

Hybrids of Two Closely Related Tropical Sea Urchins (Genus *Echinometra*): Evidence Against Postzygotic Isolating Mechanisms

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Abstract. A series of cross-fertilization experiments were conducted with two unnamed, sympatric species of sea urchins in the *Echinometra mathaei* species complex, *Echinometra* sp. A (Ea) and *Echinometra* sp. C (Ec). Heterogametic fertilization success was high when eggs of Ec and sperm of Ea were involved, and low with eggs of Ea and sperm of Ec. Hybrids produced from crosses in either direction developed normally to sexually mature adults; Ea × Ea were largest in test size, followed by Ec (ova) × Ea (sperm), Ea (ova) × Ec (sperm), and Ec × Ec, respectively. Color patterns of the hybrids were closer to the maternal coloration, whereas other characters such as relative test dimensions and spine lengths, morphology of tubefoot and gonad spicules, and gamete sizes were intermediate. Fertilization rates in F₁ backcrosses were high, minimizing the possibility that hybrid infertility is a postzygotic mechanism of reproductive isolation. On the other hand, intensive surveys failed to find individuals with hybrid characteristics in the field, suggesting that natural hybridization between the two species is rare. Prezygotic isolating mechanisms, such as microhabitat separation and gamete incompatibility, at least between Ea eggs and Ec sperm, most likely maintain the genetic integrity of these two closely related species.

Introduction

Recent morphological, biochemical, ecological, and reproductive studies have revealed the presence of four sympatric biological species of sea urchins within the *Echi-*

nometra mathaei, *sensu lato*, species complex at Okinawa, Japan. These species have been referred to as *Echinometra* species A, B, C, and D (e.g., Uehara and Shingaki, 1985; Uehara *et al.*, 1990, 1991; Arakaki and Uehara, 1991; Matsuoka and Hatanaka, 1991; Nishihira *et al.*, 1991; Palumbi and Metz, 1991; Palumbi, 1996, 1998; Aslan and Uehara, 1997; Palumbi *et al.*, 1997). *E.* sp. B is now recognized as *E. mathaei* (de Blainville, 1825), *sensu stricto*, (Arakaki *et al.*, 1998), while *E.* sp. D belongs in the *E. oblonga* (de Blainville, 1825) species complex, which may include at least three species (Arakaki and Uehara, 1999).

In sympatric echinoids like these species on Okinawa, interspecific reproductive isolation can result from different reproductive characteristics, such as timing and sites of spawning or gametic incompatibility (Lessios and Cunningham, 1990; Uehara *et al.*, 1990; Palumbi and Metz, 1991; Metz *et al.*, 1994; Palumbi, 1994, 1998; Vacquier *et al.*, 1995; Aslan and Uehara, 1997; Lessios, 1998; McCartney *et al.*, 2000). Of these factors, gamete incompatibility, preventing gametes of different species from fertilizing, may be particularly important for maintaining reproductive isolation of many animals (Dobzhansky *et al.*, 1977), including echinoderms (Palumbi and Metz, 1991; Byrne and Anderson, 1994; Vacquier *et al.*, 1995; Palumbi, 1998). On the other hand, postzygotic mechanisms such as the production of nonviable or infertile hybrids also could lead to and maintain reproductive isolation in various species (Coyne, 1992; Knowlton, 1993), including those of sea urchins (Chen and Baltzer, 1975; Lessios and Cunningham, 1990; Lessios, 1998; McCartney *et al.*, 2000).

Of the four species on Okinawa, two, Ea and Eb, appear to be reproductively isolated by gametic incompatibility,

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Table 1

Echinometra sp. A and *Echinometra* sp. C: summary of characters relevant to identification and reproductive isolation

Character	<i>Echinometra</i> sp. A	<i>Echinometra</i> sp. C	Sources*
Habitat	Moat and tide pools	Burrows in reef margin	1, 2, 6
Bathymetric range	Intertidal, below mean low water	Intertidal, above mean low water	1, 2, 6
Salinity and thermotolerance	Lower tolerance to sudden temperature and salinity changes	Higher tolerance to sudden temperature and salinity changes	4, 5
Body size	Biggest among Okinawan <i>Echinometra</i>	Moderate among Okinawan <i>Echinometra</i>	3
Wet weight (g)	50.8 ± 12.8	39.2 ± 8.7	7
Test length (mm)	47.4 ± 4.6	43.3 ± 0.1	7
Spine length (mm)	22.5 ± 1.1	16.6 ± 0.9	7
Spine color	Entirely white to greenish, or brownish-black, with white tip; distinct basal white ring	Entirely green, brown, or greenish-brown, without white tip; basal translucent, white ring.	3
Tubefoot spicules	Bihamate (C-like)	Triradiate	3
Gonadal spicules	Spindle shaped	Triradiate, curved triradiate, and bihamate	3
Breeding season	April–December (max. around late September)	April–December (max. around late September)	4, 5
Egg diameter (μm)	66.9 ± 1.3	71.8 ± 1.4	7
Sperm head length (μm)	3.9 ± 0.6	6.4 ± 0.7	7
Jelly layer thickness (μm)	23.9 ± 3.2	18.1 ± 3.5	7

* Data sources: 1, Tsuchiya and Nishihira (1984); 2, Tsuchiya and Nishihira (1985); 3, Uehara and Shingaki (1985); 4, Arakaki (1989); 5, Arakaki and Uehara (1991); 6, Nishihira *et al.* (1991); 7, this study; measurements from 25 adult individuals of each species examined for each character, mean ± standard deviation.

because hybrids are not formed when their gametes are mixed (Uehara *et al.*, 1990; Metz *et al.*, 1994). On the other hand, two other species, Ec and Ed, readily form hybrids when their gametes are mixed, while all other combinations between the species show high asymmetries in fertilization (*i.e.*, ova of one species are readily fertilized by sperm of another species, but not *vice versa*) (Uehara *et al.*, 1990). Fertilization asymmetry also has been noted for the species of *Echinometra* in the Caribbean and eastern Pacific (Lessios and Cunningham, 1990). Although fertilization asymmetry indicates gamete incompatibility in one direction, there may be little or no gamete incompatibility in the other direction, allowing for ready hybridization. Because of such a possibility for hybridization, Lessios (1998) and McCartney *et al.* (2000) suggested that postzygotic mechanisms are important in maintaining the genetic integrity of these species in the field.

