# Interspecific Hybridization and Backcrosses between Two Sibling Species of Pacific Sea Urchins (Genus *Echinometra*) on Okinawan Intertidal Reefs

M. Aminur Rahman* and Tsuyoshi Uehara*

*Department of Chemistry, Biology and Marine Science, Faculty of Science, University of the Ryukyus, 1 Senbaru, Nishihara-cho, Okinawa 903-0213, Japan*

**M. Aminur Rahman and Tsuyoshi Uehara (2004)** Interspecific hybridization and backcrosses between two sibling species of Pacific sea urchins (genus *Echinometra*) on Okinawan Intertidal Reefs. *Zoological Studies* **43**(1): 93-111. Four genetically divergent Pacific sea urchins belonging to the genus *Echinometra* occur sympatrically and abundantly along the coast of Okinawa Island. Among them, the 2 most distinct species, *Echinometra mathaei* (Em) and *Echinometra* sp. C (Ec), were examined for potential hybridization. A series of cross-fertilization experiments was conducted, and the resulting hybrids were reared. The percentage of successful heterogametic fertilization was high when egg of Ec and sperm of Em were involved, whereas it was significantly lower with eggs of Em and sperm of Ec, even at higher concentrations of sperm, suggesting that gamete incompatibility may be a prezygotic barrier that at least partially maintains reproductive isolation. Moreover, different exposure times of eggs and sperm showed that conspecific crosses reached asymptotic fertilization much sooner than did heterospecific crosses, indicating that affinity of the Em's sperm to its egg during fertilization is higher than that of Ec. Hybrids from both crosses developed normally through the larval and juvenile stages to sexually mature adults. In adults, Em x Em crosses produced the largest values of live weight, followed by Ec (ova) x Em (sperm), Em (ova) x Ec (sperm), and Ec x Ec in that order. Other indicators of growth performance such as metamorphosis, survival, and recovery of hybrids and their conspecifics also followed the same trends as live weight. Phenotypic color patterns of the hybrids were closer to the maternal coloration, whereas other characters such as test size, spine length, morphology of tubefeet and gonad spicules, pedicellaria valve length, and gamete sizes were intermediate. Fertilization rates as well as larval and juvenile performances from backcrosses using the gametes of $F_1$ progeny were high, eliminating the possibility that hybrid inviability is a postzygotic mechanism of reproductive isolation. On the other hand, intensive surveys by our team and other colleagues have failed to find individuals with such hybrid characteristics in the field, suggesting a lack of natural hybridization between the 2 species despite their physiological potential for hybridization. This strongly suggests that Em and Ec have been able to maintain their genetic distinctness due to the presence of some sort of prezygotic isolating mechanism(s) (e.g., habitat segregation, gametic incompatibility, gamete competition, and probably gamete aging) between them.
http://www.sinica.edu.tw/zool/zoolstud/43.1/93.pdf

**Key words:** Interspecific hybridization, Sibling species, *Echinometra* spp., Reproductive isolation, Speciation.

For speciation to occur, either prezygotic or postzygotic, barriers to gene exchange must arise between 2 conspecific populations (Dobzhansky et al. 1977, Gosling 1994). Prezygotic mechanisms include ecological or habitat separation, and behavioral or temporal separation in breeding and gametic incompatibility; postzygotic mechanisms include hybrid inviability, hybrid breakdown, and hybrid sterility (Dobzhansky et al. 1977, Templeton 1989). In sympatric echinoids, interspecific reproductive isolation can result from different reproductive characteristics, such as timing and sites of spawning or gametic incompatibility (Uehara et al. 1990, Metz et al. 1994, Vacquier et al. 1995, Aslan and Uehara 1997, Lessios 1998, Palumbi 1998, Rahman et al. 2001a, McCartney and Lessios

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*To whom correspondence and reprint requests should be addressed. Tel: 81-98-895-8897. Fax: 81-98-895-8576. E-mail: arahman1963@yahoo.com/ueharago@sci.u-ryukyu.ac.jp

2002). Of these factors, gametic incompatibility, which prevents gametes of different species from hybridizing due to differences in gamete recognition molecules, may be particularly important for maintaining reproductive isolation of many free-swimming animals including sea urchins (Lessios and Cunningham 1990, Vacquier et al. 1990 1995, Foltz and Lennarz 1993, Metz and Palumbi 1996, Metz et al. 1998, Palumbi 1998, Rahman et al. 2001a, McCartney and Lessios 2002). On the other hand, postzygotic mechanisms such as the production of nonviable larvae and infertile adults can also lead to and maintain reproductive isolation (Chen and Baltzer 1975, Lessios and Cunningham 1990, Coyne 1992, Knowlton 1993, Lessios 1998, McCartney et al. 2000).

Recent studies on morphological, ecological, allozymic, and gamate compatibility, as well as DNA-DNA hybridization, mtDNA, and loci coding for gamete recognition molecules have shown that 4 biological species of *Echinometra* exist in the Indo-West Pacific (IWP), distinguished as *Echinometra* spp. A, B, C, and D (Arakaki and Uehara 1991, Matsuoka and Hatanaka 1991, Nishihira et al. 1991, Palumbi and Metz 1991, Uehara et al. 1991, Palumbi 1996 1998, Aslan and Uehara 1997, Palumbi et al. 1997, Arakaki et al. 1998, Rahman et al. 2001a). Consequently, mitochondrial DNA sequence data from the 4 species of *Echinometra* show that genetic distances between these species are very small. This result as well as calibration of the rate of mtDNA evolution suggested that the *Echinometra* in the central and West Pacific diverged over the past 1-3 million years (Palumbi 1996). These values are lower than those of any pair of sea urchins separated by the rise of the Isthmus of Panama 3.5 million years ago (Lessios 1998). Although 4 distinct species of *Echinometra* are recognized, valid names for these species have been debated (Palumbi 1996, Palumbi et al. 1997). *Echinometra* sp. B is now recognized as *E. mathaei* (de Blainville, 1825) *sensu stricto* (Arakaki et al. 1998), while *Echinometra* sp. D belongs in the *E. oblonga* (de Blainville, 1825) species complex, which may include a cryptic species composed of at least three species (Arakaki and Uehara 1999). The small genetic and morphological differences among the 4 species coupled with their strong reproductive isolation make them a valuable group for studies of marine speciation (Palumbi et al. 1997).

Experimental hybridization was previously carried out on the 2 combinations of *Echinometra*

sp. A x *Echinometra* sp. D (now *E. oblonga*) and *Echinometra* sp. A x *Echinometra* sp. C (Aslan and Uehara 1997, Rahman et al. 2000 2001a). Of these combinations, all hybrids resulting from crosses in both directions developed normally into fertile adults and could be backcrossed; the researchers suggested that introgression among these combinations was probably minimized by prezygotic mechanisms, particularly separation of their respective microhabitats. Rahman et al. (2000 2001a) also reported asymmetrical fertilization in Ea-Ec crosses, with Ec ova more readily fertilized by Ea sperm than Ea ova fertilized by Ec sperm. Moreover, they found that the viability and growth of the cross of Ec (ova) x Ea (sperm) was similar to that of conspecifics, while that of Ea (ova) and Ec (sperm) was significantly lower. Of these 4 species, *E. mathaei* (Em) and *Echinometra* sp. C (Ec), while being most divergent to each other genetically (Matsuoka and Hatanaka 1991), can also be distinguished from each other by differences in adult morphology, distribution patterns, and microhabitat preferences (Table 1; Fig. 1). Although the reproductive seasons of these 2 species extensively overlap (Arakaki and Uehara 1991), no studies have addressed the mechanisms that maintain the integrity of these 2 species despite their sympatric existence in nature. To test the possibility of hybridization and backcrosses between *E. mathaei* and *Echinometra* sp. C and to assess which mechanism(s) maintain their genetic integrities in the field, detailed cross-fertilization, hybrid viability, and phenotypic characteristics were studied.
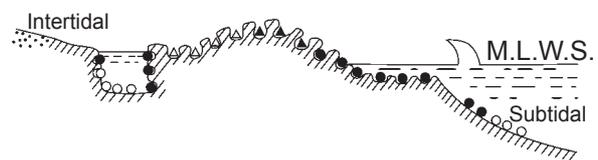


**Fig. 1.** Schematic distribution pattern of *Echinometra mathaei* (Em; ●) and *Echinometra* sp. C (Ec; △), and two other species (*Echinometra* sp. A (Ea; ○) and *Echinometra oblonga* (Eo; ▲), in a generalized form on Okinawan intertidal reefs. *Echinometra mathaei* occurs mainly in the shallow burrows on reef flats behind the reef margin and sometimes in the subtidal zone (calm water) and tidepools where *Echinometra* sp. A predominates, both above and below the mean low water level (MLWS) and shows a wider range of distribution; whereas *Echinometra* sp. C inhabits only deep burrows on the reef margin, positioned slightly above *E. mathaei* and *E. oblonga*, above the M.L.W.S. and showing a narrower range of distribution.

## MATERIALS AND METHODS

### Sample collection and maintenance

Mature adults of Em (identified on the basis of a brownish test and spines), and Ec (with an entirely green test and spines; Uehara 1990) were collected from the Sunabe coast of Okinawa Island (26°07'-N; 127°46'-E) at low tide during their natural breeding season from May to Sept.1999. Collected specimens were immediately transported to the laboratory at the Department of Chemistry, Biology, and Marine Science, University of the Ryukyus, Okinawa, where they were maintained in closed aquaria, and where they spawned within 3-4 d of collection. In order to know the exact distribution patterns, and microhabitat preferences of the 2 species on Okinawan intertidal reefs, some detailed field researches were also conducted during May to July 2003.

