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F_1 and F_2 Backcrosses in the Hybrids Between Two Unnamed Genetically Distinct Species of Tropical Sea Urchins, *Echinometra* sp. A and *Echinometra* sp. C

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Abstract: Experiments on backcrosses using the gametes of the reciprocal F_1 hybids and the conspecific controls of the two sympatric species of tropical sea urchins, *Echinometra* sp. A (Ea) and *Echinometra* sp. C (Ec) were conducted in the laboratory. The ova from the female hybrid of Ea x Ec and Ec x Ea yielded a higher percent of fertilization with Ea x Ea sperm than with Ec x Ec sperms. Conversely backcrosses by hybrid males of either crosses yielded a higher percentage of fertilization with Ec x Ec ova than with Ea x Ea ova, indicating that Ea ova appeared to be more descriminating than Ec ova due to the sequence differences in their gamete recognition alleles. On the other hand, the higher fertilization rates between the same types of hybrids versus the different types of hybrids indicated the presence of a complex sorting of gamete compatibility genes. However, the higher fertilization rates as well as higher survival rates of larvae, juveniles and adults of the F_2 hybrids eliminates the possibility that hybrid inviability/sterility is a postzygotic mechanism of reproductive isolation. In adults, EaEa x EaEc and EaEa x EcEa juveniles were consistantly larger than those of equivalent ages of the other crosses in terms of relative test dimesions and growth performances; largest to smallest were EaEa x EcEa, EaEa x EaEc, EcEa x EaEa, EaEc x EaEa, EcEa x EcEc, EcEa x EcEc, EaEc x EcEc, EcEc x EcEa and EcEc x EaEc, respectively. These results of growth performances however, indicated that the F_2 hybrids were viable in the lab-reared conditions. This experiment represents the first successful production of F_2 hybrids (Progeny of backcrosses) in the laboratory. Although, coloration patterns of F_2 hybrids tended to be maternal, other characters such as test sizes and spine lengths, growth performances and phenotypic characteristics (such as tubefoot and gonad spicules, pedicellaria valve length and gamete sizes) tended to be intermediate but closer to their maternal F_1. Despite these findings, hybrids with these morphological characters were not found in the field, indicating possible prezygotic isolating mechanism(s) that either singlely or incombination with others creating barriers to hybridize in the field. The results of this study further indicate that considerable morphological and genetic integrity is maitained between *Echinometra* sp. A (Ea) and *Echinometra* sp. C (Ec) which strongly supports their recognition as distinct evolutionary species despite the fact that they produce F_1 and F_2 hybrids under laboratory conditions.

Key words: F_1 and F_2 backcrosses, sea urchins, *Echinometra* spp., gamete compatibility, reproductive isolation, speciation

Introduction

A detailed studies on physiological adaptation and reproduction, external features of eggs and sperms, differences in cross-fertilization rates, larval and adult morphologies, karyotypes, spawning seasons and distribution patterns, it has been concluded that four closely related sympatric sea urchins belonging to the genus *Echinometra* in Okinawa should be recognized as four different species, distinguished as *Echinometra* spp. A, B, C and D (Ea, Eb, Ec and Ed) (Shingaki and Uehara, 1984; Uehara and Shingaki, 1985; Uehara and Taira, 1987; Arakaki and Uehara, 1991; Uehara, 1990 and Uehara *et al*., 1986, 1990, 1991). Recent biochemical studies on enzyme electrophoresis (Matsuoka and Hatanaka, 1991); microhabitats (Nishihira *et al*., 1991); DNA analysis (Metz *et al*., 1991; Palumbi and Metz, 1991; Palumbi, 1996 and Palumbi *et al*., 1997) and gamete recognition protein binding (Metz and Plumbi, 1996 and Palumbi, 1998) also suggested these types are separate but closely related species which have recently been speciated. Consequently, mitrochondrial DNA sequence data from the four species of *Echinometra* show that genetic distance between these species are very small. This result as well as calibration of the rate of mtDNA across sea urchins suggested that the *Echinometra* in the central and west Pacific diverged over the past 1-3 million years

(Palumbi, 1996). Although the four *Echinometra* are recognized as four distinct species, valid names for these species have been debated (Palumbi, 1996; Palumbi *et al*., 1997). *E*. sp. B is now recognized as *Echinometra mathaei Sensu stricto* (Arakaki *et al*., 1998); while *E*. sp. D belongs in the *Echinometra oblonga* species complex, which may include a cryptic species composed of at least three species (Arakaki and Uehara, 1999). The other two, "sp. A" and "sp. C" have yet to be described and named.