Sequence data of the cytochrome oxidase I gene indicates that the four species of *Echinometra* on Okinawa are of recent origin, considerably less than 3 million years old (Palumbi, 1996), and similar to the time of divergence between the Atlantic and eastern Pacific species of *Echinometra* (McCartney *et al.*, 2000). Moreover, allozyme evidence indicates that among the four species on Okinawa, Ea and Ec are the most closely related to each other, while Ea and Ed are the most divergent (Matsuoka and Hatanaka, 1991). Experimental hybridization and rearing experiments by Aslan and Uehara (1997) reported that Ed ova were readily fertilized by Ea sperm, but Ea ova were much less able to be fertilized by Ed sperm. Hybrids resulting from crosses in both directions developed normally into fertile

adults, and Aslan and Uehara (1997) suggested that introgression between Ea and Ed was probably minimized by prezygotic mechanisms, particularly separation of their respective microhabitats. Rahman *et al.* (2000) also reported asymmetrical fertilization in Ea × Ec crosses, with Ec ova being more readily fertilized by Ea sperm than Ea ova were fertilized by Ec sperm. Moreover, they found that the viability and growth of Ec (ova) × Ea (sperm) hybrids was similar to that of conspecifics, while that of Ea (ova) × Ec (sperm) hybrids was significantly lower. We report here further details of hybrids between these two species. In spite of their close relationship, individuals of Ea and Ec can be readily distinguished from each other by differences in adult morphology, as well as microhabitat preference (Table 1). On the other hand, their breeding seasons overlap extensively (Arakaki and Uehara, 1991) and because of the proximity of their microhabitats (Nishihira *et al.*, 1991), they are likely to have opportunities to hybridize in the field.

Materials and Methods

Mature adults of Ea (identified by their brownish dark test and white-tipped spines), and Ec (greenish test, spines without white tips, but with white basal ring distinguishing it from Eb) (Uehara, 1990; Arakaki *et al.*, 1998) were collected from the Sunabe coast of Okinawa Island (26°07' N; 127°46' E) at low tide within their natural breeding season from early May to late October, 1996 and 1997. Specimens were immediately transported to the laboratory, where they were maintained in closed aquaria until spawned. Cross-fertilization of the two species of *Echinometra* was con-

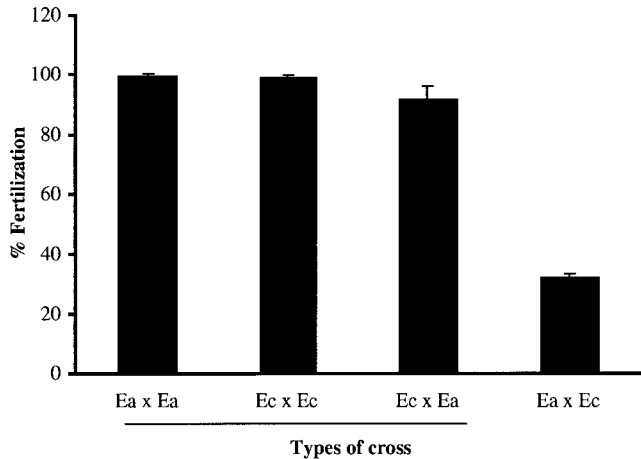


Figure 1. Mean percentage of eggs fertilized in conspecific and heterospecific crosses of *Echinometra* sp. A (Ea) and *Echinometra* sp. C (Ec); maternal species named first. Calculated from number of embryos in the 2- to 4-cell stage counted in 100 eggs 1.5 hours after gamete mixing. A total of 13 replicate crosses were done using gametes from new individuals each time; error bars indicate 1 standard deviation. Fertilization rate of Ea \times Ec significantly different from those of Ea \times Ea, Ec \times Ec, and Ec \times Ea (ANOVA, $P < 0.01$), as indicated by the horizontal bar. Data from Rahman *et al.* (2000).

ducted using all possible combinations of ova and sperm at room temperatures (26–28°C) (Rahman *et al.*, 2000). For consistency, when referring to heterospecific crosses, the maternal species is named first. As found earlier by Uehara *et al.* (1990), fertilization was high for the conspecific and Ec \times Ea crosses, but significantly lower for the Ea \times Ec crosses (Fig. 1). The juveniles produced from these crosses were used in the work reported here.