### Cross-fertilization experiments

Cross-fertilization of the 2 *Echinometra* species was conducted using all possible combinations of ova and sperm at room temperatures (27-28°C). Sperm were collected in the highest concentrated form ("dry") from the dissected testes and were stored undiluted in a refrigerator at 4-5°C until use, following the techniques described by Uehara et al. (1990) and Hata and Osanai (1994). The eggs were induced to shed by injecting a 0.5-M KCl solution into the body cavity and were collected by inverting female urchins over a glass beaker filled with filtered seawater (FSW). Sperm concentrations were maintained at $10^{-5}$ to $10^{-4}$ dilutions of "dry" sperm for the Em x Em, Ec x Ec, and Ec x Em crosses and at $10^{-2}$ to $10^{-1}$ dilutions for the Em x Ec cross (Uehara et al. 1990, Rahman et al. 2000 2001a). Fertilization was carried out by mixing 2 drops of diluted sperm

**Table 1.** Characteristics of parental *Echinometra mathaei* (Em) and *Echinometra* sp. C (Ec), relevant to their identification and reproductive isolation. Data sources are: 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, and include measurements from 25 adult individuals of each species examined for each character, mean ± SD

|  | Em | Ec | Sources |
|---|---|---|---|
| Habitat | Shallow excavated burrows on reef flats behind the reef margin, lower than Ec, and with a broader range of distribution | Deep burrows on the reef margin, positioned slightly above Em and with a narrower range of distribution | 1,2,6 |
| Bathymetric range | Intertidal, both above and below mean low water level (MLWS) | Intertidal, above MLWS | 1,2,6 |
| Salinity and thermo-tolerance | Lower tolerance to sudden temperature and salinity changes | Higher tolerance to sudden temperature and salinity changes | 4,5 |
| Body size | Bigger among Okinawan *Echinometra* | Moderate among Okinawan *Echinometra* | 3 |
| Wet weight (g) | 44.55 ± 7.38 | 39.17 ± 8.71 | 7 |
| Test length (mm) | 45.49 ± 5.66 | 43.09 ± 3.14 | 7 |
| Test width (mm) | 38.66 ± 2.89 | 34.82 ± 2.65 | 7 |
| Test height (mm) | 25.01 ± 2.21 | 24.51 ± 1.98 | 7 |
| Spine length (mm) | 18.43 ± 1.04 | 16.56 ± 0.87 | 7 |
| Color | Color highly variable, spine mostly brown and greenish brown, tip of spines not white, basal ring of spine unclear | Entirely green, brown, greenish brown, etc., with spine tip not white and basal ring of spine white and clear | 3 |
| Spicules: |  |  |  |
| a. Tubefeet | C-like or bihamate | Triradiate | 3 |
| b. Gonad | Spindle-shaped | Triradiate, curved triradiate, and bihamate | 3 |
| Breeding season | April-September (max. around late September) | April-December (max. around late September) | 4,5 |
| Egg size (μm) | 69.05 ± 1.10 | 71.83 ± 1.36 | 7 |
| Sperm head length (μm) | 4.92 ± 0.53 | 6.35 ± 0.67 | 7 |
| Thickness of jelly layer (μm) | 20.98 ± 3.55 | 18.13 ± 3.49 | 7 |

into a petri dish containing 15 ml of a conspecific egg suspension and another dish with 15 ml of a heterospecific egg suspension. Sperm were allowed to remain with the eggs for 10 min, and then excess sperm were removed with 3 consecutive washes with FSW. For consistency, when referring to heterospecific crosses, the maternal species is named first. For example, Em x Ec means that ova from Em females were fertilized by sperm from Ec males. In each heterospecific fertilization, a conspecific fertilization using ova from the same female was also conducted as a control. Fertilized eggs were then transferred to glass beakers and incubated in FSW at ambient room temperature until they attained the free-swimming blastula stage.

**Sperm dilution experiments**

The protocol and techniques used in these experiments differed from those employed for production of hybrids. To determine the fertilization rate for both homogametic and heterogametic crosses, a 0.1-ml aliquot of diluted egg suspensions (350-400 eggs) was placed in a small vial with 0.8 ml of FSW. Fresh "dry" sperm were first adjusted to $10°$ dilution and then quickly diluted in a series of seven, 10-fold dilutions. A 0.1-ml aliquot from one of these sperm solutions was then placed into the vial, to bring the final volume to 1 ml. For example, mixing a 0.1-ml aliquot of $10°$ undiluted sperm with 0.9 ml of egg suspension in a vial was called a $10^{-1}$-diluted concentration of sperm. This procedure was followed through a series until a $10^{-8}$-diluted concentration of sperm was made. After 5-10 min of gamete mixing, excess sperm were removed by 4-5 consecutive washes with FSW, and the eggs were then resuspended in 5 ml of FSW in a vial for incubation. For each heterospecific fertilization, conspecific fertilization using ova from the same female was also conducted as a control. Thirty-six replicate crosses between the 2 species were performed with gametes from new individuals each time. The fertilization rate was estimated at 1.25-1.5 h after gamete mixing by counting the number of eggs reaching the 2 to 4-cell stages among the first 100 eggs observed.

**Sperm age experiment**

In experiments to examine the effect of sperm age on fertilization success in heterospecific and conspecific crosses of Em and Ec under a limited sperm concentration ($10^{-5}$ dilution of "dry" sperm), "dry" sperm was quickly run through four, 10-fold dilutions ($10^{-4}$ dilution) using similar methods as for the sperm dilution experiment above. A 0.1-ml aliquot of the diluted sperm described above was then added to each of a series of 8 vials containing 0.8 ml FSW at time 0. A 0.1-ml aliquot of diluted egg suspension (3500-4000 eggs/ml) was added to the 1st vial at time 0, and in sequence to the remaining vials at 10-min intervals thereafter. Eggs added to each vial thus encountered sperm of different ages. Similar protocols were maintained for both conspecific and heterospecific crosses. The following steps of washing, incubating, and counting were similar to those of the sperm dilution experiments described above.

**Gamete exposure time experiment**

The effects of gamete exposure times on fertilization in Em, Ec, and their reciprocal hybrids were assessed by manipulating the duration for which eggs were in contact with a $10^{-5}$ dilution of "dry" sperm (limited sperm concentration at which conspecific crosses reached 100% or less than near 100% fertilization as proposed by Levitan 1993, Podolsky and Strathmann 1996, Palumbi 1998, and Rahman et al. 2001b). A 2-ml aliquot of diluted egg suspension (3500-4000 eggs/ml) was placed in a small beaker with 16 ml of FSW. Fresh dry sperm were diluted in a series of four, 10-fold dilutions, following methods similar to those of the sperm dilution experiment described above. A 2-ml aliquot from a $10^{-4}$ sperm suspension was then poured into the beaker containing 18 ml of an egg suspension, which ultimately constituted a $10^{-5}$ diluted concentration of sperm. At each time interval (10, 20, 30, 40, 50, 60, 90, 120, 180, 240, 300, 360, and 420 s), a 1-ml aliquot of the inseminated egg suspension was gently pipetted into plastic cylinders, the bottom of which had been replaced with a 30-$\mu$m Nitex mesh. The cylinder was then rinsed 4-5 times with FSW to remove excess sperm, and the eggs were resuspended in fresh FSW. The protocols including incubation and counting of fertilized eggs were the same as those described for the sperm dilution experiments above.

**Larval rearing and culture of juveniles and**

**adults**

Embryos and larvae produced from both homospermic and heterospermic crosses were reared through metamorphosis, as described by Rahman et al. (2000).  After 20-24 d of rearing, competent larvae were placed in small (25 x 20 x 10 cm) aquaria with aerated FSW, and pieces of coralline red algal skeletons were added to induce settlement and metamorphosis, following the method described by Rahman and Uehara (2001).  Seawater was partially changed once a week with fresh FSW.  This was continued for up to 3 mo, by which time test diameter of the juveniles were 6.0-7.0 mm.  Juveniles were then transferred to plastic (36 x 45 x 18 cm) aquaria and supplied with aerated flow-through seawater at Sesoko Marine Science Centre.  Coral skeletons covered with encrusting coralline algae served as food.  Cultures were continued for 1 yr, by which time the urchins had attained sexual maturity.  The performances of larvae, juvenile, and adults were examined and compared among the hybrid groups and their parental species controls.

**Morphological characteristics**

Detailed morphological characteristics were recorded or measured from 1-yr-old Em, Ec, and their reciprocal hybrids including test size, spine lengths, spicules in the tubefeet and gonads, pedicellaria valve length, color patterns of oral and aboral spines and test, and gamete sizes.

Tests of echinoids in the family Echinometridae are oblong, and the length, width, and height were measured with calipers after removing the spines.  For the measurement of spine length, 30 spines were randomly selected from the equator of the test.

Phenotypic coloration patterns of the body and spine in both aboral and oral views of adult urchins were observed using the color book of Kornerup and Wanscher (1978).

Spicules in the gonad and tubefoot of the urchins were thoroughly examined.  A small piece of gonadal tissue was clipped off using forceps and immersed in distilled water.  Samples were first treated with 10% KOH and then squashed on a slide glass with a cover slip and finally observed under an objective microscope (10 x 10).  Several tubefeet were clipped off with forceps, and the morphology of the spicules was investigated by the same methods described in the previous paragraph.

In order to easily collect the pedicellariae, all spines around the body were removed.  The pedicellariae were plucked form the test near the peristome and ambital area.  Soft tissues from the pedicellariae were removed by treating them with a 10% sodium hypochlorite solution (household bleach), and the clear pedicellaria were rinsed with 3 changes of distilled water.  The valve lengths (VLs) of pedicellariae were then measured under a compound microscope (10 x 10) with a preset micrometer.