Among broadcast free-spawning marine invertebrates including sea urchins gametic incompatibility is considered as particularly an important mechanism of reproductive isolation, creating barriers to gene flow (Dobzhansky *et al*., 1977; O'Rand, 1988; Minor *et al*., 1989; Lessios and Cunningham, 1990; Vacquier *et al*., 1990, 1995; Palumbi and Metz, 1991; Byrne and Anderson, 1994; Metz *et al*., 1994 and Palumbi, 1998). Molecules with species-specific composition that attract only conspecific sperm (Ward *et al*., 1985) and others that do not permit primary binding of heterospecific sperm (Summers and Hylander, 1975, 1976; Bellet *et al*., 1977; Glabe and Vacquier, 1977, 1978; Glabe and Lennarz, 1979; Vacquier, 1980; Metz *et al*., 1994; Vacquier *et al*., 1995 and Metz and Palumbi, 1996) have been found in the egg membrane of echinoids. Though the effectiveness of the barriers in relation to phylogenetic affinity between echinoid species has not yet been determined, Strathmann (1981) has found unidirectional gametic incompatibility between closely related sympatric echinoid species *Strongylocentrotus droebachiensis* and *S. pallidus*. The single species spawning have been reported for echinoderms and very recently multiple species spawning of echinoderms has been reported in which participants are sympatric congeners (Pearse *et al*., 1988 and Byrne and Barker, 1991). In these multiple species events heterospecific gamete fusion may occur, producing viable hybrids (Aslan and Uehara, 1997; Rahman, 1997, 2001 and Rahman *et al*., 2001) thereby allowing gene flow through hybridization or wasting gametes through formation of inviable hybrids (Pearse *et al*., 1988).

Hybridization have long been of interest of evolutionary biologists because they offer excellent opportunities to study speciation processes which contribute to reproductive isolation by either pre- or post-zygotic isolating mechanisms (e.g., Strathmann, 1981; Byrne and Anderson, 1994; Palumbi, 1994; Aslan and Uehara, 1997 and Rahman *et al*., 2001). This interbreeding brings together different genomes, introduces new sources of genetic variation (Willis *et al*., 1997) and experiences variability in morphological characters (e.g. Wallace and Willis, 1994; Willis *et al*., 1997; Aslan and Uehara, 1997 and Rahman *et al*., 2001). Most hybrids are usually morphologically intermediate including in sea urchins in respect of majority of the characters examined (Mayr, 1979; Campton, 1987; 1990; Uehara *et al*., 1991; Byrne and Anderson, 1994; Wallace and Willis, 1994; Aslan and Uehara, 1997 and Rahman *et al*., 2001). Laboratory hybridization experiments have been utilized extensively to confirm the probable hybrid nature to certain individuals by demonstrating that two taxa will interbreed when provided with the opportunity to do so, or that gametes from two taxa can be artificially cross-fertilized. Such information provides a valuable contribution to the natural marine hybridization. Laboratory crosses have also demonstrated that there exists considerable potential for hybridization in echinoids which live in sympatry (Uehara *et al*., 1991; Byrne and Anderson, 1994; Aslan and Uehara, 1997 and Rahman *et al*., 2001). But none of them had succeeded to find out hybrids form the field collected suspected specimens by using the hybrid characteristics as identifying markers.

