indonesia (1° 40′N; 125° 20′E) in November 1994; (5) Madang Barrier Reef, Papua, New Guinea (5° 10′S; 145° 50′E) in August to September 1988. At each locality, individuals were identified and counted as encountered while snorkeling or SCUBA diving; the survey ended when additional *Diadema* spp. became difficult to locate. In all populations, individuals of the two species were intermingled either in groups sheltered among rocks, or in aggregations of 2 to > 100 individuals clumped together in the open (see Pearse and Arch 1969).

Results

Gene frequencies are shown in Table 2. The two sampled populations of *Diadema setosum* showed a great deal of similarity in gene frequencies in all loci ($p > 0.05$ by χ^2 in all cases), even though 6000 km separate the Great Barrier Reef from the Ryukyu-Islands. Such similarity is not unexpected given that the larval life of *D. setosum* in the laboratory exceeds 6 wk (Mortensen 1937). Populations of another echinoderm, *Acanthaster planci*, have also been found to be genetically very similar between Australia and the Ryukyu Islands (Nishida and Lucas 1988). Individuals of unambiguous *D. setosum* and *D. savignyi* (or *D. paucispinum*) morphology had entirely different alleles in three loci, *Pgm*-2, *To* and *Tpi*. Calculation of probabilities of correct assignment of a genotype to each species on the basis of each locus (Ayala and Powell 1972) indicated that there was a fourth, semi-diagnostic locus, *aGlu*. The Indo-Pacific *D. savignyi* and the Hawaiian *D. paucispinum*, on the other hand, were distinguished by only one semi-diagnostic locus, *Peplt-1* (Table 3).

The alleles carried by individuals with intermediate morphologies in diagnostic and semi-diagnostic loci were used to determine whether they were hybrids or individuals with extreme morphologies. Table 4 shows that 3 of 8 specimens of suspected Hybrid Type 2 had genotypes identical to those of *Diadema setosum*, and 8 of 12 individuals of Type 3 were genotypically indistinguishable from *D. savignyi*. There is a $(\frac{1}{2})^5 = 0.03$ probability that "pure" genotypes are F_2 hybrids (based on the assumption that the three diagnostic loci segregate independently, and ignoring the semi-diagnostic locus) and a $(\frac{1}{2})^3 = 0.125$ probability that they are the offspring of backcrosses between F_1 hybrids and parental species (Avise and Van den Avyle 1984; Campton 1990). One individual was a homozygote for *Peplt-1*^{95}, an allele characteristic of *D. paucispinum*, even though this species has never been reported from the Ryukyus Islands. Five Type 2 specimens and three Type 3 individuals carried alleles characteristic of one species in one or more diagnostic loci, but in another locus they possessed alleles of another species. These could be either F_2 (or later generation) hybrids, or progeny of backcrosses of a hybrid to one of the two parental species. In addition to the individual homozygous for the *Peplt-1*^{95} allele, three other suspected hybrids carried Allele *Peplt-1*^{90}, which is otherwise only found in Hawaii in individuals of pure *D. paucispinum* morphology (Table 2). The single individual with Type 1 morphology that we were able to collect was heterozygous in the three diagnostic and also in the one semi-diagnostic locus between *D. setosum* and *D. savignyi*. Only 1/8 of F_2 and 1/8 of the offspring of a backcross are expected to be triple heterozygotes. There is a high probability, therefore, that this individual was an F_1 hybrid.

Table 2 *Diadema paucispinum*, *D. savignyi*, and *D. setosum*. Number of individuals sampled and gene frequencies in 22 loci. Only individuals unambiguously identified to species by morphology were used in calculation of gene frequencies (*Locus abbreviations* as in Table 1)

