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Absence of postzygotic isolating mechanisms: evidence from experimental hybridization between two species of tropical sea urchins*

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Abstract: Two reef margin species of tropical sea urchins, *Echinometra* sp. C (Ec) and *Echinometra oblonga* (Eo), occur sympatrically on Okinawa intertidal reefs in southern Japan. Hybridization between these species was examined through a series of cross-fertilization experiments. At limited sperm concentrations, where conspecific crosses reached near 100% fertilization, both heterospecific crosses showed high fertilization rates (81%–85%). The compatibility of the gametes demonstrated that if gamete recognition molecules are involved in fertilization of these species, they are not strongly species-specific. We found that conspecific crosses reached peak fertilization levels much faster than did heterospecific crosses, indicating the presence of a prezygotic barrier to hybridization in the gametes. Larval survival, metamorphosis, and juvenile and adult survival of hybrid groups were nearly identical to those of their parent species. Hybrids from crosses in both directions developed normally through larval stages to sexually mature adults, indicating that neither gametic incompatibility nor hybrid inviability appeared to maintain reproductive isolation between these species. In adults, Ec×Ec crosses gave the highest live weight, followed by Eo (ova)×Ec (sperm), Ec (ova)×Eo (sperm), and Eo×Eo. Other growth performance measures (viz., test size, Aristotle's lantern length, and gonad index) of hybrid groups and their parental siblings showed the same trends. The phenotypic color patterns of the hybrids were closer to the maternal coloration, whereas spine length, tube-foot and gonad spicule characteristics, pedicellaria valve length, and gamete sizes showed intermediate features. Adult F_1 hybrids were completely fertile and displayed high fertilization success in F_1 backcrosses, eliminating the likelihood that hybrid sterility is a postzygotic mechanism of reproductive isolation. Conversely, intensive surveys failed to find hybrid individuals in the field, suggesting the lack or rarity of natural hybridization. This strongly suggests that reproductive isolation is achieved by prezygotic isolating mechanism(s). Of these mechanisms, habitat segregation, gamete competition, differences in spawning times, gametic incompatibility or other genetic and non-genetic factors appear to be important in maintaining the integrity of these species.

Key words: Sea urchins, *Echinometra*, Hybridization, Gamete compatibility, Reproductive isolation, Speciation
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1 Introduction

Significant differences in morphology, ecology, molecular phylogeny, mitochondrial DNA (mtDNA), and gamete compatibility have been combined to plot the borders of four closely related *Echinometra* species common all over the tropical Pacific. These have been designated as *Echinometra* A, B, C, and D (Uehara *et al*., 1990; Matsuoka and Hatanaka, 1991; Nishihira *et al*., 1991; Metz and Palumbi, 1996; Palumbi, 1996; Rahman *et al*., 2001; 2004a; 2004b; 2005). mtDNA sequence data show that divergence among the *Echinometra* spp. in the central and west Pacific occurred over the past 1–3 million years (Palumbi, 1996). Although the four *Echinometra* species are known as four distinct species, valid names for these species have been debated. *Echinometra* sp. B is now given the name *Echinometra mathaei* (Em) (Arakaki *et al*., 1998), while *Echinometra* sp. D (Ed) belongs in the *Echinometra oblonga* (Eo) species complex, which may be composed of three cryptic species (Arakaki and Uehara, 1999). The other two species, *Echinometra* sp. A (Ea) and *Echinometra* sp. C (Ec), have yet to be formally named (Rahman *et al*., 2000; 2001).

For sympatric, sessile marine invertebrates that broadcast their gametes into the water column, an effective mechanism for reproductive isolation would be to shift spawning times or to have species-specific gamete interactions during fertilization. Among these factors, gametic incompatibility may be particularly important for maintaining reproductive isolation and species integrity in many free-swimming animals including echinoderms (Palumbi and Metz, 1991; Byrne and Anderson, 1994; Rahman *et al*., 2001; 2004a; 2004b; Zigler and Lessios, 2003; 2004; Lessios, 2007; Zigler, 2008; Palumbi, 2009). Conversely, postzygotic mechanisms such as the creation of nonviable larvae and infertile adults can also contribute to reproductive isolation (Coyne, 1992; Knowlton, 1993; Behrmann-Godel and Gerlach, 2008).

Among the Okinawan *Echinometra*, experimental hybridizations have been conducted between Eo and Ea, Ec and Ea, Eo and Em, and Ec and Em (Aslan and Uehara, 1997; Rahman *et al*., 2000; 2001; 2004a; Rahman and Uehara, 2004). In each of these combinations, ova from the former species are easily fertilized by the sperm from the latter species, but the reciprocal crosses show very low percentages of fertilization even at a very high sperm concentration. Despite these asymmetries in fertilization, hybrids originating from crosses in either direction develop normally into adults that are fertile both among themselves and in backcrosses. Conversely, gametes of the two co-occurring species on Okinawan reef flats (Ea and Em) show a strong block to fertilization in both directions, but hybrids produced at higher sperm densities grow well and exhibit parental heterosis (Rahman *et al*., 2005). These findings, as well as a lack of hybrids in the field, indicate that gene flow among these combinations is most likely to be minimized by prezygotic mechanisms, particularly segregation of their particular microhabitats and gametic incompatibility at realistic sperm concentrations. Among the four species of *Echinometra*, two reef margin species, Ec and Eo show distinct differences in adult morphologies and habitat preference (Table 1). Recent studies of the mitochondrial CO1 gene, and the entire molecule of the nuclear binding gene sequences revealed that these species are of recent origin and are probably less than three-million years old (Palumbi, 1996). The gametes of these two species are reciprocally compatible (Uehara *et al*., 1990), their breeding seasons overlap (Arakaki and Uehara, 1991), and their microhabitats intermingle. In this paper, through detailed hybridization trials and phenotypic determination of their distinct traits, we determine how reproductive isolation occurs and in particular, how these two species maintain their genetic identity in the field.