The number and percentage of various pore pairs on every ambulacral plate (of the 5 ambulacra from the apical system) in a denuded test of reciprocal hybrids and pure parental species were counted under a dissecting microscope.

Gamete sizes (egg diameter and sperm-head length) were also measured from sexually mature hybrids and their conspecifics using a differential microscope following Amy (1983) and Rahman et al. (2002) (with eggs at 400x in a slide well, and sperm at 1000x on a flat slide).

**$F_1$ backcrosses**

After 1 yr of culturing, the conspecific controls and reciprocal hybrids reached sexual maturity and contained mature gametes.  All gametes were then reciprocally backcrossed by following the methods described in the sperm dilution experiments above to determine gametic compatibility among $F_1$ hybrids and their conspecific controls.  Fertilized eggs from the above backcrosses were then cultured through larval rearing and metamorphosis until juvenile urchins were 3 mo old, following the methods employed for the production of the $F_1$ generation.  For simplicity, when referring to the backcrosses, the maternal species is named first.  For example an $F_2$ hybrid produced through backcrosses between an $F_1$ female of Ea x Ea and an $F_1$ male of Ea x Ec is denoted as EaEa x EaEc.

**Hybrids in nature**

To examine the occurrence of natural hybridization between Em and Ec, field surveys were conducted along the Sunabe coast of Okinawa and the coast of Sesoko I., where both species occur nearly sympatrically in their respective microhabitats.  On the basis of the phenotypic color patterns of the body and spines of the experimentally produced adult hybrids (see Fig. 5 and descriptions in the text), 600 specimens were collected from the Sunabe and Sesoko coasts.

These hybrid-like urchins were then compared to laboratory-cultured hybrids with respect to aboral and oral coloration, spicule characteristics of the tubefoot and gonads, pedicellaria valve length, pore pairs, and gamete sizes.

### Data analysis

Percentage data for statistical analysis were first arcsine-transformed. Those replicates in which none or all eggs were fertilized, especially in fertilization experiments were given a value of 1/4n and 1-1/4n (n = number of observations) to improve the arcsine transformation (Zar 1996). This transformation helps normalize the data and reduce heterogeneity in variances. "Bartlett's test" was used to analyze the homogeneity of variances (Bartlett 1937). When variances were not significantly heterogeneous and no major departures from normality were noted, one-way analysis of variance (ANOVA) was performed followed by Tukey's multiple comparison test. Data that did not meet the normality assumption of the parametric analysis were analyzed using non-parametric statistics. This was done by transforming values to ranks and then applying one-way ANOVA followed by Tukey's multiple comparison test. The level for statistical significance was set at 0.05. Untransformed data are presented in tables and figures.

### RESULTS

### Effects of sperm dilution, age, and exposure time on fertilization

Fertilization success of both conspecific (Em x Em, and Ec x Ec) and heterospecific (Ec x Em, and Em x Ec) crosses were highly dependent on sperm concentrations, i.e., the higher the sperm concentration, the higher the fertilization rate (Fig. 2). As shown in figure 2, cross-fertilization was highly asymmetrical. Ec ova were fertilized by both Em, as well as Ec sperm, in high percentages. By contrast, Ec sperm even at a very high sperm concentration ($10^{-1}$ dilution of "dry" sperm) yielded a very low percent of fertilization of Em ova. At higher sperm concentrations ($10^{-4}$-$10^{-1}$ dilutions), percent fertilizations were identical (100% for both conspecific crosses), but the conspecific Em x Em crosses exhibited lower percentages of fertilization than Ec x Ec crosses at sperm concentrations lower than a $10^{-5}$ dilution.

Heterospecific Ec x Em crosses yielded lower percentages of fertilization than either of the conspecific crosses at concentrations of less than $10^{-2}$ dilution. These results followed the same trends as those described by Uehara et al. (1990) and Rahman et al. (2001a). At a limited sperm concentration ($10^{-5}$ dilution), the mean fertilization rate of Ec x Em crosses (70.69% ± 1.36%) was significantly lower than those of Em x Em (96.52% ± 0.88%) and Ec x Ec (99.67% ± 0.63%) (Table 2; Fig. 2), but neither conspecific cross significantly differed in their fertilization rates (Tukey's test, *p* > 0.05). No fertilization occurred in the Em x Ec crosses at this concentration (Fig. 2), although they showed a very low percentage of fertilization (13%) at the highest sperm concentration ($10^{-1}$ dilution) (Table 2), indicating strong gametic incompatibility between Em ova and Ec sperm.

The effect of sperm aging on fertilization success in conspecific and heterospecific crosses between Em and Ec is depicted in figure 3. Fertilization rates of conspecific Ec x Ec and Em x Em, and heterospecific Ec x Em crosses noticeably decreased when eggs were added to 10 min-old sperm. With 60 min-old sperm, both conspecifics and their heterospecific Ec x Em crosses showed negligible fertilization rates (0.10%-4.0%). No fertilization occurred with Em x Ec crosses even with 0 min-old sperm. Both conspecific sperm usually lost their potency within 30-40 min,
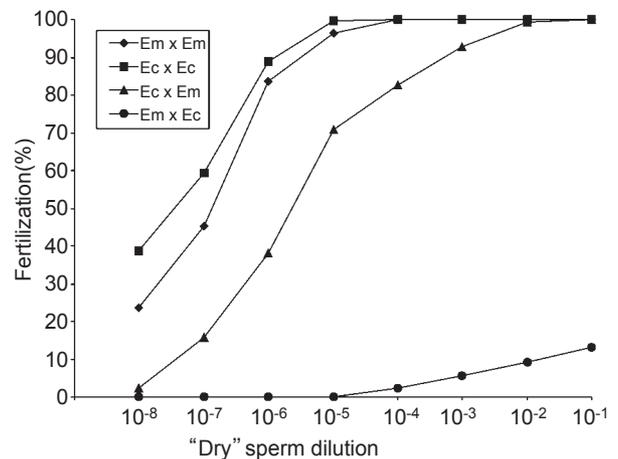


**Fig. 2.** Mean percentage of fertilization in conspecific and heterospecific crosses of *Echinometra mathaei* (Em x Em) and *Echinometra* sp. C (Ec x Ec) under various concentrations of "dry" sperm dilutions; the maternal species is named first. Fertilization as a function of sperm concentration for both conspecific and heterospecific crosses. In total, 36 replicate crosses were made for each of 4 combinations using gametes from separate male and female individuals each time.

whereas in heterospecific Ec x Em crosses, Em sperm lost their potency within 10-20 min. Overall, the longevity of sperm during conspecific Ec x Ec crosses was higher than that of Em x Em and was lowest in heterospecific Ec x Em crosses. The decreased potency of sperm in heterospecific crosses compared to that in conspecific crosses however, indicated their poor ability to produce hybrids in the field due to the combined effect of lower concentrations and aging if the distance between spawning individuals in the field was great. But if they occasionally occurred in very close to each other due to strong water currents and heavy wave action, and spawned at the same time, there would be a higher possibility to hybridize in a short period and at high sperm concentrations in the field. Fertilization successes of both hybrids and their conspecific crosses were greatly influenced by the duration of time within which egg sperm and were in contact with each other (Fig. 4). The conspecific Em x Em and Ec x Ec crosses exhibited higher percentages of fertilizations (97.0% for Em x Em and 99.8% for Ec x Ec) within 90 s of contact, whereas heterospecific Ec x Em crosses took much longer times (300-360

s) to achieve a higher fertilization rate. No fertilization was achieved by Em x Ec crosses, even after
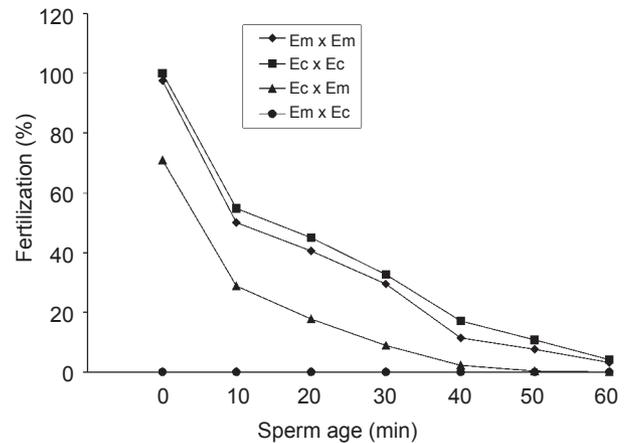


**Fig. 3.** Mean percentages of fertilization success in *Echinometra mathaei* (Em x Em), *Echinometra* sp. C (Ec x Ec), and their reciprocal hybrids at a limited sperm concentration ($10^{-5}$ dilution of "dry" sperm) using 7 levels of sperm age; the maternal species is named first. Fertilization as a function of sperm concentration and age for both conspecific and heterospecific crosses. In total, 8 replicate crosses were made using gametes from new individuals each time. No fertilization occurred with Em x Ec cross.