Locus, allele	*D. paucispinum*	*D. savignyi*	*D. setosum*	
	Hawaii	Ryukyu	Ryukyu	Australia
Acph				
(*N*)	(21)	(23)	(28)	(9)
90	0.000	0.022	0.446	0.333
100	0.881	0.565	0.482	0.667
200	0.119	0.413	0.071	0.000
aGlu				
(*N*)	(21)	(23)	(28)	(10)
95	0.024	0.000	0.839	0.950
100	0.976	1.000	0.161	0.050
bGala				
(*N*)	(21)	(22)	(28)	(10)
100	1.000	1.000	1.000	1.000
Ck				
(*N*)	(21)	(23)	(28)	(9)
100	1.000	1.000	1.000	1.000
Est-1				
(*N*)	(21)	(23)	(28)	(10)
100	1.000	1.000	1.000	1.000
Fum				
(*N*)	(21)	(23)	(28)	(10)
100	1.000	1.000	1.000	1.000
G6pdh				
(*N*)	(21)	(23)	(28)	(9)
100	1.000	1.000	1.000	1.000
Got-1				
(*N*)	(21)	(23)	(28)	(9)
100	1.000	1.000	1.000	1.000
Idh				
(*N*)	(21)	(21)	(28)	(9)
100	1.000	1.000	1.000	1.000
Lap-2				
(*N*)	(21)	(23)	(28)	(9)
100	1.000	1.000	1.000	1.000
M6pi				
(*N*)	(21)	(23)	(28)	(9)
98	0.071	0.022	0.054	0.056
100	0.452	0.087	0.107	0.056
105	0.214	0.065	0.143	0.389
106	0.095	0.217	0.536	0.278
110	0.048	0.370	0.089	0.056
111	0.071	0.196	0.018	0.000
112	0.048	0.044	0.054	0.167
Mdh-1				
(*N*)	(21)	(23)	(28)	(9)
100	0.643	0.935	0.982	0.944
103	0.357	0.065	0.018	0.056
Mdh-2				
(*N*)	(21)	(23)	(27)	(9)
100	1.000	1.000	1.000	1.000
Odh				
(*N*)	(20)	(23)	(28)	(10)
100	1.000	1.000	1.000	1.000
Peplt-1				
(*N*)	(21)	(23)	(28)	(10)
90	0.476	0.000	0.000	0.000
95	0.381	0.000	0.000	0.000
100	0.143	1.000	1.000	1.000
Peplt-2				
(*N*)	(21)	(23)	(28)	(10)
100	1.000	1.000	1.000	1.000
Pgi				
(*N*)	(21)	(23)	(28)	(9)
80	0.000	0.000	0.018	0.000
95	0.071	0.109	0.250	0.111
98	0.024	0.109	0.107	0.000
99	0.000	0.044	0.000	0.000
100	0.714	0.609	0.536	0.833
104	0.167	0.065	0.071	0.056
105	0.024	0.022	0.018	0.000
106	0.000	0.044	0.000	0.000
Pgm-1				
(*N*)	(21)	(23)	(28)	(9)
80	0.000	0.000	0.018	0.000
96	0.024	0.044	0.179	0.111
98	0.286	0.087	0.357	0.444
100	0.333	0.500	0.339	0.389
106	0.283	0.152	0.018	0.056
108	0.119	0.109	0.071	0.000
110	0.000	0.109	0.018	0.000
Pgm-2				
(*N*)	(21)	(23)	(27)	(9)
98	0.000	0.000	1.000	1.000
100	1.000	1.000	0.000	0.000
To				
(*N*)	(21)	(23)	(28)	(10)
100	0.929	1.000	0.000	0.000
110	0.071	0.000	1.000	1.000
Tpi				
(*N*)	(21)	(23)	(28)	(9)
100	1.000	1.000	0.000	0.000
105	0.000	0.000	1.000	0.833
110	0.000	0.000	0.000	0.167
Xdh				
(*N*)	(21)	(23)	(28)	(10)
100	1.000	1.000	1.000	1.000

Table 3 *Diadema paucispinum*, *D. savignyi*, and *D. setosum*. Loci diagnostic and semi-diagnostic between the three species. Probabilities of assignment of an individual to correct species on basis of its genotype in each locus, calculated according to Ayala and Powell (1972), are shown in parentheses. Gene frequencies of the two *D. setosum* populations have been pooled (*Locus abbreviations* as in Table 1)