2 Materials and methods

2.1 Sample collection and spawning

In total, 60 mature adults each of the sea urchin species Ec and Eo were collected from their respective habitats (Table 1) on the Sunabe coast of Okinawa Island, Japan (26°07′ N; 127°46′ E) at low tide by snorkeling and walking along the sea shore between July and September 2003, a period of reproductive activity for both species (Arakaki and Uehara, 1991). They were maintained in closed aquaria in the laboratory of the Department of Chemistry, Biology, and Marine Science, University of the Ryukyus, Okinawa, Japan. Gametes were obtained from each sea urchin following the injection of 0.5 mol/L KCl

Table 1 Summary of characters relevant to identification and reproductive isolation of parental *Echinometra* sp. C (Ec) and *Echinometra oblonga* (Eo)

Parameter	Ec	Eo	Ref.*
Habitat	Burrows on the reef margin, slightly above Em and with a narrower range of distribution	Deep burrows along narrow reef margin in surf break, slightly below Ec	1, 2, 5
Bathymetric range	Intertidal, above mean low water level	Intertidal, above mean low water level	1, 2, 5
Salinity and thermo-tolerance	Higher tolerance to sudden temperature and salinity changes	Lower tolerance to sudden temperature and salinity changes	4
Body size	Moderate among Okinawan species of *Echinometra*	Smallest among Okinawan species of *Echinometra*	3
Wet weight (g)	39.17±8.71	11.06±4.81	6
Test length (mm)	43.09±3.14	35.97±3.64	6
Test width (mm)	34.82±2.65	27.96±2.20	6
Spine length (mm)	16.56±0.87	18.43±1.04	6
Color	Highly variable, spines mostly green, brownish-black, greenish-brown or whitish-gray; basal translucent, white ring	Entirely black test and spines, basal ring of spine unclear	3, 6
Spicule shape			
Tube-foot	Triradiate	Triradiate, bihamate, and triradiate-bihamate	3, 6
Gonad	Triradiate, spindle, and bihamate	Triradiate, spindle, bihamate, and spindle-triradiate	3, 6
Breeding season	Apr.–Dec. (max. around late Sept.)	May–Sept. (max. around mid Sept.)	4
Egg size (μm)	72.68±1.25	75.85±1.62	6
Sperm head size (μm)	6.01±0.45	8.14±0.61	6
Jelly layer thickness (μm)	18.13±3.49	15.67±3.76	6

* Ref.: 1. Tsuchiya and Nishihira (1984); 2. Tsuchiya and Nishihira (1985); 3. Uehara and Shingaki (1985); 4. Arakaki and Uehara (1991); 5. Nishihira *et al*. (1991); 6. This study (n=25 adults of each species). Data are expressed as mean±SD

solution into the coelomic cavity. Eggs were collected in sterilized filtered seawater (SFSW). "Dry" sperm were pipetted off the genital pores and kept in concentrated form in a refrigerator at 4–5 °C for not more than 2 h.

2.2 Sperm concentration experiments

To determine the fertilization rates at different sperm concentrations for conspecific and heterospecific crosses, a 0.1-ml aliquot of diluted egg suspension (350–400 eggs) was kept in small glass vials with 0.8 ml of SFSW. Fresh "dry" sperm were quickly diluted in a series of eight 10-fold dilutions: 10^{-1}–10^{-8}. A 0.1-ml aliquot from each of these sperm solutions was then placed into the vials containing 0.9-ml egg suspensions, to bring their final volumes to 1 ml. This procedure was followed through a series ranging from 10^{-2}–10^{-9} diluted concentrations of sperm. Sperm concentrations of the 10^{-4} dilution from each species were first measured by hemacytometer counts and then adjusted with the dilution series. Percent fertilization was estimated by counting the number of embryos, reaching 2–4 cells among the first 100 eggs examined.

2.3 Gamete exposure time experiments

To assess the effects of gamete exposure time on fertilization in Ec, Eo, and their reciprocal hybrids, eggs were exposed to a limited sperm concentration at 10^{-5} dilution of "dry" sperm (1.0×10^6 sperm/ml). A 2-ml aliquot of diluted egg suspension (3 500–4 000 eggs/ml) was kept in a small glass beaker with 16 ml of SFSW. Fresh dry sperm were diluted in a series of four 10-fold dilutions, following the protocols described in the above sperm dilution experiment. A 2-ml aliquot from a 10^{-4} diluted sperm suspension

was then poured into the beaker containing 18 ml of egg suspension, which ultimately constituted a 10^{-5} diluted concentration of sperm. In each time interval (30, 60, 90, 120, 150, 180, 210, 240, 270, and 300 s), a 1-ml aliquot of the inseminated egg suspension was then gently pipetted into plastic cylinders, the bottoms of which had been fitted with 30 μ Nitex mesh. The cylinders were then rinsed 3–4 times with SFSW to remove excess sperm, and the eggs were resuspended in fresh SFSW. The method for estimating the percent fertilization was as described above.

2.4 Cross-fertilization and embryonic development

Cross-fertilizations between pairs of *Echinometra* spp. were conducted using all possible combinations of egg and sperm at ambient temperature (27–28 °C) following the protocols described by Rahman *et al*. (2000; 2005). Following convention, the maternal species is named first. In each heterospecific cross, a conspecific fertilization was conducted using the same gametes as a control. Six replicate crosses were conducted between each pair of species and percent fertilization was calculated as above. Fertilized eggs were shifted to glass beakers and incubated in SFSW at ambient temperature (27–28 °C) until they attained the free-swimming blastula stage. They were then moved to glass bottles on 10 r/min rotating paddles. Both the conspecific and heterospecific crosses attained four-armed pluteus (feeding) larvae within 48 h after fertilization.

2.5 Larval rearing and metamorphosis induction

The four-armed pluteus larvae from both conspecific and heterospecific crosses were reared in 400 or 800 ml glass bottles at a larval density of 1 individual per ml SFSW. Larvae were fed with a cultured phytoplankton, *Chaetoceros gracillis*, at densities of 1×10^4–2×10^4 cells/ml, adjusting the food level every 2 d until metamorphic competence was attained (Rahman *et al*., 2000). Six replicate trials were conducted for each cross and the survivals of hybrid and non-hybrid (conspecific) larvae were quantified and compared among the treatments. After 20–24 d of rearing, the mature larvae deemed competent were used for settlement tests. Induction of metamorphosis of competent larvae from all crosses was performed on coralline red algal stones, which were immersed in SFSW in petri dishes each containing 40 ml SFSW. Larval density was maintained at 1 individual in 2 ml SFSW using the method of Rahman and Uehara (2001). In each trial, six replicate petri dishes (each with 20 competent larvae) were used per treatment and percent metamorphosis was estimated within 24–30 h using the same environmental conditions and procedures as for larval rearing.