The embryos and larvae were reared through metamorphosis, as described by Rahman *et al.* (2000). Larval survival was 68%–73% for the conspecific and Ec \times Ea crosses, and 50% for the Ea \times Ec crosses. Larval body form and development of both conspecific controls and reciprocal hybrids were similar to each other, and no particular abnormalities were observed. After 20–24 days, larvae were placed in small (25 \times 20 \times 10 cm) aquaria with aerated, filtered seawater, and pieces of coralline algal skeletons were added to induce settlement and metamorphosis. Metamorphosis rate was high (84%–89%) and not significantly different for the conspecific and Ec \times Ea larvae, and lower (67%) for the Ea \times Ec hybrids (Rahman *et al.*, 2000). Seawater was partially changed once a week with fresh, filtered seawater, and coralline-algal-encrusted pieces of coral skeletons were provided as food. By 3 months, 65%–69% of the conspecific and Ec \times Ea juveniles, and 57% of the Ec \times Ea juveniles remained; they were all 6.0–7.0 mm in test diameter.

The cultures were continued for one year, by which time the animals were sexually mature; survival at one year was 78%–84% for the conspecifics and Ec \times Ea hybrids, and

70% for the Ea \times Ec hybrids (Rahman *et al.*, 2000). Then they were spawned, and their gametes were used for reciprocal back crosses to determine fertility among both hybrids and conspecifics. After the animals were spawned, data on animal weight, test dimensions, spine lengths, spicule characteristics, body and spine coloration, gamete sizes, and other specific characters of all the animals were recorded and compared among the treatments. The tests of echinoids in the family Echinometridae are oblong, so both length and width, as well as height, were measured. Spicule types in both the tube feet and gonads were counted in 10 replicate samples from 20 different individuals of each cross (80 animals; 800 counts). Gametes were measured using a phase contrast microscope following Amy (1983) (eggs at 400 \times in a slide well; sperm at 1000 \times on a flat slide).

Percentages were arcsine transformed to normalize the data and reduce heterogeneity in variances. Homogeneity of variances was analyzed by a Bartlett test (Bartlett, 1937); when variances were not significantly heterogeneous and showed no major departure from normality, a one-way analysis of variance (ANOVA) was done, followed by Duncan's multiple range test (Duncan, 1955). The level for statistical significance was set at 0.05. Untransformed values are presented in the tables and figures.

To examine the occurrence of hybridization between Ea and Ec in the field, searches for specimens with hybrid morphology were conducted along the Sunabe coast of Okinawa and the coast of Sesoko Island, where both species occur close together (within 1–2 m) in adjacent, interdigitating microhabitats.

Results

Comparisons of laboratory-reared adult Echinometra sp. A, Echinometra sp. C, and their reciprocal hybrids

Test length of animals 1 year after metamorphosis (Table 2) was less than for field-collected animals (Table 1), which were probably several years old. On the other hand, spine lengths of the experimental animals were greater than those of the field-collected animals, which live in rock depressions under turbulent conditions and experience considerable spine-tip abrasion.

Mean values for wet weight, test length, width, height, and spine length were greatest in Ea \times Ea progeny and smallest in Ec \times Ec progeny (Table 2). All these parameters were significantly different between Ea \times Ea and Ec \times Ec, and between each of these and the hybrids. On the other hand, there were no significant differences ($P > 0.05$) between Ea \times Ec and Ec \times Ea. Measurements in both of the reciprocal hybrids were closer to those of Ea \times Ea than to Ec \times Ec. These measurements mirror the different growth rates found for these animals by Rahman *et al.* (2000).

The aboral body coloration differed between conspecifics and hybrids (Fig. 2, upper). In Ea \times Ea specimens, test

Table 2

Weight, test size, and spine length comparison of *Echinometra* sp. A (*Ea* × *Ea*), *Echinometra* sp. C (*Ec* × *Ec*), and their reciprocal hybrids 1 year after metamorphosis

Character	<i>Ea</i> × <i>Ea</i>	<i>Ea</i> × <i>Ec</i>	<i>Ec</i> × <i>Ea</i>	<i>Ec</i> × <i>Ec</i>
Wet weight (gm)	14.7 ± 0.7 ^a (14.6–14.8)	13.6 ± 0.2 ^b (13.6–13.7)	13.9 ± 0.5 ^b (13.8–14.0)	9.2 ± 0.5 ^c (9.1–9.3)
Test length (mm)	29.4 ± 0.8 ^{a*} (28.2–31.0)	28.4 ± 0.9 ^b (27.0–30.0)	28.7 ± 0.8 ^b (27.5–30.5)	24.2 ± 1.1 ^c (23.0–26.0)
Test width (mm)	27.3 ± 0.8 ^a (26.1–29.0)	26.3 ± 0.9 ^b (25.0–28.1)	26.5 ± 0.8 ^b (25.3–28.5)	22.2 ± 1.0 ^c (21.0–23.9)
Test height (mm)	14.2 ± 0.4 ^a (13.6–15.0)	13.5 ± 0.4 ^b (12.8–13.9)	13.6 ± 0.3 ^b (13.1–14.2)	12.0 ± 0.5 ^c (11.2–13.0)
Spine length (mm)	26.8 ± 1.0 ^a (24.4–28.2)	23.4 ± 0.7 ^b (22.3–24.9)	24.3 ± 0.6 ^b (23.4–25.5)	20.7 ± 0.7 ^c (19.3–21.9)

* Thirty adult specimens were measured for each treatment; mean ± SD, ranges in parentheses. Wet weight data from Rahman *et al.* (2000). Numbers in the same row having the same superscripts are not significantly different ($P > 0.05$).

color was dark brownish, and each spine had a white tip and a translucent white ring at its base. *Ec* × *Ec* specimens were uniformly greenish, and each spine was uniformly colored except for a faded basal white ring. *Ea* × *Ec* hybrids were more similar to *Ea* × *Ea* conspecifics, having dark brownish tests and white-tipped spines with a translucent white, basal ring. On the other hand, *Ec* × *Ea* hybrids were more similar to *Ec* × *Ec* conspecifics; the tests were uniformly deep greenish, and the spines had barely detectable white tips and basal white rings.