Species	*D. paucispinum*	*D. savignyi*
D. savignyi	*Peplt-1* (0.99)	
D. setosum	*aGlu* (0.97)	*aGlu* (0.99)
	Peplt-1 (0.99)	*Pgm-2* (1.00)
	Pgm-2 (1.00)	*To* (1.00)
	To (1.00)	*Tpi* (1.00)
	Tpi (1.00)	

An estimate of the number of matings that transport alleles across species borders can be obtained from F_{ST} statistics by using Wright's (1965) formula for estimating migration (Porter 1990; Orr et al. 1994). This estimate, based on frequencies of genotypes in polymorphic loci, indicated that *Diadema setosum* and *D. savignyi* in the Ryukyu Islands exchange < 1 "propagule" per generation (Table 5). *D. paucispinum* from Hawaii and *D. savignyi* from the Ryukyu Islands on the other hand, appear to have 1.6 events of successful cross-fertilization per generation, a value marginally higher than the amount held to be sufficient for homogenization of gene pools (Chakraborty and Nei 1974; Spieth 1974; Slatkin and Maruyama 1975), but still much smaller than the migration value (6.5 migrants per generation) in the comparison of conspecific populations of *D. setosum* from the Ryukyu Islands and Australia (Table 5). Genetic exchange estimated from pairwise F_{ST} values tends to be biased upwards (Nei et al. 1977), so the true number of hybrid crosses resulting in introgression is probably even lower than estimated in Table 5. Thus, F_{ST} statistics suggested that despite the presence of hybrids, introgression between *D. setosum* and the other two species was very low, while that between *D. savignyi* and *D. paucispinum* was higher but still not substantial.

Two further analyses are relevant to the question of the extent to which the species hybridize: (a) conformity of populations to Hardy–Weinberg equilibrium, and (b) presence of linkage disequilibria between the loci. If

Table 4 *Diadema paucispinum*, *D. savignyi* and *D. setosum*. Composite genotypes of all morphologically intermediate specimens in the four diagnostic or semi-diagnostic loci listed in Table 3 (see "Materials and methods" for description of hybrid morphotypes (*Genetic inference* the most probable parentage of each individual, based on these four loci; F_2 second or later generation hybrid or backcross; – no data; *locus abbreviations* as in Table 2)

Hybrid morphotype	Loci diagnostic between:					Genetic inference
	D. setosum and *D. savignyi* (or *D. paucispinum*)				*D. paucispinum* and *D. savignyi*	
	aGlu	*Pgm-2*	*To*	*Tpi*	*Peplt-1*	
1	95 100	98 100	100 110	100 105	100 100	F_1 *D. set* × *D. sav*
2	95 95	98 98	110 110	105 105	100 100	*D. setosum*
2	95 95	98 98	110 110	105 105	100 100	*D. setosum*
2	95 95	98 98	110 110	105 105	100 100	*D. setosum*
2	95 95	100 100	110 110	105 105	100 100	F_2 *D. set* × *D. sav*
2	95 100	100 100	100 110	100 100	90 100	F_2 *D. set* × *D. sav* × *D. pauc*
2	95 95	98 98	110 110	105 105	90 100	F_2 *D. set* × *D. pauc*
2	95 100	100 100	110 110	105 105	100 100	F_2 *D. set* × *D. sav*
2	95 95	100 100	100 100	100 100	100 100	F_2 *D. set* × *D. sav*
3	100 100	100 100	100 100	100 100	100 100	*D. savignyi*
3	100 100	100 100	100 100	100 100	100 100	*D. savignyi*
3	100 100	100 100	100 100	100 100	100 100	*D. savignyi*
3	100 100	100 100	100 100	100 100	100 100	*D. savignyi*
3	100 100	100 100	100 100	100 100	100 100	*D. savignyi*
3	100 100	–	100 100	100 100	100 100	*D. savignyi*
3	100 100	100 100	100 100	100 100	100 100	*D. savignyi*
3	100 100	–	100 100	100 100	100 100	*D. savignyi*
3	95 100	100 100	100 100	100 100	90 100	F_2 *D. sav* × *D. pauc*
3	100 100	98 98	100 100	100 100	100 100	F_2 *D. set* × *D. sav*
3	100 100	100 100	100 100	100 100	95 95	*D. paucispinum*
3	95 95	100 100	100 100	100 100	100 100	F_2 *D. set* × *D. sav*