Whether all Okinawan *Echinometra* diversed in allopatry or sympatry is not known, also what mechanisms, pre-or postzygotic maintain their genetic identity today is unsure. Numerous papers have been published on artificial crosses of sea urchin showing that prezygotic isolating mechanisms for instance gamete incompatibility, usually take place (Strathmann, 1981; Uehara *et al*. 1990 and Metz *et al*., 1994) revealed by the substantial genetic divergence of sea urchin species. Up to now, only Strathmann (1981) has reported postzygotic isolating mechanisms between hybrid produced experimentally from the two species of sea urchin *Strongylocentrotus droebachiensis* and *S. pallidus* because of difficulties of rearing sea urchins (Metz *et al*., 1994). Conversely, reciprocal adult hybrids produced by using *E*. sp. A and *E*. sp. D in the laboratory revealed that the two species are isolated by prezygotic barriers in the field (Aslan and Uehara, 1997).

In our previous experiment we reported that reciprocal F_1 hybrids, produced experimentally through cross-fertilization between *E*. sp. A and *E*. sp. C were viable, fertile and morphologically intermediate between two of these species (Rahman *et al*., 2001). To determine the gametic compatibility and developmental compatibility of the F_2 hybrids and to assess the degree to which such hybridization may be responsible for genetic introgresion across species borders, a series of cross-fertilization experiments were conducted using the gametes of F_1 and F_2 hybrids and their conspecific controls.

Materials and Methods

Samples collection and maintenance: Healthy mature adults of F_1 conspecifics, Ea x Ea (brownish dark test and white-tipped spines), Ec x Ec (greenish test, spines

without white tips, but with white basal ring) and F_1 hybrids, Ea x Ec (dark brownish test and spines with a translucent white basal ring), Ec x Ea (uniformly deep greenish test and the spines had barely detectable white tips and basal white rings), produced through cross-fertilization between *Echinometra* sp. A and *Echinometra* sp. C (Rahman, 1997) were collected from the culture tanks of Sesoko Marine Research Center and were transported to the laboratory at the Dept. Marine and Environmental Sciences, University of the Ryukyus and maintained in closed aquarium before use for *in vitro* crosses. The experiment was conducted during two consecutive breeding seasons from May to October, 1998 and from May to September, 1999 in laboratory conditions.

Backcross-fertilization of gametes: Cross-fertilization among the gametes of the F_1 conspecifics and the reciprocal F_1 hybrids of two *Echinometra* species, Ea and Ec were conducted using all possible combinations of ova and sperm at room temperature (26-28°C). For simplicity, when referring to the backcrosses, the maternal species is named first. For example, F_2 hybrid produced through backcross between the F_1 female of Ea x Ea and F_1 male of Ea x Ec is denoted as EaEa x EaEc. Fertilization was done by mixing two drops of a diluted sperm into petridishes containing 15 ml egg suspensions. Sperm concentration was generally 10^{-4} to 10^{-3} of "dry sperm" (Uehara *et al.*, 1990 and Rahman, 1997). Sperms were allowed to remain with the eggs for ten minutes, excess sperms were then removed with three consecutive washes with FSW (filtered sea water). In each fertilization, a conspecific fertilization by use of ova from the same female was also conducted as a control. The egg were then layered on the bottom of petridishes and incubated at ambient room temperature (26-28°C) for one hour. The first 100 eggs encountered were classifed as "fertilized" if they had reached the 2-4 cell stage. The fertilized eggs were then transferred in glass beakers and incubated in FSW at ambient room temperature until they attained free-swimming blastula stage.

Sperm dilution experiments: The protocol and techniques used in these experiments were different from those employed for production of F_2 hybrids. To determine the fertilization rate for both F_2 conspecific and backcrosses, 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 was first adjusted to 10°C and then quickly diluted in a series of 7, 10-fold dilutions. A 0.1 ml aliquot from one of these sperm solutions was then placed into the vial, to bring the final volumes to 1 ml. For example, mixing a 0.1 ml aliquot of 10^{0} undiluted sperm with 0.9 ml egg suspensions in a vial was called 10^{-1} diluted concentration of sperm. This procedure was followed through a series until 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 into the vial for incubation. Eighteen replicate crosses were performed with gametes from new individuals each time. Fertilization rate was estimated 1.25-1.5 h after gamete mixing by counting the number of eggs reaching 2-4 cell stages among the first 100 eggs observed.