**Table 2.** Comparison of fertilization rates, and larval, juvenile, and adult performances of Em x Em, Ec x Ec, and their reciprocal hybrids. Fertilization was performed for Em x Em, Ec x Ec, and Ec x Em crosses at $10^{-5}$ dilutions of "dry" sperm, while that for Em x Ec was at a $10^{-1}$ dilution. In total, 36 crosses were made using gametes from new individuals each time. In case of larvae, juveniles, and adults, 6 replicate experiments were conducted in each cross for each type of performance. All values represent the mean ± SD with ranges in parentheses

| Performances | Em x Em | Ec x Em | Em x Ec | Ec x Ec |
|---|---|---|---|---|
| Fertilization (%) | 96.52 ± 0.88[a,1] | 70.69 ± 1.36[b] | 13.00 ± 0.86[c] | 99.67 ± 0.63[a] |
| | (95.00 - 98.00) | (68.00 - 73.00) | (12.00 - 15.00) | (98.00 - 100.00) |
| Larva and Juveniles | | | | |
| Survival (%) | 80.54 ± 2.27[a] | 75.75 ± 2.02[a] | 60.04 ± 2.64[b] | 78.71 ± 2.02[a] |
| | (77.50 - 83.75) | (72.75 - 79.00) | (57.50 - 62.50) | (76.25 - 81.75) |
| Metamorphosis (%) | 89.17 ± 3.76[a] | 85.00 ± 4.47[a] | 64.17 ± 3.76[b] | 87.50 ± 5.24[a] |
| | (85.00 - 95.00) | (80.00 - 90.00) | (65.00 - 70.00) | (80.00 - 95.00) |
| Recovery (%)[2] | 72.15 ± 1.42[a] | 68.23 ± 1.15[a] | 57.35 ± 1.62[b] | 70.25 ± 1.31[a] |
| | (70.75 - 75.38) | (66.50 - 69.98) | (55.70 - 59.92) | (69.01 - 72.10) |
| Adults | | | | |
| Wet weight (g) | 11.28 ± 0.62[a] | 10.93 ± 0.58[a] | 10.77 ± 0.60[a] | 8.98 ± 0.57[b] |
| | (10.30 - 12.30) | (10.22 - 12.00) | (10.00 - 11.80) | (8.25 - 10.00) |
| Wet gonad weight (g) | 1.99 ± 0.08[a] | 1.68 ± 0.06[b] | 1.59 ± 0.05[b] | 1.32 ± 0.03[c] |
| | (1.85 - 2.20) | (1.44 - 1.84) | (1.45 - 1.75) | (1.22 - 1.45) |
| Survival (%) | 86.45 ± 3.27[a] | 82.36 ± 2.72[a] | 70.25 ± 2.52[b] | 84.85 ± 2.30[a] |
| | (84.67 - 91.33) | (79.33 - 87.33) | (67.67 - 76.33) | (83.33 - 91.67) |

[1]Values in the same row with the same superscript do not significantly differ (by Tukey's test, $p > 0.05$).
[2]Three month-old juvenile urchins that were transferred to flow-through seawater system for advanced culture.

a prolonged time (420 s) of contact (Fig. 4).

## Larval, juvenile, and adult performances

Survival of competent larvae of Ec x Em hybrids did not significantly differ (by Tukey's test, $p > 0.05$) from that of conspecific parents, but that of the reciprocal hybrid (Em x Ec) was significantly lower (Tukey's test, $p < 0.05$) (Table 2). Laboratory-raised larvae of the parental species and hybrids reached a state of metamorphosis at about
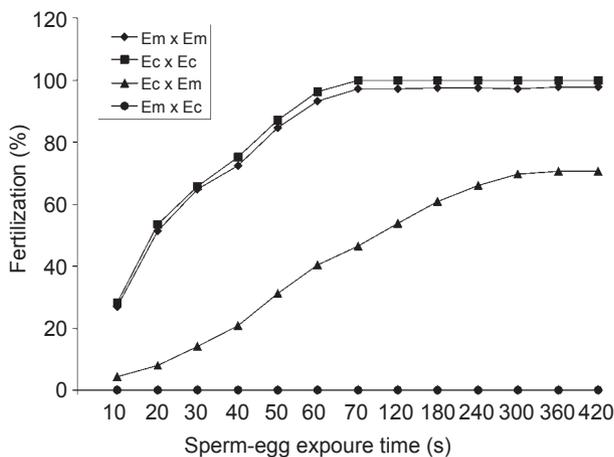


**Fig. 4.** Mean percentage of fertilization success in *Echinometra mathaei* (Em x Em), *Echinometra* sp. C (Ec x Ec), and their reciprocal hybrids in a series of timed crosses at a limited sperm concentration ($10^{-5}$ dilution); the maternal species is named first. In total, 8 replicates of conspecific and heterospecific crosses were made using gametes from new individuals each time. Fertilization as a function of sperm-egg exposure time for both conspecific and heterospecific crosses. No fertilization occurred in Em x Ec crosses, even with longer sperm exposure times.

20-24 d of age as evidenced by possession of a large rudiment. The majority of larvae metamorphosed to young juveniles on coralline red algal stone (CRAS) collected from the intertidal zone of the tropical Pacific within 1 d and there were no particular deformities/defects observed in the metamorphosed juvenile Ec x Em and Em x Ec hybrids. Em x Ec hybrids showed a significantly (Tukey's test, $p < 0.05$) lower metamorphosis rate compared with those of other groups, but the rate of Ec x Em hybrids did not differ significantly (Tukey's test, $p > 0.05$) from those of their pure parental species (Table 2). The recovery rate of 3 mo-old juvenile urchins of conspecific parents and their reciprocal hybrids followed the same trends as the metamorphosis rate (Table 2).

The growth and survival of hybrids and their parental species at the end of the experimental period are summarized in table 2. The mean wet weight attained by Em x Em was significantly higher than that of Ec x Ec, while both reciprocal hybrids, Ec x Em and Em x Ec, attained intermediate sizes (Table 2) which did not significantly differ (Tukey's test, $p > 0.05$) from the conspecific Em x Em. Wet gonad weight was significantly lower for Ec x Ec, while the hybrids of either direction contained a large amount of gonad tissue, but less than that of Em x Em, although the values did significantly differ (Tukey's test, $p < 0.05$) (Table 2). Survival was highest in Em x Em followed by Ec x Ec, and Ec x Em in that order, which did not significantly differ (Tukey's test, $p > 0.05$), but that of the hybrid, Em x Ec, differed significantly (Tukey's test, $p < 0.05$) from all other groups (Table 2). Therefore, the results of larval, juvenile, and adult growth and survival indicated that the hybrids in both

**Table 3.** Comparison of test sizes and spine lengths of *Echinometra mathaei* (Em x Em), *Ecchinometra* sp. C (Ec x Ec) and their reciprocal hybrids, 1 yr after metamorphosis. Twenty adult specimens were measured for each treatment. All values represent the mean ± SD with ranges in parentheses

| Treatment | Length of tests (mm) | Width of tests (mm) | Height of tests (mm) | Volume of tests (cm³) | Length of spines (mm) |
|---|---|---|---|---|---|
| Em x Em | 27.13 ± 0.61[a,1] (25.95 - 28.10) | 25.23 ± 0.56[a] (24.10 - 26.20) | 13.04 ± 0.45[a] (12.32 - 13.95) | 8.96 ± 0.63[a] (7.96 - 10.16) | 22.76 ± 0.92[a] (20.20 - 24.80) |
| Em x Ec | 26.38 ± 0.75[a] (25.10 - 27.75) | 24.85 ± 0.51[a] (23.90 - 25.85) | 12.64 ± 0.44[a] (12.15 - 13.55) | 8.29 ± 0.66[a] (7.48 - 9.61) | 21.87 ± 0.77[a] (19.80 - 24.50) |
| Ec x Em | 26.61 ± 0.75[a] (25.75 - 27.00) | 25.02 ± 0.58[a] (24.00 - 26.00) | 12.77 ± 0.46[a] (12.25 - 13.65) | 8.60 ± 0.69[a] (7.77 - 9.68) | 21.99 ± 0.79[a] (19.90 - 24.60) |
| Ec x Ec | 24.27 ± 0.73[b] (23.00 - 25.30) | 22.38 ± 0.82[b] (21.20 - 23.75) | 11.85 ± 0.47[b] (11.20 - 12.70) | 6.55 ± 0.77[b] (5.61 - 6.89) | 20.51 ± 0.79[b] (18.90 - 21.85) |

[1]Mean values in the same column with the same superscript do not significantly differ (by Tukey's test, $p > 0.05$).

directions were viable but showed intermediate features under lab-reared conditions. Similar results were also evidenced in hybrids between *Echinometra* sp. A and *Echinometra* sp. C (Rahman et al. 2000 2001a).

**Comparisons of phenotypic characteristics of conspecific Em x Em, Ec x Ec, and their reciprocal hybrids**

The length, width, height, and volume of the test, and spine length of Em x Em, Ec x Ec, and their reciprocal hybrids were measured 1 yr after metamorphosis (Table 3). Mean values for all of these parameters were highest in Em x Em progeny and smallest in Ec x Ec progeny. Although sta-

tistically significant differences were recognized in all parameters between Em x Em and Ec x Ec, the hybrids did not differ significantly from Em x Em but differed from Ec x Ec. On the other hand, both reciprocal hybrids were of intermediate sizes, and no significant differences (Tukey's test, $p > 0.05$) were evidenced between them (Table 3). Similar results were also obtained for hybrids between *Echinometra* sp. A and *Echinometra* sp. D (now *E. oblonga*) (Aslan and Uehara 1997), and between *Echinometra* sp. A and *Echinometra* sp. C (Rahman et al. 2000 2001a).