Table 5 *Diadema paucispinum*, *D. savignyi* and *D. setosum*. Values of Wright's standardized variance, F_{ST} (*below diagonal*) and of estimated number of individuals carrying genes between populations, *Nm* (*above diagonal*), calculated in pairwise fashion from all polymorphic loci. Individuals with suspected hybrid morphotypes have not been included in calculations

		D. paucispinum,	*D. savignyi*,	*D. setosum*	
		Hawaii	Ryukyu	Ryukyu	Australia
D. paucispinum,	Hawaii		1.602	0.340	0.331
D. savignyi,	Ryukyu	0.135		0.344	0.317
D. setosum,	Ryukyu	0.424	0.421		6.507
	Australia	0.430	0.441	0.037	

Table 6 *Diadema paucispinum*, *D. savignyi* and *D. setosum*. Heterozygote deficiencies (*D*) and significance of deviations from Hardy–Weinberg expectations based on exact tests of most common vs rare alleles. Individuals with suspected hybrid morphotypes have not been included. Probabilities corrected for multiple tests with Bonferroni inequality (Lessios 1992) (*Locus abbreviations* as in Table 1)

Locus	*D. paucispinum*, Hawaii	*D. savignyi*, Ryukyu	*D. setosum*	
			Ryukyu	Australia
Acph	− 0.335 NS	0.085 NS	− 0.439 NS	− 0.056 NS
aGlu	< 0.001 NS	0.000	− 0.610 NS	< 0.001 NS
M6pi	− 0.295 NS	− 0.443 NS	− 0.159 NS	− 0.286 NS
Peplt-1	− 0.541 NS	0.000	0.000	0.000
Pgi	0.020 NS	− 0.003 NS	0.052 NS	− 0.638 NS
Pgm-1	− 0.179 NS	− 0.203 NS	− 0.512 NS	− 0.505 NS
To	0.051 NS	0.000	0.000	0.000
Tpi	0.000	0.000	0.000	0.133 NS

a substantial number of backcrosses were occurring in every generation, the injection of alien alleles into the gene pool of each species should cause genotype frequencies to deviate from Hardy–Weinberg expectations. This was not the case. No statistically significant deviations could be detected in any of the populations (Table 6). Appreciable hybridization should also have caused gametic disequilibrium between loci, especially those that differed between species in allele frequencies, because offspring of hybrids are likely to carry alien alleles in more than one locus (Campton 1987). Unlike deviations from Hardy–Weinberg expectations, which would disappear in one generation of random mating, gametic disequilibria decay only gradually (Campton 1987, 1990; Väinölä and Hvilsom 1991). However, as Table 7 indicates, only one significant deviation from random association of alleles in different loci was observed. This case, in the comparison of *M6pi* and *Pgm-1* of *Diadema savignyi* was clearly caused by the distortion of χ^2 values due to low expected cell frequencies, because both of these loci had many alleles, each with low frequency (Table 2). It should be kept in mind, however, that lack of significant deviations from Hardy–Weinberg equilibrium and the absence of gametic disequilibrium constitute only circumstantial evidence against the hypothesis that introgression is extensive, since both analyses have low statistical power (Ward and Sing 1970; Brown 1975; Lessios 1992; Zapata and Alvarez 1992, 1993).

The conclusion that hybridization between Indo-Pacific species of *Diadema* is limited also emerges from the low proportions of putative hybrid morphotypes in Japan, Indonesia, and Papua New Guinea, (Table 8). The only region in which the Type 1 morphotype, that of F_1 hybrids between *D. setosum* and *D. savignyi* was found to exceed 1% is Shirahama, where both species were abundant, very intermingled and had overlapping lunar spawning cycles (Pearse and N. Kobayashi unpublished data). The same region, however, had a very low incidence of the other two suspected hybrid morphotypes, a small proportion of which represents (by our electrophoretic evidence) backcrosses or F_2 hybrids. Relatively high proportions of Type 2 morphotypes were found in three populations in the Madang Barrier Reef, but no clear Type 3 morphotypes were seen in either Indonesia or Papua New Guinea. Many of the suspected hybrids in Madang, however, had small indistinct spots on their interambulacra and could thus be said to be closer to Type 3 individuals.