Orally (Fig. 2, lower), *Ea* × *Ea* conspecifics had white-tipped spines around the mouth and brownish dark tests, whereas *Ec* × *Ec* conspecifics had yellowish green spines around the mouth and a greenish test color. *Ea* × *Ec* hybrids were more similar to *Ea* × *Ea*, whereas *Ec* × *Ea* hybrids were more similar to *Ec* × *Ec*; that is, hybrid coloration was most similar to maternal coloration.

Tube foot spicules in *Ea* × *Ea* were always bihamate (C-shaped), whereas those in *Ec* × *Ec* were always triradiate (Fig. 3). Tube foot spicules of *Ea* × *Ec* hybrids were bihamate (58%), bihamate-like (17%), triradiate-bihamate (20%), and triradiate (5%), whereas those of *Ec* × *Ea* were bihamate (32%), bihamate-like (12%), triradiate-bihamate (44%), and triradiate (13%). Therefore, the tube foot spicule morphologies of the hybrids were intermediate and tended toward maternal affinities.

There was little intraspecific variation in gonadal spicule morphology (Fig. 4). The spicules in *Ea* gonads were almost all spindle-shaped (98%; other spicules seen: bihamate, <1%; irregular, 1%; no triradiates were found). In contrast, those in *Ec* gonads were nearly all triradiate (94%; other spicules seen: spindle, 5%; bihamate, 1%). Gonads in both *Ea* × *Ec* and *Ec* × *Ea* had high proportions of spindle-shaped spicules (62% and 46%, respectively), with spindle-like (16% and 11%), triradiate-spindle (13% and 25%), triradiate (7% and 15%), bihamate (1% in both), and irregular (1% in both) spicules in smaller proportions.

Gamete sizes of conspecific and hybrid individuals were different. Egg diameters of *Ea* × *Ea* were smallest among the four treatments, whereas the *Ec* × *Ec* eggs were largest

(Table 3). Hybrids contained intermediate-sized eggs that were significantly different from the eggs of either of the conspecifics. The sizes of sperm heads were also smallest in *Ea* × *Ea*, and were significantly different among all the crosses, including between the hybrids.

Existence of natural hybrids

The characteristics of reproductively mature hybrids are summarized in Table 4. Two hundred individuals with coloration more-or-less intermediate between the two species were collected from the field. However, detailed comparisons of spicule shapes and gamete sizes revealed that none actually had character combinations common to the experimentally obtained hybrids; that is, all could be assigned to either *Ea* or *Ec* on the basis of both spicule shapes and gamete sizes.

Similarly, Aslan and Uehara (1997) did not find any natural hybrids between *Ea* and *Ed* in the field, even though they were able to rear hybrids of these species in the laboratory.

Backcrosses

There was no indication that the hybrids were any less fertile than either of their conspecific half-siblings (Table 5). Eggs from both *Ea* × *Ec* and *Ec* × *Ea* yielded higher percentages of fertilization with *Ea* × *Ea* sperm (93% and 98%), than with *Ec* × *Ec* sperm (82.2% and 89.2%), similar to the findings with the parental crosses in which *Ea* sperm more readily fertilized *Ec* eggs than *Ec* sperm fertilized *Ea* eggs (Fig. 1). Moreover, backcrosses by sperm from males of *Ea* × *Ec* and *Ec* × *Ea* yielded higher percentages of fertilization with *Ec* × *Ec* ova (99.3% and 99.7%) than with *Ea* × *Ea* ova (84% and 86.5%); *Ea* ova appear to be more discriminating than *Ec* ova. The higher fertilization rates between the same types of hybrids *versus* the different types of hybrids (99.5% and 99% *versus* 95% and 86%) indicate the presence of a complex sorting of gamete compatibility genes.