Major phenotypic color patterns of hybrids and their conspecific parents were examined at the end of the experiment. The aboral body coloration differed between conspecific and hybrid speci-
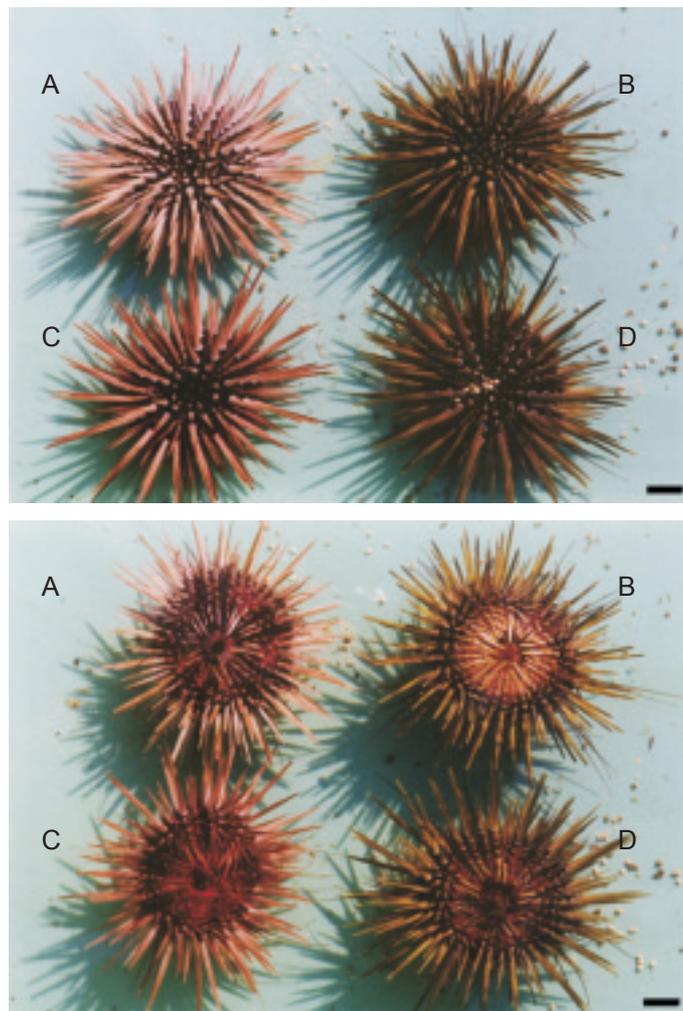


**Fig. 5.** Aboral (upper) and oral (lower) color patterns of experimentally produced 1-yr-old adult *Echinometra mathaei* (Em), *Echinometra* sp. C (Ec), and their reciprocal hybrids; the maternal species is named first: (A) Em x Em; (B) Ec x Ec; (C) Em x Ec; (D) Ec x Em. Scale bars indicate 1.0 cm.

mens (Fig. 5, upper). Test color of Em x Em was dominated by dark brown and each spine was uniformly brown with an indistinct white basal ring. The test and spine of Ec x Ec were uniformly greenish and each spine had a clear basal white ring. The Em x Ec hybrid was more similar to Em x Em in having a dark-brown test with uniformly brown spines. On the other hand, Ec x Em was closer to Ec x Ec in having a greenish-brown test with greenish spines. As to oral body coloration (Fig. 5, lower), Em x Em had pale-brown spines around the mouth and dark-brown test, whereas Ec x Ec had yellowish-green spines around the

mouth and a greenish test color. The characters of the Em x Ec hybrid were more similar to those of Em x Em, whereas those of the Ec x Em hybrid were more similar to those of Ec x Ec. Therefore, the coloration of hybrids was maternally inherited. Similar color patterns were also observed in hybrids produced from other cross combinations of *Echinometra* spp. (Aslan and Uehara 1997, Aslan 2000, Rahman et al 2001a).

Tubefoot spicules in Em x Em were bihamate (85.56%), bihamate-like (3.19%), triradiate (9.15%), and triradiate-bihamate (1.11%); whereas those in Ec x Ec were always triradiate (100%)
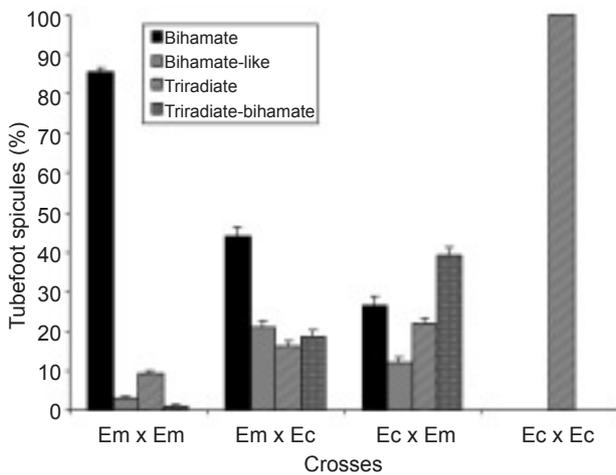


**Fig. 6.** Types and mean percentages of spicule characteristics in tubefoot of 1-yr-old Em x Em, Ec x Ec, and their reciprocal hybrids. Twenty individuals were randomly examined for each treatment with 10 tubefeet per individual. Error bars indicate the standard deviation.
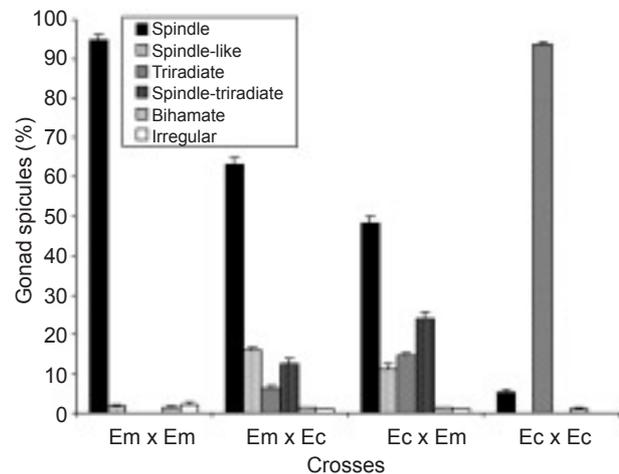


**Fig. 7.** Types and mean percentages of spicule characteristics in gonads of 1-yr-old Em x Em, Ec x Ec, and their reciprocal hybrids. Twenty individuals were randomly examined for each treatment with 10 gonadal tissues per individual. Error bars indicate the standard deviation.

**Table 4.** Valve length (VL) of 4 types of pedicellaria in *Echinometra mathaei* (Em x Em), *Echinometra* sp. C (Ec x Ec), and their reciprocal hybrids. Twenty individuals were examined from each cross with 10 pedicellariae of each type from each individual. All values represent the mean ± SD in μm followed by the ranges in parentheses

| Pedicellaria type | Em x Em | Em x Ec | Ec x Em | Ec x Ec |
|---|---|---|---|---|
| Tridentate | 980.33 ± 18.34[a,1] | 942.66 ± 16.35[b] | 911.45 ± 16.92[c] | 877.64 ± 13.32[d] |
| | (950.00 - 1050.00) | (890.00 - 100.00) | (870.00 - 980.00) | (840.00 - 930.00) |
| Globiferous | 735.42 ± 21.41[a] | 708.31 ± 18.34[b] | 680.45 ± 17.92[c] | 645.22 ± 19.63[d] |
| | (690.00 - 790.00) | (660.00 - 770.00) | (640.00 - 740.00) | (590.00 - 680.00) |
| Ophiocephalous | 610.44 ± 22.31[a] | 587.72 ± 19.98[b] | 566.92 ± 20.36[c] | 536.4 3 ± 20.54[d] |
| | (560.00 - 670.00) | (540.00 - 660.00) | (510.00 - 640.00) | (490.00 - 590.00) |
| Triphyllous | 172.43 ± 12.72[a] | 161.38 ± 12.77[b] | 150.89 ± 11.38[c] | 140.21 ± 10.87[d] |
| | (150.00 - 220.00) | (140.00 - 200.00) | (130.00 - 190.00) | (110.00 - 180.00) |

[1]Mean values in the same row with the same superscript do not significantly differ (by Tukey's test, $p > 0.05$).

(Fig. 6). Tubefoot spicules of Em x Ec hybrids were bihamate (43.98%), bihamate-like (21.13%), triradiate (16.32%), and triradiate-bihamate (18.62%), but they were dominated by the bihamate type; whereas those of Ec x Em were bihamate (26.62%), bihamate-like (12.12%), triradiate (21.99%), and triradiate-bihamate (38.65%) but were dominated by the triradiate-bihamate type (Fig. 6). Moreover, the proportions of spicules in the Em x Ec and Ec x Em hybrids significantly differed (Tukey's test, $p < 0.05$) and tended towards maternal affinities.

Spicules of the gonad in Em x Em were almost spindle-shaped (94.95%), with a very few other types such as spindle-like (1.61%), bihamate (1.29%), and irregular (2.10%) (Fig. 7), but gonad spicules in Ec x Ec were almost all triradiate (93.75%), spindle-shaped (5.13%) and a very negligible proportion (1.12%) bihamate (Fig. 7). Gonadal spicules of Em x Ec were mostly spindle-shaped (63.04%), spindle-like (15.81%), and spindle-triradiate (12.52%) with a very few triradiate (6.32%), bihamate (1.15%), and irregular (1.80%) ones (Fig. 7); whereas spicules in Ec x Em were spindle-shaped (48.17%), spindle-triradiate (24.03%), and triradiate (14.43%) with fewer bihamate (1.23%) and irregular (0.95%) types (Fig. 7). Significant differences were recognized among the

hybrid groups and their reciprocal hybrids in the proportions of the major types of spicules. Therefore, the ratio of gonad spicules in the hybrids were intermediate.

The pedicellariae found in both conspecifics and their reciprocal hybrids were tridentate, globiferous, ophiocephalous and triphyllous. Only the valve length (VL) of all 4 types was measured and compared among conspecifics and their hybrid groups. As shown in table 4, all 4 types of pedicellaria VL of Em x Em were significantly (Tukey's test, $p < 0.05$) larger than those of their corresponding types of Ec x Ec. Both hybrids had intermediate sizes, but differed significantly from the controls and among themselves (Table 4).