2.6 Culture of juveniles and adults

The newly produced juveniles of hybrids and their conspecific controls were reared in small aquaria (25 cm×20 cm×10 cm) with aerated seawater, and pieces of dead coral with coralline algae were provided as food (Rahman *et al*., 2000; 2005). This was continued for up to three months. The juveniles were then shifted to plastic aquaria (46 cm×55 cm×25 cm) supplied with aerated flow-through seawater. Dead coral covered with encrusting coralline algae was supplied as food. The stocking density was maintained at 30 individuals in each replicate aquarium. The cultures were continued for one year by which time the urchins attained sexual maturity. The survival rate (%) and growth performances of juveniles and adults were examined and compared among the hybrid groups and their parental controls.

2.7 Morphological characteristics

Detailed morphological characteristics were recorded from one-year-old Ec×Ec, Eo×Eo, and their hybrids, including color patterns of oral and aboral spines and test, sizes of test, spine length, and Aristotle's lantern, morphology of spicules in the tube-foot and gonad, pedicellariae valve length, and gamete size following the detailed procedures described by Rahman *et al*. (2004a; 2004b).

2.8 F_1 backcrosses

After one year of rearing, the majority of reciprocal hybrids and their conspecific parents attained sexual maturity and contained mature gametes. To determine gametic compatibility among hybrids and conspecific controls, all gametes were reciprocally backcrossed at a limited sperm concentration (1.0×10^6 sperm/ml) following the methods in the above sperm concentration experiments and protocols described by Rahman and Uehara (2001) and Rahman *et al*. (2004a; 2004b). Following convention, when

referring to the backcrosses, the maternal species is named first. Six replicate crosses among the F_1 conspecifics and their F_1 hybrids were performed and the protocols including incubation and counting of fertilized eggs were the same as those described in the sperm concentration experiments.

2.9 Hybrids in nature

To investigate the incidence of any natural hybridization between the two *Echinometra* species, field surveys were conducted along the Sunabe coast of Okinawa and the west coast of Sesoko Island, where both species occur sympatrically in adjacent microhabitats. About 400 individuals, suspected to be hybrids on the basis of color patterns, were collected and compared to the lab-cultured hybrids with respect to the above morphological characteristics.

2.10 Data analysis

Percentage data were arcsine transformed, and replicates in which no eggs or all eggs were fertilized were given a value of $0.25n$ and $1-0.25n$ (n is the number of observations) to improve the arcsine transformation (Zar, 1996). A "Bartlett's test" was used to analyze the homogeneity of variances (Bartlett, 1937). When variances were not significantly heterogeneous and there were no major departures from normality, a one-/two-way analysis of variance (ANOVA) was carried out followed by the Tukey's multiple comparison test. All statistical analyses were performed with the computerized statistical package SPSS 15.0. Untransformed data are presented in tables and figures.

3 Results

3.1 Effects of sperm concentration and exposure time on fertilization success

Fertilization success of conspecific and heterospecific crosses was highly dependent on sperm concentrations (Fig. 1). Fertilization rates in conspecific (Eo×Eo and Ec×Ec) and heterospecific (Ec×Eo and Eo×Ec) crosses at higher sperm concentrations (1.0×10^7–1.0×10^9 sperm/ml) were 100% or very near to 100%. At a limited (intermediate) sperm concentration (1.0×10^6 sperm/ml), where conspecific crosses reached nearly 100% fertilization, mean fertilization rates in Ec×Eo (85.33%) and Eo×Ec (81.83%) crosses were significantly (Tukey's test, $P<0.05$) lower than in either conspecific cross. Under lower sperm concentrations ($\leq1.0\times10^5$ sperm/ml), heterospecific crosses showed similar trends but progressively lower fertilization rates compared to conspecific crosses.

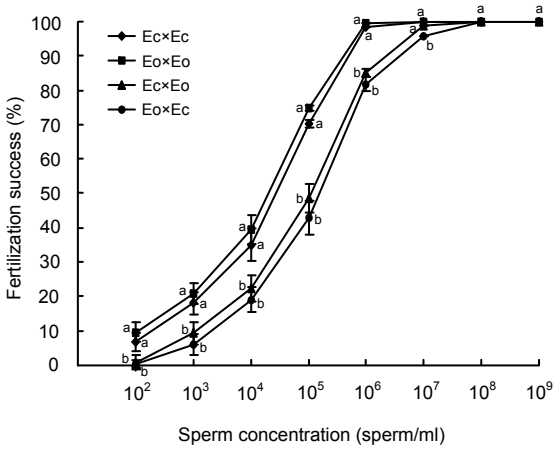

Fig. 1 Percentages of fertilization success in conspecific and heterospecific crosses of *Echinometra* sp. C (Ec) and *Echinometra oblonga* (Eo) under various concentrations of sperm

Maternal species is named first in each cross. Data are expressed as mean±SD (n=6). Mean values for each concentration data point with the same letters are not statistically significantly different (Tukey's test, $P>0.05$)

Fertilization success of both conspecific and heterospecific crosses was significantly influenced by sperm-egg exposure time (i.e., the greater the exposure time, the higher the fertilization rate) (Fig. 2). At a limited sperm concentration (1.0×10^6 sperm/ml), the conspecific crosses reached high percentages of fertilization (98.0% in Ec×Ec and 99.2% in Eo×Eo) within a contact period of 90 s, whereas heterospecific Ec×Eo and Eo×Ec crosses took much longer (180 s for Ec×Eo and 210 s for Eo×Ec) to reach asymptotic levels of fertilization.