Table 7 *Diadema paucispinum*, *D. savignyi* and *D. setosum*. Analysis of linkage disequilibrium using Burrows's composite measure (Weir 1979) in individuals of "pure" morphology of each species and every locus combination for which it could be performed. Australian population of *D. setosum* was not included. Individuals carrying alleles with frequencies <0.01 have been ignored. Values for global χ^2 for entire locus combinations are shown. Probabilities have been adjusted for number of tests performed with Holmes's sequential Bonferroni test (Rice 1989) (*N* number of comparisons; *r* common correlation coefficient; *df* degrees of freedom; * $p < 0.05$)

Species, loci	(*N*)	*r*	χ^2	*df*	*p*
D. paucispinum					
Acph-M6pi	(12)	0.457	5.71	1	NS
Acph-Mdh-1	(21)	0.330	3.21	1	NS
Acph-Peplt-1	(21)	0.142	1.76	2	NS
Acph-Pgi	(16)	0.049	0.06	1	NS
Acph-Pgm-1	(20)	0.350	12.18	3	NS
M6pi-Mdh-1	(12)	0.047	0.05	1	NS
M6pi-Peplt-1	(12)	0.234	2.95	2	NS
M6pi-Pgi	(8)	0.623	4.64	1	NS
M6pi-Pgm-1	(12)	0.198	2.82	3	NS
Mdh1-Peplt-1	(21)	0.221	3.20	2	NS
Mdh1-Pgi	(16)	0.153	0.30	1	NS
Mdh1-Pgm-1	(20)	0.119	1.65	3	NS
Peplt-1-Pgm-1	(20)	0.165	7.54	6	NS
Pgi-Pgm-1	(15)	0.317	6.55	3	NS
D. savignyi					
Acph-M6pi	(17)	0.145	1.68	3	NS
Acph-Pgm-1	(16)	0.390	8.27	3	NS
M6pi-Pgm-1	(12)	0.427	35.59	9	*
D. setosum					
Acph-aGlu	(25)	0.236	3.18	1	NS
Acph-M6pi	(18)	0.153	1.51	2	NS
Acph-Pgi	(21)	0.053	0.15	2	NS
Acph-Pgm-1	(20)	0.325	11.61	2	NS
aGlu-M6pi	(20)	0.242	3.24	2	NS
aGlu-Pgi	(24)	0.143	2.17	2	NS
aGlu-Pgm-1	(23)	0.234	8.09	2	NS
M6pi-Pgi	(19)	0.134	1.91	4	NS
M6pi-Pgm-1	(18)	0.249	10.10	4	NS
Pgi-Pgm-1	(20)	0.157	4.49	4	NS

Table 8 *Diadema savignyi* and *D. setosum*. Percent of the two species and three suspected hybrid morphotypes in various populations in tropical west Pacific (see "Materials and methods" for definition of the three morphotypes (*N* number of individuals counted; + additional *Diadema* spp. were present, but were not included in sample) At Sesoko Island, two counts were performed at different times, but they may have included many of same individuals