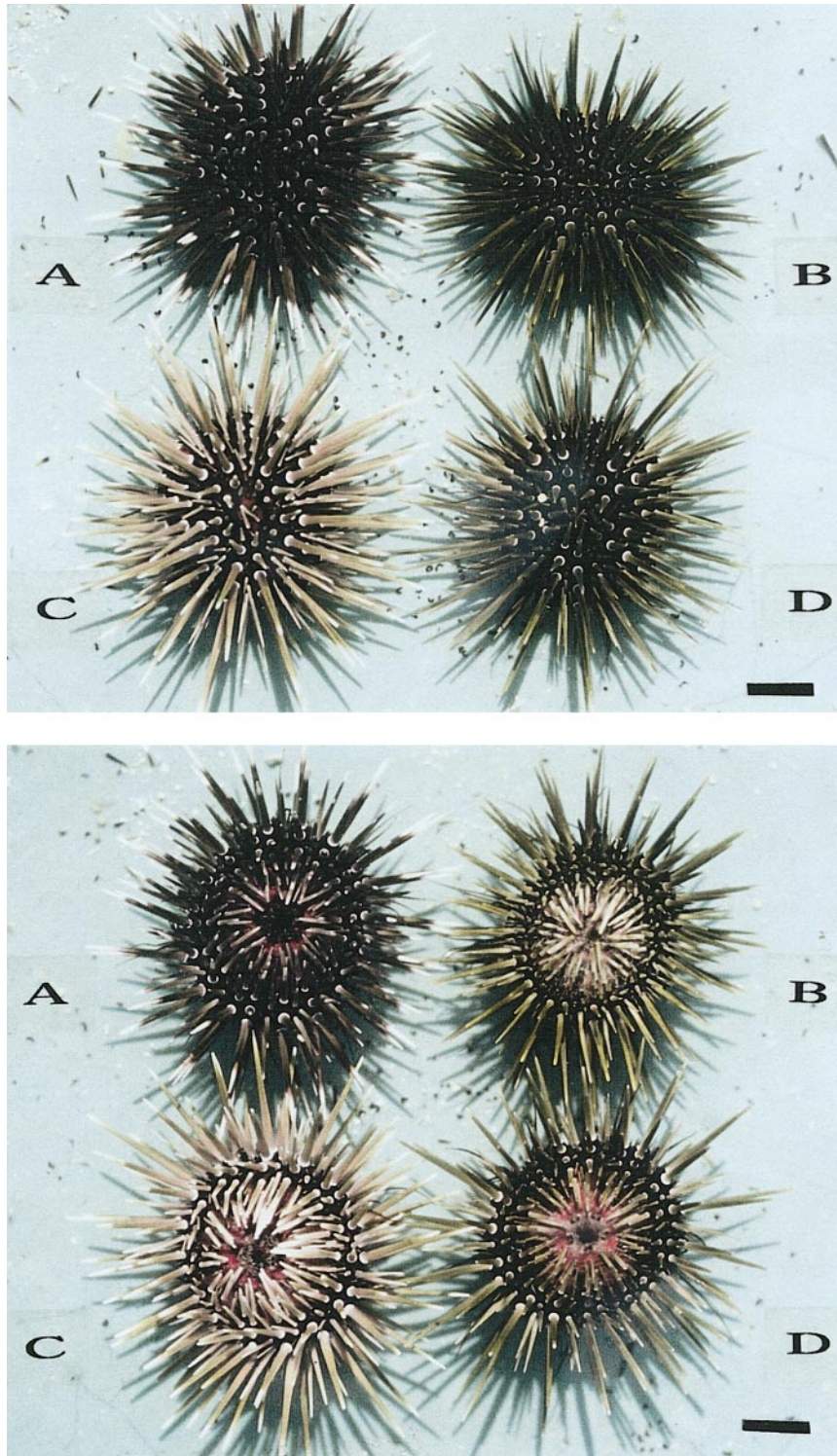


Figure 2. Aboral (upper) and oral (lower) color patterns of *Echinometra* sp. A and *E.* sp. C, and their reciprocal hybrids, 1 year after metamorphosis; maternal species named first. (A) Ea × Ea; (B) Ec × Ec; (C) Ea × Ec; (D) Ec × Ea.

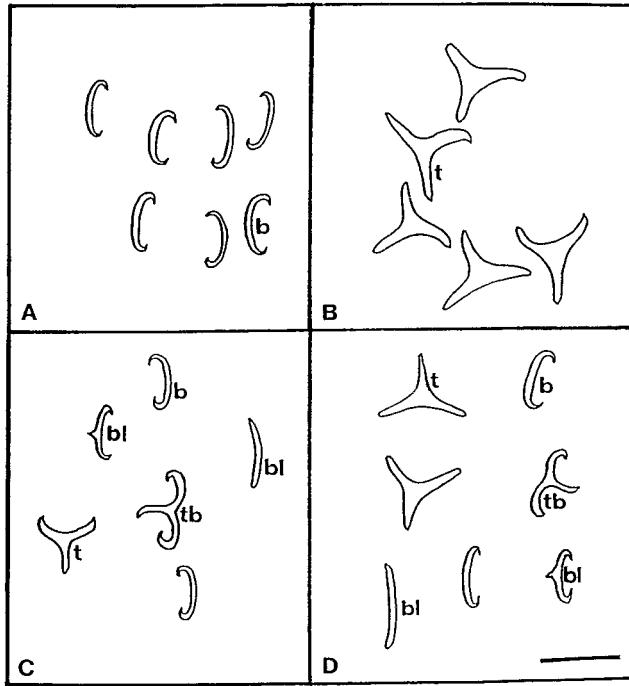


Figure 3. Morphology of tubefoot spicules of *Echinometra* sp. A and *Echinometra* sp. C and their reciprocal hybrids, 1 year after metamorphosis; maternal species named first. (A) Ea \times Ea; (B) Ec \times Ec; (C) Ea \times Ec; (D) Ec \times Ea. b, bihamate; bl, bihamate-like; t, triradial; tb, triradial-bihamate. Scale bar, 50 μ m.

Discussion

The crosses between Ea-Ec showed a distinct asymmetry in fertilization success, as reported previously by Uehara *et al.* (1990) and Rahman *et al.* (2000). Heterogamic fertilization rate was high when eggs of Ec were mixed with sperm of Ea, but much lower when eggs of Ea were mixed with sperm of Ec. The reduction in fertilization with the Ea egg \times Ec sperm crosses indicates the presence of a protein-binding system for gamete recognition, as reported by Metz *et al.* (1994), and Metz and Palumbi (1996). Such a system might eventually lead to gamete incompatibility and reproductive isolation, and thus provide a mechanism for maintaining species integrity, as proposed by Metz *et al.* (1994), Vacquier *et al.* (1995), Metz and Palumbi (1996), and Palumbi (1998). However, with the high fertilization of Ec eggs by Ea sperm, prezygotic isolation by gamete incompatibility hardly appears to be present between these two species, or among most of the other species of *Echinometra* on Okinawa, most of which, like the two sympatric species of Caribbean *Echinometra*, also show high asymmetry in fertilization among hybrid crosses (Uehara *et al.*, 1990; Lessios and Cunningham, 1990). Consequently, it seems unlikely that gamete incompatibility, by itself, provides a mechanism for reproductive isolation in *Echinometra*. Reproductive isolation and speciation apparently occurred be-

fore gametic incompatibility closed off the possibility of gene flow, and as in two sympatric species of asteroids in the genus *Patriella* that do not have gamete incompatibility (Byrne and Anderson, 1994), other mechanisms need to be found to explain how species integrity is maintained in these closely related, interfertile species.