3.2 Larval, juvenile, and adult performances

Survival percentages of competent larvae of Ec×Eo and Eo×Ec hybrids were not significantly different from those of larvae of conspecific crosses (Tukey's test, $P>0.05$). Both reciprocal hybrids showed lower metamorphosis rates than either of the

parental crosses but the values did not differ significantly (Table 2). The recovery rate of three-month-old juveniles of conspecific parents and their hybrids followed the same trends as metamorphosis (Table 2).

The mean live weight attained by one-year-old Ec×Ec urchins was significantly higher than the weight gained by Eo×Eo urchins (Tukey's test, $P<0.05$), while both reciprocal hybrids attained intermediate sizes. These values did not differ significantly from each other (Tukey's test, $P>0.05$). Wet gonad weight was significantly lower in the slow-growing Eo×Eo urchins than in the faster-growing Ec×Ec urchins. Both reciprocal hybrids had gonads of intermediate weight but did not differ significantly from each other (Tukey's test, $P>0.05$). The gonad index followed the same trends seen for wet gonad weight. Survival was the highest in Eo×Eo crosses followed by Ec×Ec, Eo×Ec, and Ec×Eo. Neither reciprocal hybrid showed significant differences in survival (Tukey's test, $P>0.05$) from their parental siblings and among themselves. However, survival and growth performance indicated that hybrids in both directions were viable but intermediate to their parents, under lab-reared conditions.

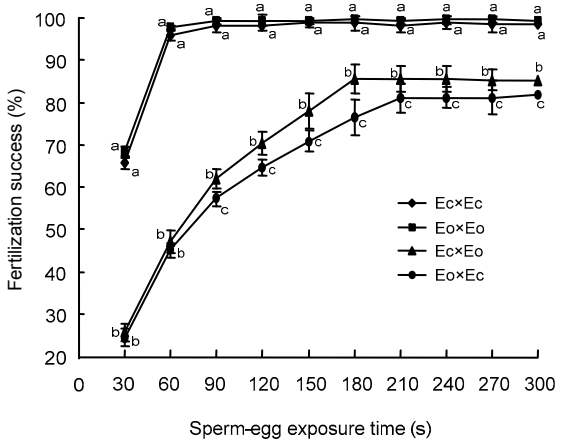

Fig. 2 Percentages of fertilization success in crosses of *Echinometa* sp. C (Ec×Ec), *Echinometra oblonga* (Eo×Eo) and their reciprocal hybrids at a limited sperm concentration (1.0×10^6 sperm/ml) for various exposure time

Maternal species is named first in each cross. Fresh gametes from new individuals were used for each replicate. Data are expressed as mean±SD (n=6). Mean values for each exposure time data point with the same letters are not statistically significantly different (Tukey's test, $P>0.05$)

3.3 Comparisons of phenotypic characteristics

Tests, spines, and Aristotle's lanterns of one-year-old urchins were the largest in Ec×Ec crosses followed by Eo×Ec, Ec×Eo, and Eo×Eo progenies. Statistically significant differences (Tukey's test, $P<0.05$) were found in all parameters between Ec×Ec and Eo×Eo, but the hybrids showed intermediate sizes and did not differ significantly (Tukey's test, $P>0.05$) from each other (Table 3).

The body coloration differed between adult conspecifics and hybrids. In Ec×Ec specimens, test and spine color was uniformly green, and each spine had a clear white ring at its base. Eo×Eo specimens had entirely dark tests and spines, and each spine had a faded basal white ring. The Ec×Eo hybrids were more similar to Ec×Ec conspecifics in having a

Table 2 Performances of larval, juvenile, and adult urchins from conspecific and hybrid crosses of *Echinometra* sp. C (Ec) and *Echinometra oblonga* (Eo)

Cross	Larva and juvenile urchins			Adult urchin (one-year-old)			
	Survival (%)	Metamorphosis (%)	Recovery* (%)	Wet weight (g)	Wet gonad weight (g)	Gonad index (%)	Survival (%)
Ec×Ec	78.50±2.08^a (75.25–81.00)	88.33±5.16^a (80.00–95.00)	71.46±2.23^a (68.75–74.25)	9.21±0.83^a (8.25–10.90)	1.33±0.06^a (1.26–1.50)	14.52±0.70^a (13.51–15.33)	86.67±3.34^a (83.33–90.00)
Ec×Eo	76.63±1.98^a (73.50–78.75)	85.83±4.92^a (80.00–90.00)	69.88±2.13^a (66.50–72.75)	6.59±0.42^b (6.01–7.23)	0.75±0.07^b (0.66–0.85)	11.41±0.40^b (10.79–11.98)	83.33±3.34^a (80.00–86.67)
Eo×Ec	77.08±2.22^a (74.25–79.50)	86.67±4.08^a (80.00–90.00)	70.33±2.04^a (67.75–73.50)	6.77±0.45^b (6.20–7.43)	0.79±0.08^b (0.69–0.90)	11.68±0.35^b (10.99–12.11)	84.45±3.35^a (80.00–86.67)
Eo×Eo	79.30±2.02^a (76.50–81.75)	89.16±5.85^a (80.00–95.00)	72.67±2.26^a (69.50–75.50)	5.02±0.50^c (4.40–5.90)	0.51±0.05^c (0.42–0.60)	10.19±0.45^c (9.38–11.13)	87.78±1.92^a (86.67–90.00)

Six replicate experiments were conducted in each cross for each performance measure. Data are expressed as mean±SD (range). Mean values in the same column having the same superscripts are not significantly different (Tukey's test, $P>0.05$). * Three-month-old juvenile urchins that were transferred to a flow-through sea water system for advanced culture

greenish-dark test with greenish spines and each spine having a clear basal white ring. On the other hand, Eo×Ec specimens were closer to Eo×Eo specimens in having a quite dark test and spines with faded basal white rings. In terms of oral body coloration, Ec×Ec urchins had yellowish-green spines around the mouth and a greenish test color, whereas Eo×Eo urchins had an entirely dark test and spines around the mouth. Ec×Eo hybrids were more similar to Ec×Ec conspecifics, and Eo×Ec hybrids were similar to Eo×Eo conspecifics in these characters; i.e., the coloration of hybrids was maternally inherited.