Larval rearing and culture of juveniles and adults: Only the embryos and larvae of F_2 conspecific controls (EaEa x EaEa and EcEc x EcEc) and backcrossed F_2 hybrids (EaEc x EaEa, EaEa x EaEc, EcEa x EaEa, EaEa x EcEa, EaEc x EcEc, EcEc x EaEc, EcEa x EcEc and EcEc x EcEa) produced from the above fertilization experiments were continued to rear through metamorphosis, as described by Rahman *et al.* (2000). After 22-24 days of rearing, the competent larvae were placed in small (25 x 20 x 10 cm) aquaria with aerated filtered sea water and pieces of coralline red algal skeletons were added to induce settlement and metamorphosis (Rahman and Uehara, 2001). Sea water was partially changed once a week with fresh filtered sea water. This was continued for up to three months, by which time the juveniles were 6.0-7.0 mm in test diameter. The juveniles were then transferred to plastic aquaria (36 x 45 x 18 cm) supplied with aerated flow through sea water at Sesoko Marine Research Centre. Coral skeletons covered with encrusting coralline algae, served as food. The cultures were continued for one year by which time the urchins attained sexual maturity. The performances of larvae, juvenile and adults were examined and compared among the F_2 hybrid groups and their parental species controls.

Morphological characteristics: Detailed morphological characteristics for describing the differences were recorded or measured from the above one year old F_2 conspecifics, EaEa x EaEa, EcEc x EcEc and their F_2 hybrid groups were: test size and spine lengths, spicules in the tubefeet and gonads, pedicellaria valve length, color patterns of oral and aboral spines and test and gamete sizes.

The tests of echinoids in the family Echinometridae are oblong so after removing the spines, length, width and height were measured with calipers. 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 of

both aboral and oral view of adult urchins were observed by the color book of Kornerup and Wanscher (1978).

The spicules in gonad and tubefoot of the urchins were thoroughly examined. A small piece of the gonadal tissue was clipped off using a forceps and immersed in distilled water. The 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 a forceps and the morphology of the spicules was investigated by the same methods as above.

In order to collect the pedicellarias easily, all spines around the body were removed. The pedicellaria were plucked form the test near peristome and ambital area. Soft tissues from the pedicellaria were removed by treating with 10% sodium hypochloride solution (household bleaching). After bleaching, the pedicellaria were rinsed with three changes of distilled water. The valve length (VL) of pedicellaria were then measured under a compound microscope (10 x 10) with presetting micrometer.

Gamete sizes (egg diameter and sperm-head length) were also measured from the sexually matured hybrids and their conspecifics using a differential microscope (eggs at 400x in a slide well, sperm at 1000x on a flat slide), following the methods of Amy (1983) and Rahman *et al.* (2001, 2002).

F_2 backcrosses: After 1 year of culturing, the F conspecifics and F_2 hybrids reached sexual maturity and contained mature gametes. Gametes were obtained, following the similar method of gamete shedding experiment. Their gametes were then reciprocally backcrossed, following the methods of F_1 backcrosses.

Hybrids in nature: To examine the occurrence of natural hybridization between Ea and Ec, field surveys were conducted along Sunabe coast of Okinawa and coast of Sesoko Island, where both of the species occur nearly sympatrically in adjacent microhabitats. On the basis of the phenotypic color patterns of the spines and tests, about 400 suspected hybrids were collected from Sunabe and Sesoko coasts and compared to the laboratory cultured hybrids with respect to coloration patterns of the body and the spines, spicule characteristics and other possible morphological features.