Percentages of each pore pair on the ambulacral plates of denuded tests are summarized in table 5. There were no significant differences (Tukey's test, $p > 0.05$) in the very small proportions of 1-, 2-, 6-, 7-, and 8-pore pairs among the conspecifics and their hybrid groups (Table 5). The percentages of 4-pore pairs among all 4 groups were the highest (60.14% to 70.85%), while the 3-pore pairs (16.85% to 25.87%) were the next highest, followed by 5-pore pairs (8.02 to 8.73%). The major striking and significant differences (Tukey's test, $p < 0.05$) were recognized in the proportions of each of the 3- and 4-pore pairs

**Table 5.** Proportion of each pore pairs in denuded tests of adult Em x Em, Ec x Ec, and their reciprocal hybrids. Twenty tests from each cross were thoroughly examined for the various pore pairs. All values represent the mean ± SD in percentage with ranges in parentheses

| Pore pairs | Em x Em | Em x Ec | Ec x Em | Ec x Ec |
|---|---|---|---|---|
| One-pore pair | 1.38 ± 0.48[a,1] | 1.35 ± 0.46[a] | 1.29 ± 0.49[a] | 1.27 ± 0.58[a] |
| | (0.72 - 2.61) | (0.70 - 2.60) | (0.68 - 2.55) | (0.00 - 2.24) |
| Two-pore pair | 1.31 ± 0.58[a] | 1.74 ± 0.63[a] | 1.85 ± 0.60[a] | 2.16 ± 0.99[a] |
| | (0.68 - 2.52) | (0.76 - 2.85) | (0.79 - 2.92) | (0.00 - 3.42) |
| Three-pore pair | 16.85 ± 1.33[d] | 19.73 ± 1.48[c] | 22.76 ± 1.68[b] | 25.87 ± 1.47[a] |
| | (14.20 - 18.25) | (17.30 - 21.40) | (19.95 - 24.88) | (22.40 - 28.22) |
| Four-pore pair | 70.85 ± 1.73[a] | 66.91 ± 1.63[b] | 63.64 ± 1.89[c] | 60.14 ± 1.44[d] |
| | (68.25 - 73.20) | (64.44 - 69.30) | (61.14 - 66.42) | (57.33 - 62.70) |
| Five-pore pair | 8.02 ± 1.02[a] | 8.59 ± 1.29[a] | 8.66 ± 1.32[a] | 8.73 ± 0.89[a] |
| | (6.70 - 8.86) | (6.99 - 10.13) | (7.12 - 10.22) | (7.26 - 10.30) |
| Six-pore pair | 0.85 ± 0.45[a] | 0.88 ± 0.48[a] | 0.92 ± 0.60[a] | 0.94 ± 0.49[a] |
| | (0.00 - 1.44) | (0.00 - 1.50) | (0.00 - 1.58) | (0.00 - 1.65) |
| Seven-pore pair | 0.42 ± 0.32[a] | 0.41 ± 0.38[a] | 0.40 ± 0.42[a] | 0.37 ± 0.39[a] |
| | (0.00 - 0.96) | (0.00 - 0.92) | (0.00 - 0.90) | (0.00 - 0.81) |
| Eight-pore pair | 0.32 ± 0.36[a] | 0.39 ± 0.42[a] | 0.48 ± 0.45[a] | 0.52 ± 0.43[a] |
| | (0.00 - 0.80) | (0.00 - 0.95) | (0.00 - 1.05) | (0.00 - 1.25) |

[1]Mean values in the same row with a common superscript do not significantly differ (by Tukey's test, $p > 0.05$).

among the conspecifics and their reciprocal hybrids. The 5-pore pairs did not significantly differ, despite the fact that the hybrids had intermediate proportions.

The egg diameter of the conspecific Em x Em was the smallest while that for Ec x Ec was the largest. Hybrids contained intermediate-sized eggs that significantly differed (Tukey's test, $p < 0.05$) from either of the conspecifics, but the hybrids did not significantly differ (Tukey's test, $p > 0.05$) from each other (Table 6). The size of sperm heads also significantly differed (Tukey's test, $p < 0.05$) from that of Ec x Ec. Although the values did not differ significantly between Em x Ec and Ec x Em hybrids, both were intermediate between values of Em x Em (the smallest) and Ec x Ec (the largest) (Table 6). Aslan and Uehara (1997) and Rahman et al. (2001a) also found similar results in hybrids produced from other cross combinations of *Echinometra* spp.

## Existence of natural hybrids

Six hundred individuals with more or less intermediate coloration of the 2 species as well as with the experimentally produced maternal coloration patterns of both reciprocal hybrids were collected from the Sunabe and Sesoko coasts, where Em and Ec are sympatrically and abundantly intermingled. However, detailed comparisons of the above morphological characters revealed that none of these individuals actually had common character combinations to the experimentally produced hybrids; all were either *Echinometra mathaei* or *Echinometra* sp. C. Similarly, neither Aslan and Uehara (1997) nor Rahman et al. (2001a) found any natural hybrids between *Echinometra* sp. A and *Echinometra oblonga*, or between *Echinometra* sp. A and *Echinometra* sp. C in the field even though they had succeeded in producing sexually mature hybrids of these species in the

**Table 6.** Gamete sizes of sexually matured conspecific *Echinometra mathaei* (Em x Em), *E.* sp. C (Ec x Ec), and their reciprocal hybrids, 1 yr after metamorphosis. Twenty individuals were examined from each cross with 25 eggs and 25 sperms from each individual; mean ± SD in μm, ranges in parentheses

| Gametes | Em x Em | Em x Ec | Ec x Em | Ec x Ec |
|---|---|---|---|---|
| a. Egg diameter | 69.02 ± 1.05[c] | 71.85 ± 1.20[b] | 71.38 ± 1.17[b] | 72.63 ± 1.29[a,1] |
| Ranges | (67.50 - 71.25) | (70.50 - 73.50) | (70.25 - 73.25) | (71.25 - 74.50) |
| b. Sperm-head length | 4.89 ± 0.82[c] | 5.79 ± 0.80[b] | 5.41 ± 0.90[b] | 6.45 ± 0.77[a,1] |
| Ranges | (3.72 - 6.20) | (5.40 - 6.70) | (5.20 - 6.40) | (5.96 - 7.44) |

[1]Mean values in the same row with different superscripts were statistically significant (by Turkey's test, $p < 0.05$).

**Table 7.** Percentage of eggs fertilized in backcrosses among 1-yr-old laboratory-reared F$_1$ generation of conspecifics and hybrids of *Echinometra mathaei* (Em) and *Echinometra* sp. C (Ec) at limited sperm concentration ($10^{-5}$ dilution). Each value represents 9 replicate crosses with gametes from new individuals in each replicate; mean ± SD, ranges in parentheses

| Sperm from | Eggs from | | | |
|---|---|---|---|---|
| | Em x Em | Em x Ec | Ec x Em | Ec x Ec |
| Em x Em | 97.44 ± 0.73 | 87.11 ± 1.45 | 84.89 ± 1.05 | 72.56 ± 1.42 |
| | (96.00 - 98.00) | (85.00 - 90.00) | (83.00 - 86.00) | (70.00 - 75.00) |
| Em x Ec | 80.04 ± 1.22 | 93.11 ± 0.78 | 95.44 ± 0.88 | 95.89 ± 1.27 |
| | (78.00 - 82.00) | (92.00 - 94.00) | (94.00 - 97.00) | (94.00 - 98.00) |
| Ec x Em | 80.35 ± 1.22 | 98.56 ± 1.01 | 94.87 ± 1.27 | 93.11 ± 0.78 |
| | (79.00 - 82.00) | (97.00 - 100.00) | (93.00 - 97.00) | (92.00 - 94.00) |
| Ec x Ec | 0 | 74.33 ± 1.41 | 77.11 ± 1.90 | 99.56 ± 0.73 |
| | | (73.00 - 77.00) | (74.00 - 80.00) | (99.00 - 100.00) |

laboratory.

## Fertilization rates in $F_1$ backcrosses

Fertilization rates of backcrosses using gametes of $F_1$ hybrids and their conspecific parental species under a limited sperm concentration ($10^{-5}$ dilution) are shown in table 7. Ova from both hybrids, Em x Ec and Ec x Em yielded higher percentages of fertilization with Em x Em sperm (87.11% and 84.89%) than those with Ec x Ec sperm (74.33% and 77.11%), demonstrating that Ec sperm were more discriminating than Em sperm. Conversely, backcrosses by males from Em x Ec and Ec x Em yielded higher percentages

of fertilization with Ec x Ec ova (95.89% and 99.44%) than with Em x Em ova (80.04% and 80.35%, respectively), demonstrating that Em ova appeared to be more discriminating than Ec ova. Thus, differences in fertilization rates of gametes among $F_1$ conspecifics and their $F_1$ hybrids clearly indicate sequential differences in their gamete recognition alleles. On the other hand, higher fertilization rates between the different types and within the same types of hybrids (Table 7), however, indicated the near loss of any specificity in the gamete-binding loci. Similar results were also obtained in $F_1$ backcrosses among hybrids and the $F_1$ conspecifics of Ea and Ec by Rahman et al. (2001a).