Furthermore, the Ea-Ec crosses had higher fertilization rates in the F₁ backcrosses than Aslan and Uehara (1997) found for Ea-Ed F₁ backcrosses. The higher fertilization rate may be due to the higher genetic similarity between Ea and Ec than between Ea and Ed, as proposed by Matsuoka and Hatanaka (1991). If this is true, it provides evidence for the eventual evolution of complete gamete incompatibility in these species. Supporting the idea of a transition of gamete compatibility to incompatibility after speciation, Uehara *et al.* (1990) reported that fertilization between Ec and Ed was high in both directions, while crosses between Ea and Eb were completely infertile; other combinations among the four species were asymmetrical. Whether these differences reflect evolutionary distances among the species, or indicate that other factors—such as some sort of reinforcement selection coupled to microhabitat proximity—are involved, or

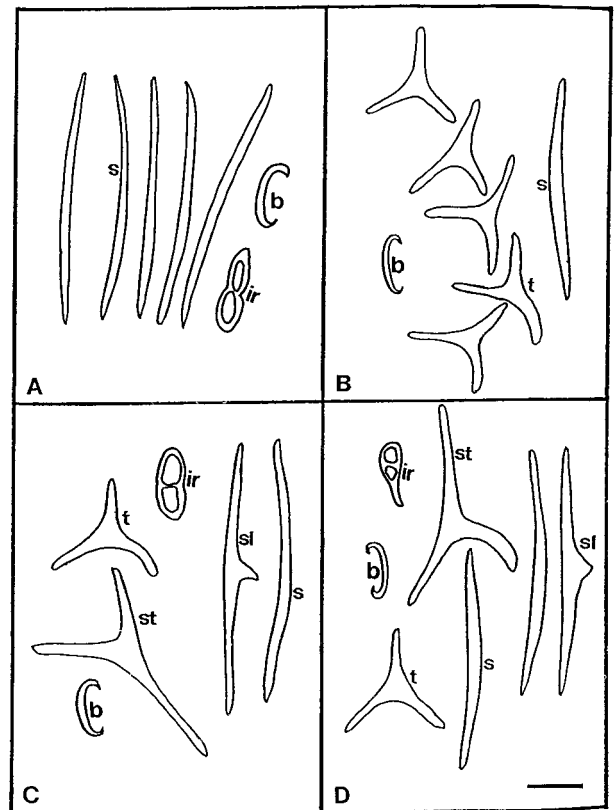


Figure 4. Morphology of gonadal spicules of *Echinometra* sp. A and *Echinometra* sp. C and their reciprocal hybrids, 1 year after metamorphosis; maternal species named first. (A) Ea \times Ea; (B) Ec \times Ec; (C) Ea \times Ec; (D) Ec \times Ea. b, bihamate; ir, irregular; s, spindle; sl, spindle-like; st, spindle-triradial; t, triradial. Scale bar, 100 μ m.

Table 3

Gamete sizes of *Echinometra* sp. A ($Ea \times Ea$), *Echinometra* sp. C ($Ec \times Ec$), and their reciprocal hybrids 1 year after metamorphosis

Measurement	$Ea \times Ea$	$Ea \times Ec$	$Ec \times Ea$	$Ec \times Ec$
Egg diameter	66.9 \pm 1.0 ^{a*} (65.0–67.5)	71.0 \pm 1.2 ^b (70.0–73.8)	70.2 \pm 1.1 ^b (67.5–72.5)	72.6 \pm 1.3 ^c (71.3–75.0)
Sperm head length	4.1 \pm 0.5 ^a (3.1–5.0)	5.7 \pm 0.7 ^b (4.3–6.8)	5.2 \pm 0.8 ^c (4.3–6.8)	6.5 \pm 0.8 ^d (5.0–7.4)

* Twenty individuals were examined from each cross with 25 eggs and 25 sperm from each individual; mean \pm SD in μm , ranges in parentheses. Figures in the same row having the same superscripts are not significantly different ($P < 0.05$).

suggest that gametic incompatibility in one or the other species “simply happened by accident” (Lessios and Cunningham, 1990, p. 938) remains to be determined.

The successful culturing of hybrids to fertile adults in laboratory conditions suggests that postzygotic isolating mechanisms, such as hybrid inviability or sterility, are not involved in maintaining species integrity. However, Rahman *et al.* (2000) found that $Ea \times Ec$ hybrids had lower larval survival, metamorphosis success, and juvenile survival than either $Ec \times Ea$ hybrids or conspecific half-siblings. Consequently, not only gamete incompatibility but postzygotic fitness parameters act against $Ea \times Ec$ hybrids. Nevertheless, the surviving $Ea \times Ec$ hybrids grow at the same rate as conspecifics (Rahman *et al.*, 2000), and as shown in the present study, are as fertile as the conspecifics in backcrosses. Moreover, $Ec \times Ea$ hybrids are as viable and fertile as the conspecifics, indicating that there are neither gametic nor postzygotic blocks to introgression for at least half of the possible hybrid events.