Data analysis: Percentage data where statistical analysis conducted were arcsine transformed. Those replicates in which none or all eggs 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 helped to normalized the data and reduce heterogeneity in variances. A "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, a one way analysis of variance (ANOVA) was done followed by Tukey's multiple comparison test. Data that did not meet the normality assumption of 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

Fertilization rates in F_1 backcrosses: Fertilization rates of backcrosses using the gametes of F_1 hybrids and their conspecific parental species under various sperm concentrations are depicted in Fig. 1. It is evident that gametes from both the hybrids were completely fertile and reciprocally compatible with the gametes of their F_1 conspecifics and vise versa, though the percentages of fertilization showed asymmetrical values in some crosses under lower sperm concentrations (10^{-8} to 10^{-3} dilutions) and the identical values for all crosses under higher sperm concentrations (10^{-2} to 10^{-1} dilutions) (Fig. 1). Under limited sperm concentration (10^{-5} dilution), eggs from both Ea x Ec and Ec x Ea yielded higher percentages of fertilization with Ea x Ea sperms (82.27 and 84.11%) than with Ec x Ec sperms (74.56 and 80.39%) (Table 1), similar to the findings with parental crosses where Ea sperm more readily fertilized Ec eggs than Ec sperms fertilized Ea eggs (Rahman *et al.*, 2001). The results obtained from backcrosses among the F_1 conspecifics and F_1 hybrids however, indicated that gene flow through female hybrids should run predominantly back to *E.* sp. A rather than to *E.* sp. C but backcrosses by hybrid males altered the direction of gene flow between these species (i.e., back to *E.* sp. C rather than to *E.* sp. A). However, the higher fertilization rates in all backcrosses eliminates the possibility that hybrid infertility/sterility is a postzygotic mechanism of reproductive isolation of these two species. Moreover, backcrosses by sperms from males of Ea x Ec and Ec x Ea yielded higher percentages of fertilization with Ec x Ec ova (86.94 and 93.44%) than with Ea x Ea ova (74 and 74.83%) (Table 1); Ea ova appears to be more discriminating than Ec ova. The fertilization rates between the same types of hybrids versus the different types of hybrids (85.89% versus 76.39% and 83.94% versus 80%) indicate a complex sorting of gamete recognition genes (Table 1). The higher fertilization rates in F_1 backcrosses,

Table 1: Percentage of eggs fertilized in backcrosses among laboratory-reared F_1 generation of conspecifics and hybrids of *Echinometra* sp. A (Ea) and *Echinometra* sp. C (Ec) at limited sperm concentration (10^{-5} dilution). Counts made 1.5 hours after gamete mixing of number of eggs out of 100 that had reached 2-4 cell stage (fertilized). Each value represents 18 replicate crosses with gametes from new individuals in each replicate; mean±SD, ranges in parentheses

	Egg from			
Sperm from	Ea x Ea	Ea x Ec	Ec x Ea	Ec x Ec
Ea x Ea	90.33±1.68	82.27±1.60	84.11±1.23	83.28±2.16
	(88.00-93.00)	(79.00-85.00)	(80.00-86.00)	(79.00-86.00)
Ea x Ec	74.00±1.68	83.94±1.80	76.39±1.50	86.94±1.30
	(72.00-78.00)	(79.00-86.00)	(74.00-79.00)	(85.00-90.00)
Ec x Ea	74.83±1.38	80.00±1.71	85.89±2.08	93.44±0.98
	(73.00-78.00)	(78.00-83.00)	(83.00-89.00)	(92.00-95.00)
Ec x Ec	0	74.56±1.23	80.39±1.46	99.00±1.02
		(72.00-77.00)	(80.00-86.00)	(97.00-100.00)

Table 2: Comparison of larval, Juvenile and adult performances of F_2 hybrids, produced experimentally through backcrossing among F_1 conspecifics and the reciprocal hybrids of *Echinometra* sp. A (Ea) and *Echinometra* sp. C (Ec). Five replicate experiments were conducted in each cross for each type of performance. All values represent mean±SD with ranges in parentheses. Mean values within each experiment with common superscripts in the same column are not significantly different ((Tukey's test, $P > 0.05$)