Tube-foot spicules in Ec×Ec urchins were always triradiate (100%), whereas those in Eo×Eo urchins were triradiate (90.29%), bihamate (3.67%), and triradiate-bihamate (6.04%) (Table 4). Tube-foot spicules of Ec×Eo hybrids were triradiate (83.38%), bihamate (2.19%), bihamate-like (3.86%), and triradiate-bihamate (10.57%), whereas those in Eo×Ec hybrids were triradiate (74.12%), bihamate (4.30%), bihamate-like (6.60%), and triradiate-bihamate (14.98%) (Table 4). The proportions of various types of tube-foot spicules in Ec×Eo and Eo×Ec hybrids differed significantly (Tukey's test, $P<0.05$) from their conpecific controls and showed higher proportions of intermediate features.

Table 3 Test sizes, spine lengths, and Aristotle's lantern characteristics of urchins from conspecific and hybrid crosses of *Echinometra* sp. C (Ec) and *Echinometra oblonga* (Eo), one year after metamorphosis

Cross	Test length (mm)	Test width (mm)	Test height (mm)	Test volume (cm^3)	Spine length (mm)	Aristotle's lantern length (mm)	Aristotle's lantern diameter (mm)
Ec×Ec	24.24±0.98[a]	22.25±0.93[a]	12.01±0.48[a]	6.50±0.79[a]	20.13±0.75[a]	8.71±0.59[a]	8.35±0.70[a]
	(23.05–25.95)	(21.20–23.82)	(11.35–12.90)	(5.55–7.97)	(18.68–21.56)	(7.60–9.80)	(7.00–9.40)
Ec×Eo	22.17±0.58[b]	20.56±0.78[b]	10.91±0.40[b]	5.01±0.44[b]	17.81±0.64[b]	8.04±0.57[b]	7.71±0.58[b]
	(20.90–22.92)	(18.88–21.71)	(10.03–11.39)	(4.01–5.66)	(16.25–19.22)	(6.80–9.10)	(6.60–8.90)
Eo×Ec	22.30±0.62[b]	20.69±0.73[b]	11.05±0.41[b]	5.11±0.47[b]	18.02±0.68[b]	8.17±0.56[b]	7.93±0.57[b]
	(21.00–23.15)	(19.00–21.95)	(10.10–11.65)	(4.12–5.92)	(16.50–19.45)	(7.00–9.20)	(6.80–9.00)
Eo×Eo	20.80±0.68[c]	19.35±0.42[c]	10.20±0.60[c]	4.07±0.43[c]	15.10±0.50[c]	7.33±0.57[c]	7.20±0.64[c]
	(19.80–21.92)	(18.62–19.90)	(9.32–11.20)	(3.50–4.83)	(13.25–17.02)	(6.40–8.40)	(6.20–8.20)

Twenty adult specimens were measured for each treatment. Data are expressed as mean±SD (range). Mean values in the same column having the same superscripts are not significantly different (Tukey's test, $P>0.05$)

Table 4 Percentages of different shapes of the tube-foot and gonad spicules of one-year-old urchins from conspecific and hybrid crosses of *Echinometra* sp. C (Ec) and *Echinometra oblonga* (Eo)

Cross	Tube-foot spicule (%)				Gonad spicule (%)					
	Triradiate	Bihamate	Bihamate-like	Triradiate-bihamate	Triradiate	Spindle	Spindle-like	Spindle-bihamate	Bihamate	Bihamate-like
Ec×Ec	100[a]	0[d]	0[c]	0[d]	93.75±0.88[a]	5.13±0.69[c]	0[c]	0[d]	1.12±0.22[d]	0[c]
					(92.50–95.02)	(3.85–6.18)			(0.86–1.64)	
Ec×Eo	83.38±1.76[c]	2.19±0.40[c]	3.86±0.72[b]	10.57±1.19[b]	80.06±1.41[c]	4.22±0.59[d]	3.12±0.66[b]	7.67±0.66[b]	2.07±0.23[c]	2.88±0.45[b]
	(80.90–86.21)	(1.53–2.86)	(2.72–5.06)	(8.59–12.96)	(78.02–82.69)	(2.94–5.43)	(2.17–4.18)	(6.67–8.96)	(1.68–2.56)	(2.07–3.59)
Eo×Ec	74.12±1.71[d]	4.30±0.66[a]	6.60±0.65[a]	14.98±1.08[a]	72.05±1.74[d]	5.98±0.43[b]	4.31±0.50[a]	10.45±0.74[a]	3.36±0.50[a]	3.85±0.57[a]
	(70.25–76.71)	(3.02–5.79)	(5.16–7.79)	(13.58–17.26)	(69.89–76.14)	(5.03–6.74)	(3.08–4.94)	(8.90–12.25)	(2.33–4.10)	(2.46–4.60)
Eo×Eo	90.29±1.14[b]	3.67±0.46[b]	0[c]	6.04±0.78[c]	84.01±1.80[b]	8.12±0.71[a]	0[c]	4.91±0.76[c]	2.96±0.55[b]	0[c]
	(88.21–92.38)	(2.86–4.37)		(4.76–7.69)	(81.07–86.67)	(6.93–9.85)		(3.87–5.97)	(2.10–3.69)	

Twenty individuals were randomly examined for each treatment with 10 tube-feet and 10 gonadal tissues per treatment. Data are expressed as mean±SD (range). Mean values in the same column having the same superscripts are not significantly different (Tukey's test, $P>0.05$)

Gonad spicules in Ec×Ec urchins were nearly all triradiate-shaped (93.75%; other spicules seen: spindle, 5.13%; bihamate, 1.12%), whereas those in Eo×Eo urchins were triradiate (84.01%), spindle (8.12%), spindle-triradiate (4.91%) and bihamate (2.96%) (Table 4). Gonad spicules in Ec×Eo hybrids were triradiate (80.06%), spindle (4.22%), spindle-like (3.12%), spindle-triradiate (7.67%) with a few bihamate (2.07%), and bihamate-like (2.88%) types, whereas spicules in Eo×Ec hybrids were triradiate (72.05%), spindle (5.98%), spindle-like (4.31%), spindle-triradiate (10.45%), bihamate (3.36%), and bihamate-like (3.85%). Although significant differences (Tukey's test, $P<0.05$) were recognized among the hybrids and their parental species, the proportions in the hybrids were intermediate.