Table 4

Characterization of hybrids produced experimentally through cross fertilization between *Echinometra* sp. A (Ea) and *Echinometra* sp. C (Ec)

Distinctive features	$Ea \times Ec$	$Ec \times Ea$
Body color		
a. Oral	Ea-like	Ec-like
b. Aboral	Ea-like	Ec-like
Test sizes		
a. Length	Intermediate	Intermediate or Ea-like
b. Width	Intermediate	Intermediate or Ea-like
c. Height	Intermediate	Intermediate or Ea-like
Spines		
a. Length	Intermediate	Intermediate or Ea-like
b. Color	Ea-like with white tip or intermediate	Ec-like or intermediate with faint white spine tip
c. Milled ring color	Ea-like	Ea-like
Spicules		
a. Tubefoot	Intermediate and Ea-like	Intermediate and Ec-like
b. Gonad	Intermediate and Ea-like	Intermediate and Ea-like
Gametes		
a. Sperm sizes/form	Intermediate	Intermediate
b. Egg sizes	Intermediate	Intermediate

Besides gamete incompatibility, potential prezygotic mechanisms that may occur between these two species include ecological separation and asynchronous reproductive cycles (Mayr, 1970; Palumbi, 1994). The two species of sea urchins in this study, Ea and Ec , live relatively close to each other but occupy different microhabitats; Ea inhabits the calmer moat and tidepools of the reef-flat, generally below the mean low water level, whereas Ec inhabits burrows or crevices in the wave-swept intertidal of the reef margin (Table 1). These differences in microhabitats may be enough to prevent most chances of cross fertilization. In broadcast spawning invertebrates such as echinoids, for example, fertilization success drops dramatically with distance between spawning individuals (Pennington, 1985; Levitan, 1998a, b). However, individuals of Ea are occasionally found near and within the reef margin where Ec predominates, and it remains unclear, whether microhabitat separation, by itself, is sufficient to prevent introgression between the two species, or at least through Ec ova and Ea sperm where there is little or no gamete incompatibility, hybrid viability, or hybrid fertility.

In addition to the problem of being separated enough in adjacent microhabitat to prevent gamete mixing after spawning, there is the problem of maintaining microhabitat differentiation that could assure reproductive isolation. This problem is especially acute for species with long-lived, widely dispersing larvae, such as species of *Echinometra*. The different larvae would have to have exquisite settling cues that assured that they would be established in their exact microhabitats or suffer highly selective postsettlement mortality outside their particular microhabitats. There is little evidence of highly selective habitat selection in sea urchin larvae (Pearse and Cameron, 1991). Indeed, Rahman and Uehara (2001) found no discrimination in settling preferences by competent larvae of the four species of *Echinometra* on Okinawa; all showed settlement rates of 86%–89% on coralline red algae, and much lower rates on other algae. The mechanism resulting in and maintaining microhabitat differentiation among broadcast spawning species such as those of *Echinometra* in the tropical west Pacific remain unknown and unexplored.

Echinoid sperm are active for only a short time after spawning, much less than an hour in temperate species

Table 5

Percentage of eggs fertilized in backcrosses among laboratory-reared F_1 generation of conspecifics and hybrids of *Echinometra* sp. A (*Ea*) and *Echinometra* sp. C (*Ec*)

Sperm from	Eggs from			
	<i>Ea</i> × <i>Ea</i>	<i>Ea</i> × <i>Ec</i>	<i>Ec</i> × <i>Ea</i>	<i>Ec</i> × <i>Ec</i>
<i>Ea</i> × <i>Ea</i>	99.7 ± 0.5 (99–100)	93.0 ± 1.4 (91–95)	98.0 ± 1.4 (96–100)	86.8 ± 1.7 (84–88)
<i>Ea</i> × <i>Ec</i>	84.0 ± 1.6 (82–86)	99.0 ± 0.9 (98–100)	95.0 ± 1.4 (93–97)	99.3 ± 0.8 (98–100)
<i>Ec</i> × <i>Ea</i>	86.5 ± 1.9 (84–88)	86.0 ± 1.8 (83–88)	99.5 ± 0.8 (98–100)	99.7 ± 0.5 (99–100)
<i>Ec</i> × <i>Ec</i>	30.7 ± 2.0 (28–33)	82.2 ± 1.8 (80–84)	89.2 ± 2.1 (87–93)	99.8 ± 0.4 (99–100)

Each value represents six replicate crosses with gametes from different individuals in each replicate; mean ± SD, ranges in parentheses.

(Hinegardner, 1975; Levitan *et al.*, 1991), and probably even less in tropical species. Asynchrony in spawning, therefore, would ensure that these two species could exist in sympatry as separate species whether or not their gametes are capable of fertilization (Lessios, 1984). However, *Ea* and *Ec* mature at the same time and their spawning periods overlap extensively (Arakaki and Uehara, 1991; Table 1). Consequently, seasonal separation of spawning is not a mechanism of reproductive isolation. On the other hand, gametes may be released at different times of the day or specific pheromonal spawning cues may differ between the species, and these factors may prevent near simultaneous spawning that could lead to hybridization. Natural spawning in the field has not yet been observed.

The sperm concentration used in this study was probably much higher than would be encountered under usual natural conditions, especially considering the dilution that would occur if individuals were separated by 1 m or more (Pennington, 1985; Levitan, 1998b). However, Uehara *et al.* (1990) provide data showing that fertilization rates of eggs of both *Ea* and *Ec* mixed with sperm from *Ea* were similar over a wide range of sperm concentrations, whereas the sperm of *Ec* had very low fertilization success with *Ea* eggs, even at high concentrations, up to 10^{-2} . Consequently, it is equivocal whether inappropriate sperm concentrations were used in this study, and *Ea* sperm, at least, appear as likely to fertilize *Ec* eggs as *Ea* eggs over concentrations found in the field.