**Table 8.** Comparisons of larval survival, metamorphosis, and the recovery of the $F_2$ progenies produced from backcrosses among the $F_1$ generation of conspecifics and hybrids of Em and Ec. All values represent the mean ± SD with number of replicate experiments (upper) and ranges (lower) in parentheses

| Backcrosses | Larval survival (%) | Metamorphosis (%) | Recovery (%) |
|---|---|---|---|
| EmEm x EmEm | 79.33 ± 1.94[a,1] (3) | 89.00 ± 4.18[a] (5) | 73.25 ± 1.97[a] (3) |
| | (77.75 - 81.50) | (85.00 - 90.00) | (71.22 - 75.52) |
| EcEc x EcEc | 78.25 ± 1.98[a] (3) | 88.00 ± 2.73[a] (5) | 71.56 ± 1.88[a] (3) |
| | (76.75 - 80.50) | (85.00 - 90.00) | (69.25 - 73.60) |
| EcEc x EmEm | 75.91 ± 1.53[a] (3) | 86.00 ± 4.18[a] (5) | 70.05 ± 2.02[a] (3) |
| | (74.25 - 77.25) | (80.00 - 90.00) | (68.21 - 73.03) |
| EcEm x EmEm | 77.75 ± 2.06[a] (3) | 87.00 ± 2.74[a] (5) | 71.04 ± 1.78[a] (3) |
| | (75.50 - 78.25) | (85.00 - 90.00) | (69.02 - 73.75) |
| EmEc x EmEm | 78.08 ± 2.08[a] (3) | 88.00 ± 2.74[a] (5) | 71.33 ± 1.85[a] (3) |
| | (75.75 - 79.75) | (85.00 - 90.00) | (69.24 - 73.35) |
| EcEm x EcEc | 76.58 ± 1.01[a] (3) | 86.00 ± 4.18[a] (5) | 70.25 ± 1.98[a] (3) |
| | (75.50 - 77.50) | (80.00 - 90.00) | (68.30 - 72.88) |
| EmEc x EcEc | 76.42 ± 0.95[a] (3) | 86.00 ± 4.18[a] (5) | 69.98 ± 1.82[a] (3) |
| | (75.75 - 77.50) | (80.00 - 90.00) | (68.10 - 72.24) |
| EmEm x EcEm | 77.42 ± 1.28[a] (3) | 87.00 ± 4.47[a] (5) | 70.85 ± 2.13[a] (3) |
| | (76.00 - 78.50) | (80.00 - 90.00) | (68.68 - 72.96) |
| EmEm x EmEc | 77.25 ± 0.90[a] (3) | 87.00 ± 4.47[a] (5) | 70.68 ± 1.92[a] (3) |
| | (76.50 - 77.00) | (80.00 - 90.00) | (68.50 - 72.80) |
| EcEc x EcEm | 78.42 ± 1.42[a] (3) | 90.00 ± 4.47[a] (5) | 72.89 ± 1.97[a] (3) |
| | (77.25 - 80.00) | (85.00 - 95.00) | (70.30 - 75.10) |
| EmEc x EmEc | 78.00 ± 1.15[a] (3) | 89.00 ± 4.18[a] (5) | 72.34 ± 1.75[a] (3) |
| | (77.00 - 79.25) | (85.00 - 95.00) | (70.21 - 74.85) |
| EmEc x EcEm | 78.58 ± 1.28[a] (3) | 91.00 ± 6.52[a] (5) | 72.72 ± 1.88[a] (3) |
| | (77.50 - 80.00) | (80.00 - 95.00) | (70.25 - 74.90) |
| EcEc x EmEc | 78.67 ± 1.88[a] (3) | 89.00 ± 2.23[a] (5) | 72.50 ± 2.03[a] (3) |
| | (76.75 - 80.50) | (85.00 - 90.00) | (70.35 - 75.00) |
| EcEm x EmEc | 78.33 ± 1.38[a] (3) | 90.00 ± 5.00[a] (5) | 72.66 ± 1.67[a] (3) |
| | (77.00 - 79.75) | (85.00 - 95.00) | (70.45 - 74.95) |
| EcEm x EcEm | 77.42 ± 1.18[a] (3) | 89.00 ± 4.18[a] (5) | 72.30 ± 1.90[a] (3) |
| | (76.50 - 78.25) | (85.00 - 95.00) | (70.20 - 74.69) |

[1]Mean values in the same column with the same superscript do not significantly differ (by Turkey's test, $p > 0.05$).

**Larval and juvenile performances of F$_2$ hybrids**

Performances of F$_2$ hybrids and their con-specifics in terms of larval survival, metamorphosis and recovery (survival of 3 mo-old juvenile urchins) in all combinations (except for the EmEm x EcEc cross as no fertilization occurred at 10$^{-5}$ dilution of "dry" sperm) were assessed and compared among treatments (Table 8). Survival of competent larvae in all combinations was high and no significant differences (Tukey's test, $p > 0.05$) were recognized among them. Metamorphosis and recovery rates also followed the same trends as larval survival. These results, however, indicated that F$_2$ hybrids were viable which further indicates the close genetic relationship between Em and Ec.

## DISCUSSION

Cross-fertilization rates between the 2 sibling species of *Echinometra*, Em and Ec, showed a distinct asymmetry as previously reported by Uehara et al. (1990). The heterogamic fertilization rate was high when sperm of Em was involved; yet a comparatively lower percent of Em eggs was fertilized by Ec sperm, even at a higher sperm concentration. This strong impediment to fertilization, at least with Em (egg) x Ec (sperm) crosses, indicates the presence of a gamete recognition protein binding system, as reported by Metz et al. (1994) and Metz and Palumbi (1996). This reduction in fertilization rates might eventually lead to gamete incompatibility and reproductive isolation. It could, at some point, provide a mechanism for maintaining species integrity of these *Echinometra* species (Metz et al. 1994, Vacquier et al. 1995, Metz and Palumbi 1996, Aslan and Uehara 1997, Palumbi 1998, Rahman et al. 2001a). However, the higher fertilization rate of Ec eggs by Em sperm indicates that prezygotic isolation by gamete incompatibility hardly appears to be present between these 2 species, or among most of the other putative mor-phospecies of *Echinometra* that coexist in Okinawa (Uehara et al. 1990, Aslan and Uehara 1997, Aslan 2000, Rahman 2000, Rahman et al. 2000 2001a). These findings run contrary to the predictions from the "speciation by reinforcement" model (Dobzhansky 1940, Butlin 1989, Liou and Price 1994). The model envisions that populations, which have acquired a degree of reproductive isolation in allopatry will, when they become sympatric, develop prezygotic isolation to avoid gamete wastage in inferior hybrids. Therefore, it

seems unlikely that unidirectional gamete incompatibility alone provides a mechanism for speciation in *Echinometra*. Two sympatric species of asteroids in the genus *Patiriella* do not show gamete incompatibility (Byrne and Anderson 1994). Reproductive isolation and speciation may have occurred before the evolution of gametic incompatibility because sperm-egg recognition molecules do accumulate over time as species diverge (e.g., McCartney and Lessios 2002).

Similar asymmetric gametic incompatibility between 2 sympatric species of Caribbean *Echinometra* such as that observed between *E. lucunter* and *E. viridis* has been found several times (Lessios and Cunningham 1990, McCartney and Lessios 2002). A high percentage of eggs of *Strongylocentrotus droebachiensis* is fertilized by sperm of its sympatric (but bathymetrically displaced) congener, *S. pallidus* but the reciprocal cross of *S. pallidus* eggs and *S. droebachiensis* sperm produces a very low percentage of fertilization (Strathmann 1981). Oyster species in the genus *Crassostrea* that co-occur in Japan show an asymmetric blockage to fertilization (Banks et al. 1994). Percent fertilization of eggs from white abalone (*Haliotis sorenseni*) by sperm of another California species, *H. rufescens*, is close to 100%, but is much lower in the reciprocal cross (Leighton and Lewis 1982). In 1 case, asymmetric incompatibility exists between sympatric species that are distantly related. Close to 100% of the eggs of the Hawaiian sea urchin, *Colobocentrotus atratus*, can be fertilized by sperm of the sea urchins *Echinometra mathaei*, *Pseudobolatia indiana*, and *Tripneustes gratilla* (the latter of 2 of which are in a different family). In contrast, the same concentration of *C. atratus* sperm fertilizes a much smaller percentage of eggs in the reciprocal crosses (Branham 1972). Kaneshiro (1980) suggested that behavioral isolation evolves as a byproduct of disruptive or directional selection in allopatry, and that its asymmetry is due to drift and founder effects in one but not both of the descendent populations. In an analogous fashion, asymmetric barriers to fertilization could emerge as an accident of history, such as a bottleneck in one of the 2 sister species, then becomes exaggerated as selection within the bottlenecked population promotes co-evolutionary changes of egg and sperm, the mechanisms of which need to be examined in order to explain how species integrity is maintained in these closely related species.

Furthermore, the successful rearing of hybrids to fertile adults in laboratory conditions eliminates

the likelihood that hybrid inviability or sterility is a post-zygotic mechanism of reproductive isolation. However, Em x Ec hybrids had lower survival, metamorphic success, and juvenile survival than did Ec x Em hybrids or both conspecific controls. Nevertheless, those Em x Ec hybrids that did survive and grow at the same rate as conspecifics were as fertile as conspecifics in the backcrosses. Moreover, Ec x Em hybrids were as viable and fertile as conspecifics, indicating that there is neither gametic nor postzygotic blockages to introgression present at least in Ec x Em hybrids. Similarly, Rahman et al. (2000 2001a) found no existence of postzygotic blockages to introgression in Ec x Ea hybrids produced experimentally from the less-distinct pair of undescribed Ea and Ec.