Site	(*N*)	*D. savignyi*	*D. setosum*	Type 1	Type 2	Type 3
Shirahama, Honsu Island, Japan						
Kamiyaka-ta	(102)	56.9	42.2	1.0	0	0
Okinoshima Island	(114)	62.3	35.1	2.6	0	0
Tô Island	(291+)	31.6	67.0	1.0	0	0.3
Tanaka Island	(246+)	27.2	72.4	0.4	0	0
All localities	(753)	38.2	60.6	1.1	0	0.1
Okinawa, Ryukyu Islands, Japan						
Minna Island	(26)	57.7	34.6	0	0	7.7
Sesoko Island (Apr)	(282+)	8.5	91.5	0	0	0
Sesoko Island (Aug)	(351)	6.0	94.0	0	0	0
Motobu Harbor	(166)	30.0	68.7	0.6	0	0.6
Minatogawa	(269+)	5.6	94.4	0	0	0
All localities	(1094)	11.4	88.2	0.1	0	0.3
Iriomote Island, Japan						
Amitori Bay	(49)	83.7	12.2	0	0	4.1
NE Sulawesi/Sangihe Islands, Indonesia						
Bitung	(189)	11.6	87.8	0.5	0	0
Pahepa Island	(544)	98.9	0.9	0	0.2	0
Pasige Island	(318)	65.4	33.0	0.3	1.3	0
Cape Torowitan	(178 +)	33.7	65.7	0	0.6	0
All localities	(1339)	67.4	32.0	0.2	0.5	0
Madang Barrier Reef, Papua New Guinea						
Madang Resort	(567)	32.6	67.4	0	0	0
Kranket Island	(793+)	27.9	71.9	0.1	0	0[a]
Nagada Harbor	(56)	7.1	57.1	3.6	32.1	0
Demasa Island	(183)	0	91.3	0	8.7	0
Malapau Island	(29)	0	82.8	0	17.2	0
All localities	(1628)	25.2	72.2	0.2	2.4	0

[a] One individual of another possible hybrid morphotype was noted. It had an orange anal ring, no white spots, a pattern of blue dashes, and spoon-shaped pedicellariae similar to those of *D. savignyi*

Examination of the gonads of these suspected hybrids indicated that they were not in reproductive synchrony with either *D. setosum* or *D. savignyi* populations nearby (Pearse unpublished data). One dark individual, with no interambulacral white spots but with an orange anal ring and a pattern of blue dashes, was found in Kranket Island. This individual resembles two others noted in Shirahama outside the counts on which Table 8 is based. These specimens suggest that there may be an additional intermediate morphotype, and that the Madang Barrier Reef should receive further examination.

Discussion

This is the first time that two species of echinoids have been unequivocally demonstrated to hybridize in nature. Our data from the Ryukyu Islands indicate that the single individual judged to be morphologically most intermediate between *Diadema setosum* and *D. savignyi* is also genetically very likely to be an F_1 hybrid, and that some (although by no means all) of the other specimens which could not be easily assigned to either species are either F_2 (or later-generation) hybrids or backcross progeny. Thus, at least some of the previous reports, based on morphology, of echinoid hybrids in nature (e.g. Mortensen 1940, 1943; Vasseur 1952; Swan 1953; Mayr 1954; Hagström and Lönning 1961; Kelso 1970; Strathmann 1981; Palumbi and Metz 1991) are likely to have identified true hybrids as well, and suggest that natural hybridization between sympatric species of echinoids does take place.

Do the three *Diadema* species hybridize extensively enough for introgression to be important in their evolution? The existence of individuals that could be F_2 (or later-generation) hybrids between *D. setosum*, *D. savignyi* and *D. paucispinum* or progeny of backcrosses implies that F_1 hybrids are viable to sexual maturity and capable of reproduction. This is consistent with T. Uehara's finding that artificially produced hybrids between *D. setosum* and *D. savignyi* developed into males that spawned motile sperm (T. Uehara, personal communication to J.S. Pearse). Despite this, however, levels of introgression appear to be low. The genetic data suggest strongly that little interspecific gene flow occurs between *D. setosum* and the other two species. Although 8 of the 21 individuals of intermediate morphology sampled electrophoretically appear to have a *D. setosum* progenitor, and although the data suggest that back-crossing does occur, the existence of three loci in which individuals of pure *D. setosum* morphology have completely different alleles from the other

two species clearly indicates that such crosses are infrequent, or that their offspring have reduced viability or fertility. The same conclusion, however, cannot be reached as easily from the genetic data with respect to the amount of introgression that might be taking place between *D. savignyi* and *D. paucispinum*, because only one semi-diagnostic locus distinguishes these species and because F_{ST} estimates of gene flow indicate an exchange of more than one propagule per generation.