Four types of pedicellariae, tridentate, globiferous, ophiocephalous and triphyllous, were observed in both conspecifics and their reciprocal hybrids. Valve lengths of all four types of pedicellariae of Ec×Ec urchins were significantly (Tukey's test, $P<0.05$) larger than those of their corresponding types from Eo×Eo urchins (Table 5). Both the hybrids had intermediate sizes, but they differed significantly from each other.

Egg diameters of Ec×Ec urchins were the smallest among the four crosses while the Eo×Eo eggs were the largest. Hybrids had intermediate-sized eggs that differed significantly (Tukey's test, $P<0.05$) in size from the eggs of the conspecifics. But the hybrids did not differ significantly in size (Tukey's test, $P>0.05$) from each other (Table 6). The sizes of sperm heads were also the smallest in Ec×Ec urchins, and were significantly different (Tukey's test, $P<0.05$) among crosses, including between the hybrid groups. Therefore the gamete sizes of hybrids were intermediate between their parental controls.

3.4 Existence of natural hybrids

Four hundred individuals with coloration patterns more or less intermediate between those of the two species were collected from the reef flats of the Sunabe and Sesoko coasts, where Ec and Eo were abundant and found close together. However, detailed comparisons of the above morphological characters revealed that none of these individuals were hybrids; that is, all could be assigned to either Ec or Eo. Molecular analyses (e.g., nuclear DNA) are needed to find out whether hybrids and their backcross

Table 5 Valve lengths of four types of pedicellariae in urchins from conspecific and hybrid crosses of *Echinometra* sp. C (Ec) and *Echinometra oblonga* (Eo), one year after metamorphosis

Cross	Valve length (μm)			
	Tridentate	Globiferous	Ophiocephalous	Triphyllous
Ec×Ec	880.9±34.2[a]	649.5±29.9[a]	546.4±27.7[a]	145.3±20.1[a]
	(840.0–950.0)	(590.0–680.0)	(490.0–590.0)	(110.0–180.0)
Ec×Eo	833.7±32.7[b]	628.9±28.6[b]	517.2±27.5[b]	133.6±18.1[b]
	(780.0–900.0)	(580.0–670.0)	(460.0–570.0)	(100.0–170.0)
Eo×Ec	783.3±30.7[c]	609.0±28.5[c]	484.9±27.7[c]	122.5±18.1[c]
	(750.0–840.0)	(570.0–660.0)	(440.0–550.0)	(90.0–160.0)
Eo×Eo	732.4±29.7[d]	590.7±27.7[d]	452.4±26.9[d]	110.5±16.3[d]
	(680.0–800.0)	(530.0–630.0)	(410.0–500.0)	(80.0–140.0)

Twenty individuals were examined from each cross with 10 pedicellariae of each type from each individual. Data are expressed as mean±SD (range). Mean values in the same column having the same superscripts are not significantly different (Tukey's test, $P>0.05$)

Table 6 Gamete sizes of sexually mature urchins from conspecific and hybrid crosses of *Echinometra* sp. C (Ec) and *Echinometra oblonga* (Eo), one year after metamorphosis

Cross	Gamete size (μm)	
	Egg diameter	Sperm-head length
Ec×Ec	72.36±1.27[c] (70.68–75.64)	6.04±0.54[d] (5.00–7.00)
Ec×Eo	74.36±1.28[b] (72.54–76.88)	7.16±0.57[c] (6.50–8.50)
Eo×Ec	73.98±1.23[b] (71.92–76.26)	6.83±0.59[b] (6.00–8.00)
Eo×Eo	75.74±1.54[a] (73.16–78.12)	8.24±0.53[a] (7.50–9.50)

Six individuals were examined from each cross for each sex with 25 eggs and 25 sperm from each individual. Data are expressed as mean±SD (range). Mean values in the same column having different superscripts are significantly different (Tukey's test, $P<0.05$)

individuals are occurring in the field, which have not been identified by morphological characteristics.

3.5 Fertilization rates in F_1 backcrosses

Fertilization rates in F_1 backcrosses using the gametes of F_1 hybrids and their conspecific parents at a limited sperm concentration are shown in Table 7. Eggs from both hybrids, Ec×Eo and Eo×Ec, yielded higher fertilization rates with Ec×Ec sperm (93.33% and 91.83%) than with Eo×Eo sperm (92.83% and 89.00%), indicating that Eo sperm were more discriminating than Ec sperm. However, backcrosses by sperm from Ec×Eo and Eo×Ec hybrids produced higher percentages of fertilization with Ec×Ec ova (85.83% and 81.17%) than with Eo×Eo ova (83.0% and 78.83%), again indicating that Eo ova appeared to be slightly less attractive than Ec ova. Thus, differences in fertilization rates among the F_1 conspecifics and their F_1 hybrids indicate discrimination differences in their gamete recognition genes.

4 Discussion

The gametes of two reef margin species, Ec and Eo, are reciprocally compatible (Uehara *et al*., 1990). However, at a sperm concentration where conspecific crosses achieved 100% or near 100% fertilization, both heterospecific crosses showed 81%–85% fertilization success, and these values declined significantly with decreasing sperm concentrations. These impediments to fertilization in both heterospecific crosses indicate the presence of a protein-binding system for gamete recognition. Incompatibility of bindin and bindin-receptors might eventually lead to reproductive isolation, as proposed by Metz *et al*. (1994), Metz and Palumbi (1996), Biermann *et al*. (2004), Lessios (2007), Zigler (2008), and Palumbi (2009). These two species typically do not co-occur throughout their respective ranges, and Geyer and Palumbi (2003) found more genetic separation of bindin genes in their sympatric than in their allopatric populations. In Okinawa, where they are sympatric, fertilization percentages over a broad range of sperm densities are almost identical between heterospecific crosses, while other combinations of gametes from the four *Echinometra* species show high asymmetries in fertilization: the ova of reef margin species are readily fertilized by sperm of reef flat species, but not vice versa (Uehara *et al*., 1990; Rahman *et al*., 2001; 2004a; Rahman and Uehara, 2004). In contrast, gametes of the two reef flat species, Ea and Em, are nearly incompatible (Rahman *et al*., 2004b) which in this case suggests positive selection for gamete incompatibility to prevent hybridization in sympatry.