Potential prezygotic isolating mechanisms that may occur between these 2 species include ecological separation and asynchronous reproductive cycles (Mayr 1970, Palumbi 1994, McCartney et al. 2000, Rahman et al. 2001a, McCartney and Lessios 2002). The 2 species of sea urchins, Em and Ec, in this study live relatively close to each other but occupy different microhabitats; Em inhabits shallower excavated burrows on rocky reef flats, positioned below those of Ec, whereas Ec prefers to inhabit deeper burrows on the reef margin, positioned above those of Em (Nishihira et al. 1991). In their earlier study, Tsuchiya and Nishihira (1985) found that Em was more aggressive than Ea and always demonstrated remarkable agonistic behavior towards intruders by driving them away if the latter species was brought into a borrow which the former inhabits. Whether or not this agonistic behavior is responsible for the microhabitat segregation in Em and Ec remains to be examined. However, the distance between individuals in the field may be great enough to prevent hybridization, because as shown in other species of echinoids, fertilization success drops dramatically with distance between spawning individuals (Pennington 1985, Levitan 1998a b). Moreover, individuals of Em 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 these 2 species.

In our laboratory experiment, the sperm concentration used to fertilize the eggs of Em and Ec was probably higher than would be encountered under natural conditions, especially considering the dilution that would occur when individuals were separated by 1 m or more (Pennington 1985, Levitan 1998b). Unfortunately, we know very little

about the fertilization kinetics of free-spawning marine invertebrates in the field. However, results from our cross-fertilization experiments revealed that the fertilization rates of eggs of either Em and Ec mixed with sperm from Em were similar over a wide range of sperm concentrations, while the sperm of Ec had very low fertilization success with Em eggs, even at high concentrations of up to $10^{-1}$ dilution (Fig. 2). The hybrid crosses had a lower average fertilization rate compared with the controls possibly because heterospecific gametes have a lower affinity for each other. Under conditions of lower sperm concentrations that would be expected in nature, this decreased fertilization rate may contribute to the reproductive isolation of Em and Ec.

During the field survey on the shallow rocky intertidal zone of the Sesoko and Sunabe coasts of subtropical Okinawa, Rahman and Uehara (2001) observed that juveniles as well as adults of all 4 species of *Echinometra*, despite their microhabitat segregation, formed dense aggregations on coralline red algal substratum rather than on brown or green algal rocks. In addition to the problem of being sufficiently separated in adjacent microhabitats to prevent gamete mixing after spawning, there is a problem of maintaining microhabitat differentiation that could assure reproductive isolation. This problem is especially acute for species of *Echinometra* with widely dispersing larvae. 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 microhabitat selection in sea urchin larvae. Cameron and Schroeter (1980) also found that larvae of *Stongylocentrotus purpuraturs* settled indiscriminately on bacterial-covered substrates, and later microhabitat differentiation of the juveniles probably occurred to prevent high selective mortality or to avoid predation. The mechanism resulting in and maintaining microhabitat differentiation among species of *Echinometra* in the tropical West Pacific deserves further study.

Sperm of echinoids remain active for a short period of time when they are diluted after spawning (Rahman et al. 2001b), much less than the hour in temperate species (Hinegardner 1975, Levitan et al. 1991), and possibly less in tropical species like *Echinometra* (Rahman et al. 2001b). The results of our sperm-age experiment under laboratory conditions demonstrated that sperm lost their potency within 20-30 min in conspecific cross-

es and much sooner in heterospecific crosses under a limited sperm concentration ($10^{-5}$ dilution of "dry" sperm). As sperm lose their potency within a short period of time, the decreased longevity of diluted sperm might decrease the likelihood of cross-fertilization, when spawning is completely asynchronous and if the distance between spawning individuals in the field is great. Reduced fertilization rates in heterospecific crosses compared to those in conspecific crosses due to the aging of diluted sperm may possibly be responsible for maintaining reproductive isolation between Em and Ec. Asynchrony in spawning, therefore, would ensure that these 2 species could exist in sympatry as separate species whether or not their gametes are capable of fertilization (see Lessios 1984). However, the annual spawning period of Em and Ec overlap extensively, starting from Apr./May through Sept. (Arakaki and Uehara 1991; Table 1), and they can readily spawn during this period. Therefore, temporal separation of spawning seasons is an unlikely mechanism of reproductive isolation. Nevertheless, peak spawning periods, as well as salinity and temperature tolerances, differ between the 2 species (Arakaki 1989). On the other hand, different stages of gametogenetic cycles observed through histological examinations by Aslan (2000) revealed that different individuals of *Echinometra* spp. became ripe or spawned at different times as similarly found for *Echinometra mathaei* at Okinawa (Nishihira 1975), in the Gulf of Suez (Pearse 1969), and at Rottnest I. (Pearse and Philips 1968), and for *Echinometra* sp. A (Yabiku and Nishihira pers. comm.). These factors, in addition to possible separation in diel spawning times or specific pheromonal spawning cues, provide exogenous factors underlying reproductive isolation between Em and Ec.

As with other free-spawning marine invertebrates, sea urchins are known to have no courtship or very few premating behaviors or communication between adults before reproduction (e.g., Lessios and Cunningham 1990, Palumbi and Metz 1991, Metz et al. 1994, Lamare and Stewart 1998), especially when habitats and spawning seasons may overlap such as in *Echinometra* spp. (Arakaki and Uehara 1991). During these hybridization experiments, we kept both sexes of mature Em and Ec in the same aquarium and closely observed them. But the urchins neither spawned nor showed any breeding behavior within or between them. Similarly, during the peak spawning period, Nishihira (1975) and Aslan (2000) in their field studies observed no particular mating

behavior in *Echinometra* spp. rather than mass-spawning, when they aggregated in their respective habitats. Instead, gametes are spawned into the water column, and the most important interaction is between egg and sperm at fertilization. In these cases, reproductive isolation may arise by changes in timing of gamete release (see Lessios 1984 and above) or in the clumping of conspecific adults (Billet and Hausen 1982). However, the behavioral components of reproductive isolation that are thought to drive rapid speciation in other taxa are largely absent from *Echinometra* spp. (Palumbi and Metz 1991).

A possible elucidation of the lack of introgression in the field may be due to interspecific gamete competition between these species. Palumbi (1998), for example, showed that at low concentrations where less than near 100% fertilization is achieved, sperm from different males of the same species 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 proposed by Rice (1998), and which was demonstrated to occur in multiple-mating, internally fertilizing species such as the ground crickets, *Allonemobius fasciatus* and *A. socius* (Howard et al. 1998).

Information on how such sperm competition could be achieved in broadcast-spawning, externally fertilizing species such as sea urchins, has been lacking. However our findings from the sperm-egg exposure (contact) time experiment revealed that conspecific Em x Em and Ec x Ec crosses achieved higher percentages of fertilization much sooner than did the heterospecific Ec x Em crosses under a limited sperm concentration (Fig. 4). As Em and Ec mature at the same time and their breeding seasons extensively overlap (Arakaki and Uehara 1991; Table 1), there might be a possibility of their gametes meeting in the water column. However, if it does occur in the field, and if conspecific sperm outcompete heterospecific sperm for fertilization (Howard et al. 1998) and are always at an advantage when mixed at low concentrations with sperm from another species because of more-compatible gametes than heterospecific gametes, a mechanism for maintaining species integrity in sympatric Em and Ec may be present.

Characteristics of the hybrid progeny between

Em and Ec, shown in the present paper are inherited by either the maternal or bi-parental genome. Color patterns of both hybrids, for instance, tended to be maternally inherited. Similar maternally inherited color patterns were also observed in hybrids between *Strongylocentrotus nudus* and *S. intermedius* (Osanai 1974), between Ea and Eo (Aslan and Uehara 1997), and between Ea and Ec (Rahman et al. 2001a). On the other hand, these hybrid phenotypes are important markers to find hybrids in the field (e.g., Menge 1986, Aslan and Uehara 1997, Aslan 2000, Rahman et al. 2001a). Other remarkable characters such as test size, spine length, spicule morphology, pedicellaria valve length, pore pair ratio, and gamete sizes showed intermediate features. The intermediate phenotypes that appeared in hybrids between Em and Ec were used to discover the hybrids in the field, but we have been unsuccessful in finding any hybrid genotypes. This may be due to the fact that there might be some isolating mechanisms that prevent these 2 species from hybridizing in the field such that no introgression takes place despite their sympatric existence. If genetic analyses, using allozymes and DNA markers, also fail to find evidence of hybrids in the field, then there must be some effective isolating mechanisms that separate these 2 congeners into distinct species.

Although there are very few differences in gonad development of these 2 species, the adult $F_1$ hybrids were completely fertile and the $F_2$ hybrids produced from the $F_1$ backcrosses were as viable (in terms of larval survival, metamorphosis, and juvenile survival) as $F_1$ conspecifics, indicating that nuclear genetic differences between these 2 species are not large enough to cause developmental incompatibility, in spite of their morphological differences and habitat segregation. In other words, these species are genetically very close to each other, and perhaps no effective introgression takes place. Moreover, from the results of hybridization obtained from the combination of having closer genetic affinities, as between Ea and Ec (Rahman et al. 2000 2001a), and more-distant affinities, as between Ea and Ed (Aslan and Uehara 1997), as well as from this study (between Em and Ec), it could be predicted that viable hybrids will also be produced in other combinations of *Echinometra* spp. if they are hybridized in the laboratory.

From the above discussion, it is evident that Em and Ec are almost certainly recent derivatives from a single ancestral species, and introgression is either very low or non-existent between them; that is, they are effectively reproductively isolated. The presence of prezygotic isolating mechanisms such as gametic incompatibility and possibly gametic competition, as well as habitat segregation, most likely maintain the genetic distinctness of Em and Ec. Although, Em has already been recognized as *E. mathaei* (Arakaki et al. 1998), new taxonomic descriptions and species name should be given to Ec.

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