To decide whether the genetic similarity of *Diadema savignyi* and *D. paucispinum* results from recent common descent or from reticulate evolution, one needs to consider the possibility of their geographic overlap, because cross-fertilization between gametes can only result from adults of the two species spawning in close proximity to each other. There are no published reports of *D. savignyi* in Hawaii, but Clark (1954) mentions three specimens of *D. paucispinum* from Onota Atoll in Kiribati (Gilbert Islands). These, along with another specimen also identified by A.H. Clark as *D. paucispinum*, were deposited in the National Museum of Natural History of the United States. Clark provided no information on the characters he used to identify these specimens. One of us (JSP) examined one of these specimens (USNHM #EO-8465). No tridentate pedicellariae could be found. The specimen bore no evidence of spine removal, necessary to count the plates (a semi-diagnostic character). Clark examined preserved specimens, so he could not have observed whether there were blue lines on the test. It is possible that Clark's specimens belonged to *D. savignyi*, which predominates in Polynesia (Pearse unpublished data). Nevertheless, our own data from the Ryukyu Islands are consistent with the hypothesis that *D. paucispinum* may be more widespread than previously thought. The reason *Peplt-1* is not entirely diagnostic between *D. savignyi* (or *D. setosum*) and *D. paucispinum* is that the latter species carries, in relatively low frequencies, alleles that are common in the other two species (Table 2). However, Alleles *Peplt-1*^{90} and *Peplt-1*^{95} were never observed in "pure" *D. setosum* or *D. savignyi* and should thus be considered as characteristic of *D. paucispinum*. The presence in the Ryukyu samples of a Type 3 specimen that lacked blue lines and was homozygous for *Peplt -1*^{95} and of three others carrying the *Peplt -1*^{90} allele indicates that *D. paucispinum* may occasionally be present in the Ryukyus Islands, and that it may cross with *D. setosum* and *D. savignyi*. It may be that *D. paucispinum* is primarily allopatric from the other two species, but its larvae are occasionally carried by the North Equatorial Current from Hawaii to Japan, where they reach sexual maturity and hybridize with the locally much more abundant *D. setosum* and *D. savignyi*. This influx does not result in homogenization of the *D. paucispinum* and *D. setosum* gene pools; it is rather unlikely to have this effect with regards to *D. savignyi* either. According to this logic, the genetic similarity between *D. savignyi* and *D. paucispinum* is more likely to reflect a recent split in their phylogency than reticulate evolution, a conclusion also supported by the existence of close but distinct mitochondrial DNA lineages in each of these species (Lessios et al. 1996).

That no major introgression is occurring between any of the species, at least in Okinawa, is also suggested by the population-genetics analyses of our data. Although tests for deviations from Hardy–Weinberg and di-locus gametic equilibria cannot be taken as strong evidence for lack of introgression, their lack of significance in *Diadema* spp. is consistent with the other evidence in suggesting low gene flow between the species. Finally, the low incidence of suspected hybrid morphotypes in all localities of the western tropical Pacific also suggests that hybridization between the species is infrequent, particularly if it is kept in mind that only a small proportion of Type 2 and Type 3 morphotypes could be shown to be genetic hybrids. Thus, despite occasional events of hybridization between sympatric species, reticulate evolution is probably not of major consequence in the genus *Diadema*. How the two sympatric species maintain their separate genetic identities despite the presence of viable and non-sterile hybrids and the lack of any obvious prezygotic isolation mechanism (Uehara et al. 1990) remains to be determined.

Acknowledgements V. Pearse, D. Pearse, R. Chock and A. Tomita helped with the collections; M.G. Hadfield, G. Walker and F.A. Neva made transportation of frozen specimens from the western Pacific to Panamá possible; K. Yamazato and T. Uehara provided invaluable help in Okinawa, and P. Crewes facilitated the trip to Indonesia. A. Calderon, L. Calderon, A. Graybeal and M. Soriano helped in the laboratory, and E. Bermingham, B. Kessing and G. Pogson commented on the manuscript. D. Pawson permitted examination of specimens at the US National Museum of Natural History. P. Garnier-Gere provided a copy of his program LINKADES. The work was supported by funds from the Molecular Evolution Program of the Smithsonian Institution and by NSF Grang INT-8817807 and a Christensen Research Institute Fellowship to JSP.

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