Moreover, in F_1 backcrosses the Ec-Eo hybrids exhibited higher fertilization rates than Ea-Eo (Aslan and Uehara, 1997), Ea-Ec (Rahman *et al*., 2001), Em-Eo (Rahman *et al*., 2004a), Em-Ec (Rahman and Uehara, 2004), and Ea-Em (Rahman *et al*., 2004b) hybrids. In other words, the two species show a high divergence in their bindins (Landry *et al*., 2003; Geyer and Palumbi, 2005). Similarly, although the two sympatric species of Caribbean sea urchins, *Lytechinus willamsi* and *Lytechinus variegatus* show a high divergence in bindins (Zigler and Lessios, 2004), their gametes are reciprocally as compatible as those in F_1 backcrosses within a wide range of sperm concentrations. However, the lack of evidence for gamete incompatibility between Ec and Eo suggests that the observed differences in bindin do not significantly affect gamete interactions. There may be subtle

Table 7 Percentage of eggs fertilized in backcrosses among lab-reared F_1 generation urchins from conspecific and hybrid crosses of *Echinometra* sp. C (Ec) and *Echinometra oblonga* (Eo) at a limited sperm concentration (1.0×10^6 sperm/ml)

Egg	Fertilization rate (%)			
	Sperm (Ec×Ec)	Sperm (Ec×Eo)	Sperm (Eo×Ec)	Sperm (Eo×Eo)
Ec×Ec	98.67 ± 1.63^{a} (96.0–100.0)	85.83 ± 2.48^{c} (83.0–90.0)	81.17 ± 2.99^{b} (77.0–85.0)	85.67 ± 3.86^{c} (79.0–89.0)
Ec×Eo	93.33 ± 1.51^{b} (92.0–96.0)	97.67 ± 1.21^{a} (96.0–99.0)	93.17 ± 2.31^{a} (90.0–96.0)	92.83 ± 1.72^{b} (90.0–95.0)
Eo×Ec	91.83 ± 1.60^{b} (90.0–94.0)	91.33 ± 2.17^{b} (88.0–94.0)	95.17 ± 2.32^{a} (92.0–98.0)	89.00 ± 2.37^{c} (86.0–92.0)
Eo×Eo	81.0 ± 3.79^{c} (75.0–86.0)	83.0 ± 2.37^{c} (80.0–86.0)	78.83 ± 3.43^{b} (75.0–83.0)	99.67 ± 0.52^{a} (99.0–100.0)

Each value represents six replicate crosses with gametes from new individuals in each replicate. Data are expressed as mean±SD (range). Mean values in the same column having different superscripts are statistically significant (Tukey's test, $P<0.05$)

fertilization effects that are not detected, but it is also possible that the monophyly of bindin is simply a result of the stochasticity of coalescence processes (Hudson and Turelli, 2003; Zigler and Lessios, 2004). Two sympatric species of asteroids belonging to the genus *Patiriella*, *P. calcar* and *P. gunni*, are reciprocally compatible and do not show any gametic incompatibility in either cross, despite the fact that they are morphologically and genetically distinct (Byrne and Anderson, 1994). Therefore, reproductive isolation between Ec and Eo may have occurred before the evolution of gametic incompatibility because gamete recognition molecules accumulate over time as species diverge (McCartney and Lessios, 2002; Lessios, 2007; Zigler, 2008). If this is true, it provides evidence for the eventual evolution of gamete incompatibility and speciation in these two species (Rahman *et al*., 2001).

The percentages of larval survival, metamorphosis, and juvenile and adult survival of the hybrid groups were similar to those of their parents, eliminating the probability that developmental incompatibility or hybrid inviability is a postzygotic mechanism of reproductive isolation. The surviving hybrids grew at the same rate as the conspecifics. Moreover, hybrids were as viable and fertile as the conspecifics, demonstrating that there are neither gametic nor postzygotic obstructions to introgression between them. These similarities are correlated with their higher genetic affinities compared to other closely related pairs of Okinawan *Echinometra* spp., where hybrids in one direction were as viable as conspecifics while hybrids in the other direction were less viable (Rahman *et al*., 2000; 2001; 2004a; Rahman and Uehara, 2004). The hybrids of two reef flat species, Ea and Em, showed inferior performances in larval and juvenile traits, but better performances in advanced stages (Rahman *et al*., 2004b; 2005). The progeny of reciprocal crosses between *Strongylocentrotus droebachiensis* and *Strongylocentrotus pallidus* produced viable gametes, and backcrosses to the parent species indicated that gene flow would run predominantly back to *S. pallidus* (Strathmann, 1981). However, Addison and Hart (2005) reported only small amounts of introgression from the reciprocal cross (*S. pallidus*×*S. droebachiensis*) and none at all from the more fertile cross of *S. droebachiensis*×*S. pallidus*.