In addition, the eggs in this study were exposed to sperm at relatively high concentrations for 1.5 h, much longer than they would likely be so exposed in the field. Levitan *et al.* (1991) found that fertilization increases in sea urchins with time of exposure of eggs to sperm over a range of 10–60 s. It is possible, therefore, that fertilization rates would be lower in the *Ec* × *Ea* crosses if the gametes had been kept together for shorter times. Preliminary experiments indicate that fertilization reaches the asymptote in less than 2 min in conspecific crosses of *Ea* and *Ec*, within 4 min in *Ec* × *Ea* crosses, and by about 6 min in *Ea* × *Ec* crosses (Rahman, unpubl. data). Therefore, more extended exposure times,

like those used in the study, would not increase the fertilization rates. However, if gametes are spawned in the field at the same time by equal numbers of adjacent individuals of both sexes of both species, most conspecific gametes would probably be fertilized before much hybridization could occur. On the other hand, where the populations intermingle it seems as likely that a spawning *Ec* female could be closer to a spawning *Ea* male than to spawning *Ec* males, so that hybrids could be expected to occur.

To date there have been no experiments with mixed populations of gametes. Palumbi (1998) showed that at low concentrations where less than near 100% fertilization is achieved, sperm from different males of the same species of sea urchins fertilized different proportions of eggs from the same female, and eggs from different females were fertilized in different proportions by sperm from the same male. These differences were further related to differences in the sequence of the bindin alleles. This opens the possibility of some sort of interlocus antagonistic coevolution between gametes, as was proposed by Rice (1998) and which was demonstrated to be occurring in multiple-mating, internally fertilizing species such as crickets (Howard *et al.*, 1998). How such sperm competition could be achieved in broadcast spawning, externally fertilizing species such as sea urchins, if it occurs at all, remains to be determined. However, if it does occur, and if conspecific sperm are always at an advantage when mixed at low concentrations with sperm from another species because of more compatible gametes, a mechanism for maintaining species integrity in sympatric species may be present.

Some characteristics of the hybrid progeny found in the present study were mainly maternal. Color patterns of both hybrids, for instance, tended to be maternally inherited. Maternal inheritance of color pattern also was observed in hybrids between *Strongylocentrotus nudus* and *S. intermedius* (Osanaï 1974). On the other hand, other characteristics, such as spine length and spicule shapes, especially those from the gonads, showed intermediate features, and can be used to distinguish the hybrids from either parent. Such distinct hybrid phenotypes are important for finding hybrids

in the field (*e.g.*, Hagström and Lønning, 1961; Menge, 1986; Palumbi and Metz, 1991; Lessios and Pearse, 1996). We searched for distinctive intermediate phenotypes in the field, and although suggestive color morphs were found, examination of the spicules revealed no hybrids. Similarly, although hybrids with distinguishing characteristics can be formed in the laboratory between Ea and Ed, Aslan and Uehara (1997) were unable to find such hybrids in the field. Moreover, although two species of the tropical long-spined sea urchins, *Diadema savignyi* and *D. setosum*, readily hybridize in the laboratory (Uehara *et al.*, 1990) and often occur in mixed populations in the field (Pearse, 1998), genetic analyses, using allozyme analyses, showed that hybrids only occur rarely in the field and there is limited introgression (Lessios and Pearse, 1996). Genetic analyses using allozyme or DNA markers should now be done with sympatric species of *Echinometra* to determine whether some introgression is occurring that has not been detectable by morphological characters.

The adult hybrids of these two species were completely fertile, indicating that genetic differences between them are not large enough to cause developmental or gamete incompatibility, in spite of their morphological differences and microhabitat segregation. The high fertilization rates in backcrossing further suggests that they are genetically very close to each other. Similar results were obtained from crosses that produced fertile hybrids between Ea and the putatively more distantly related Ed (Aslan and Uehara, 1997). There is no reason to believe that fertile hybrids could not be produced in crosses between most other species of *Echinometra* on Okinawa, including between Ec and Ed, which show high fertilization with each other in both directions (Uehara *et al.*, 1990). Only Ea and Eb (= *Echinometra mathaei*) showed very low fertilization with each other in either direction, and may be isolated by gamete incompatibility (Uehara *et al.*, 1990; Metz *et al.*, 1994). These latter two species co-occur in pools and channels below mean low water on the reef flats of Okinawa, and they may have the greatest opportunity for gamete mixing in the field. Consequently, for these two species, gamete incompatibility may be necessary to maintain species integrity. Reproductive isolation of the other species, however, probably depends on additional prezygotic isolating mechanisms, such as microhabitat separation.

New biological species are formed when populations become reproductively isolated from other, previously conspecific populations. How and when reproductive isolation develops has yet to be established for broadcast spawning marine invertebrates such as sea urchins (Lessios, 1984; Knowlton, 1993; Palumbi, 1994). Although not isolated by full gamete incompatibility, or by hybrid inviability or infertility, Ea and Ec are both morphologically and genetically distinct, and hybridization between them appears to be either very low or nonexistent—that is, they appear to be

effectively reproductively isolated. The morphological distinctness and apparent genetic integrity maintained between them warrants their recognition as distinct biological species despite their ability to produce viable and fertile hybrids in the laboratory. They should be given taxonomic descriptions and appropriate species names that are distinct from *Echinometra mathaei*, *sensu stricto* (=Eb; Arakaki *et al.*, 1998) and Ed (=the Okinawan component of the *Echinometra oblonga* species complex; Arakaki and Uehara, 1999).

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