Crosses	Larval survival*(%)	Metamorphosis (%)	Recovery**(%)	Adult survival (%)	Adult live weight (g)
EaEa x EaEa	79.08±1.66^a	89.00±4.18^a	72.50±1.89^a	86.67±2.72	14.90±0.80^a
	(78.00-81.00)	(85.00-90.00)	(70.25-74.43)	(83.33-90.00)	(13.85-15.90)
EaEa x EcEa	77.00±1.15^a	86.00±4.18^a	70.14±1.82^a	83.33±2.72^a	14.72±0.72^a
	(76.00-78.25)	(80.00-90.00)	(68.41-72.95)	(80.00-86.67)	(13.65-15.62)
EcEa x EaEa	78.25±1.00^a	88.00±5.70^a	71.20±1.77^a	84.17±3.19^a	14.23±0.55^a
	(77.25-79.25)	(80.00-95.00)	(69.20-73.80)	(80.00-86.67)	(13.28-15.38)
EaEa x EaEc	76.92±1.01^a	85.00±5.00^a	69.95±1.59^a	82.67±1.92^a	14.30±0.65^a
	(76.00-78.00)	(80.00-90.00)	(68.00-72.14)	(80.00-83.33)	(13.35-15.55)
EaEc x EaEa	77.58±1.01^a	87.00±2.75^a	71.08±1.83^a	84.17±3.19^a	13.82±0.60^a
	(76.50-78.50)	(85.00-90.00)	(69.11-73.22)	(80.00-83.33)	(12.65-14.72)
EcEc x EcEa	78.58±1.05^a	88.00±5.70^a	71.44±1.90^a	85.83±3.19^a	0.94±0.64^b
	(77.50-80.00)	(80.00-95.00)	(69.20-73.45)	(83.33-90.00)	(10.02-12.12)
EcEa x EcEc	78.08±0.88^a	86.00±4.18^a	70.01±1.83^a	82.50±5.00^a	11.31±0.62^b
	(76.25-78.00)	(80.00-90.00)	(68.21-72.80)	(76.67-86.67)	(10.35-12.36)
EcEc x EaEc	78.25±1.15^a	87.00±5.70^a	71.30±1.68^a	85.00±1.93^a	10.85±0.68^b
	(77.25-79.50)	(80.00-95.00)	(69.08-73.29)	(83.33-86.67)	(9.90-12.02)
EaEc x EcEc	76.75±1.15^a	85.00±5.00^a	70.04±1.98^a	82.50±5.00^a	11.10±0.58^b
	(75.75-78.00)	(80.00-90.00)	(68.30-72.82)	(76.67-86.67)	(11.15-12.18)
EcEc x EcEc	78.42±1.66^a	88.00±4.47^a	71.60±1.92^a	85.83±3.19^a	9.20±0.86^c
	(77.00-80.25)	(85.00-95.00)	(69.50-73.58)	(83.33-90.00)	(8.32-11.11)

*Matured larvae that were deemed competent for metamorphosis after a 22-24 days culture period in laboratory condition.
**Three months old juvenile urchins that were transferred to flow-through sea water system for advanced culture.

Table 3: Comparison of test sizes and spine lengths of F_2 hybrids, produced experimentally through backcrossing among F_1 conspecifics and the reciprocal F_1 hybrids of *Echinometra* sp. A (Ea) and *Echinometra* sp. C (Ec) one year after metamorphosis. A total of 20 adult individuals were measured for each cross. All values represent (mean±SD) in mm with ranges in parentheses