The higher compatibility of the gametes of Ec and Eo demonstrated that if gamete recognition molecules are involved in fertilization in these species, they are not strongly species-specific. Genetic divergence and speciation of Ec and Eo have occurred without gametic incompatibility and perhaps arose through ecological factors. The two species in the present study live comparatively close to each other but inhabit different microhabitats: Ec inhabits burrows in the reef margins whereas Eo inhabits deep burrows in surf breaking reef margins, positioned below those of Ec. During our field studies, we observed that Ec was more aggressive than Eo and always showed notable antagonistic behavior towards intruders by driving them away if the Eo was placed into a burrow inhabited by Ec. Tsuchiya and Nishihira (1985) observed a similar phenomenon in two reef flat species, Ea and Em. There is little evidence of highly selective microhabitat assortment in sea urchin larvae. Cameron and Schroeter (1980) observed that competent larvae of *S. purpuratus* settled to metamorphose indiscriminately on bacterial-covered substrates, and later microhabitat segregation of the juveniles occurred to avoid high selective mortality or to avoid predation. It was also observed that the majority of the larvae of *Echinometra* spp. settled indiscriminately on stones covered with coralline algae in the field and their juveniles were found in their respective microhabitats. However, this discrimination probably decreases most chances of cross-fertilization because, as shown in other broadcast spawning invertebrates, fertilization success decreases with the distance between spawning individuals (Levitan, 1998). If gametes of one species are swept over spawning individuals of another species in adjacent areas, sperm concentration has a major effect on fertilization (Rahman *et al*., 2004a). Moreover, individuals of Ec are occasionally found to intermingle with those of Eo, and in this case sperm concentration will not be a factor in avoiding hybridization. Consequently, it is still unclear whether microhabitat segregation, by itself, is enough to prevent introgression in these two congeners.

The annual breeding seasons of Ec and Eo overlap extensively (Arakaki and Uehara, 1991) (Table 1) and they can readily spawn during this time. There are no data on the precise spawning times of any echinoid (Pearse and Cameron, 1991), nor is there

any evidence or indication that different species of *Echinometra* spawn in different lunar cycles, as do some closely related sympatric species of *Diadema* (Muthiga, 2003; Coppard and Campbell, 2005). Therefore, temporal separation of spawning seasons is an unlikely mechanism for maintaining reproductive isolation (Byrne and Anderson, 1994; Rahman and Uehara, 2004). Nevertheless, peak spawning periods, as well as salinity and temperature tolerances differ between the two species (Arakaki and Uehara, 1991) (Table 1). Different gametogenetic cycles observed through histological examinations (Aslan, 2000), revealed that different individuals of *Echinometra* spp. spawned at different times as found for Em in Okinawa (Nishihira, 1975), the Gulf of Suez (Pearse, 1969), and at Rottnest Island (Pearse and Philips, 1968). These factors, in addition to possible separation in daily spawning times or specific pheromonal spawning cues, could prevent hybridization between the two species (Rahman and Uehara, 2004).

Free-spawning invertebrates (e.g., coelenterates, polychaetes, mollusks, and echinoderms), including sea urchins, are recognized as having no obvious courtship or little premating behavior between sexes before reproduction (Metz *et al*., 1994; Lamare and Stewart, 1998; Landry *et al*., 2003), even when habitats and spawning seasons may overlap as in *Echinometra* spp. (Arakaki and Uehara, 1991; Rahman and Uehara, 2004). Instead, gametes are released into the water column, and the most essential interaction is between eggs and sperm at fertilization. In these instances, reproductive isolation may arise by changes in the timing of gamete release (Lessios, 1984) or clumping of conspecific adults (Billett and Hausen, 1982). However, the behavioral components in reproduction that are considered to force rapid speciation in other animals are largely absent in sea urchins including those within the *Echinometra* spp. complex (Palumbi and Metz, 1991; Rahman and Uehara, 2004).

If gametes of both species are in the water together and species specificity operates in gamete recognition and sperm-binding genes, there could be interspecific competition for fertilization success (Rahman *et al*., 2004a). Our findings from the gamete exposure (contact) time experiment revealed that conspecific Ec×Ec and Eo×Eo crosses reached asymptotic levels of fertilization much faster than the heterospecific Ec×Eo and Eo×Ec crosses under a limited sperm concentration (Fig. 2). How such sperm competition could be achieved in broadcast spawning, externally fertilizing species such as *Echinometra* spp. is unclear. However, if it does occur in the field, and if conspecific sperm outcompetes heterospecific sperm for fertilization (Howard *et al*., 1998), a mechanism for maintaining species integrity in sympatric Ec and Eo may be present. Evidence for such a system of gamete competition was found recently when gametes of these two species were mixed at low concentrations and nearly all parents of the resulting embryos were identified as conspecifics by the use of DNA markers (Geyer and Palumbi, 2005).

Although the expression of an intermediate phenotype by the lab-reared hybrids might be used to discover hybrids in the field, it would be hard to identify hybrids if the phenotype of only one parent is expressed or if the two parental species are morphologically similar. Coloration patterns of the hybrids tended to be maternally inherited. Conversely, other characters, such as test sizes, spine lengths, Aristotle's lantern length, spicule morphology of the tube-foot and gonad, pedicellariae valve length, and gamete sizes, tended to be intermediate and could be used to distinguish easily hybrids from either parent. We searched for the distinctive intermediate phenotypes in the field, and although suggestive color morphs were observed, none were identified as hybrids and all of them were assigned to either Ec or Eo. However, without doing any genetic analysis (e.g., mtDNA analysis), we cannot rule out that some of these intermediate phenotypes were backcross individuals. As in other *Echinometra*, Ec and Eo are sympatric but hybrids are rare or absent (Geyer and Palumbi, 2005). The only evidence of hybridization in the field is a single specimen out of 97 examined that had similar color and spicules characteristics of Em, but with the mtDNA of Eo (Palumbi *et al*., 1997). This specimen may have represented a backcross, perhaps of several generations (Rahman *et al*., 2004a). Moreover, although two species of the tropical long-spined sea urchins, *Diadama savignyi* and *Diadama setosum*, readily hybridize in the laboratory (Uehara *et al*., 1990), and often occur in mixed populations in the field (Pearse, 1998), allozyme analyses revealed that there is restricted introgression and hybrids occur only rarely in the field (Lessios and

Pearse, 1996). Based on mtDNA analyses, these two species separated between 6 and 10 million years ago (Lessios *et al*., 2001) and are at least twice as old as the Pacific species of *Echinometra* (Rahman *et al*., 2004a). Since the lab-reared hybrids are fully fertile in all these species, there is almost certainly some kind of effective isolating mechanism(s) separating them that does not involve hybrid viability and fertility. Of these mechanisms, habitat segregation, gamete competition and probably differences in spawning times, gametic incompatibility, and other genetic and non-genetic factors appear to be essential in maintaining reproductive isolation and speciation in these recently diverged species of tropical sea urchins.

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