Crosses	Length of tests	Width of tests	Height of tests	Length of spines
EaEa x EaEa	29.48±0.83^{a*}	27.38±0.81^a	14.28±0.37^a	26.81±1.01^a
	(28.30-31.20)	(26.15-29.10)	(13.65-15.00)	(24.43-28.30)
EaEa x EcEa	29.11±0.89^a	27.16±0.73^a	14.13±0.50^a	25.44±0.88^a
	(28.00-31.01)	(25.98-28.98)	(13.45-14.88)	(24.10-26.35)
EcEa x EaEa	29.01±0.91^a	27.05±0.86^a	13.98±0.57^a	24.78±0.72^a
	(27.89-30.95)	(25.85-28.75)	(13.40-14.60)	(23.85-25.85)
EaEa x EaEc	29.14±0.90^a	27.13±0.83^a	14.05±0.65^a	25.65±0.87^a
	(27.96-31.04)	(25.95-28.89)	(13.50-14.70)	(24.30-26.53)
EaEc x EaEa	28.51±0.76^a	26.39±0.66^a	13.61±0.41^a	24.56±0.77^a
	(27.10-30.21)	(25.08-28.24)	(12.85-14.00)	(23.60-25.53)
EcEc x EcEa	25.73±0.68^b	24.16±0.64^b	12.29±0.57^b	21.64±0.75^b
	(24.88-26.75)	(23.10-25.28)	(10.65-13.00)	(20.05-23.00)
EcEa x EcEc	27.02±0.62^b	25.00±0.56^b	12.85±0.59^b	22.77±0.78^b
	(25.80-28.01)	(24.00-26.08)	(11.10-13.35)	(20.80-24.75)
EcEc x EaEc	25.65±0.67^b	24.11±0.63^b	12.26±0.62^b	21.59±0.67^b
	(24.80-26.80)	(23.05-25.20)	(10.60-12.95)	(20.0-22.96)
EaEc x EcEc	26.88±0.65^b	24.85±0.55^b	12.79±0.60^b	22.67±0.72^b
	(25.65-27.78)	(23.88-25.75)	(11.01-13.25)	(20.20-24.66)
EcEc x EcEc	24.25±1.03^c	22.22±1.01^c	11.49±0.58^c	20.35±0.69^c
	(23.05-26.04)	(21.10-23.98)	(10.25-12.25)	(19.30-21.76)

*Mean values within each experiment with common superscripts in the same column are not significantly different (Tukey's test, $P > 0.05$)

Table 4: Comparison of types and percentages of tubefoot spicules of F_2 hybrids, produced experimentally through backcrossing among F_1 conspecifics and the reciprocal F_1 hybrids of *Echinometra* sp. A (Ea) and *Echinometra* sp. C (Ec) one year after metamorphosis. A total of 20 adult individuals were measured for each treatment. All values represent (mean±SD) in percentage with ranges in parentheses

Crosses	Bihamate	Bihamate-like	Triradiate	Triradiate-bihamate
EaEa x EaEa	100.00^{a}**	0.00^{i}	0.00^{j}	0.00^{i}
EaEa x EcEa	65.73±1.07^{b}	24.72±1.25^{d}	2.12± 0.24^{g}	7.42±0.65^{h}
	(63.92-67.43)	(22.47-26.80)	(1.75-2.53)	(6.00-8.27)
EcEa x EaEa	58.60±2.13^{e}	29.08±1.83^{a}	3.12±0.19^{f}	9.20±0.59^{f}
	(54.73-61.19)	(27.06-32.64)	(2.73-3.43)	(7.99-10.12)
EaEa x EaEc	82.62±1.85^{c}	6.25±1.10^{h}	1.01± 0.52^{i}	10.12±1.04^{e}
	(80.44-84.88)	(4.65-8.02)	(0.54-1.58)	(8.40-12.47)
EaEc x EaEa	76.56±1.57^{d}	8.19±1.14^{g}	1.56±0.95^{h}	13.69±1.08^{c}
	(74.40-79.84)	(6.65-10.08)	(0.89-2.10)	(11.64-15.61)
EcEc x EcEa	13.09±0.84^{i}	15.82±1.15^{f}	51.50±1.55^{b}	19.59±0.76^{a}
	(11.76-14.75)	(14.29-18.52)	(48.75-54.29)	(17.86-20.58)
EcEa x EcEc	17.60±0.94^{h}	22.80±1.75^{e}	44.70±1.18^{c}	14.90±0.76^{b}
	(14.33-17.42)	(20.93-25.80)	(42.37-46.61)	(13.68-16.41)
EcEc x EaEc	19.54±1.10^{g}	25.60±1.68^{c}	42.04±2.09^{d}	12.72±1.01^{d}
	(18.13-19.63)	(21.27-27.98)	(39.56-46.24)	(11.31-14.93)
EaEc x EcEc	25.44±0.96^{f}	27.57±0.98^{b}	38.92±1.48^{e}	8.07±1.18^{g}
	(24.00-27.88)	(26.14-29.44)	(36.31-41.45)	(5.90-10.91)
EcEc x EcEc	0.00^{i}	0.00^{i}	100.00^{a}	0.00^{i}

*Mean values within each experiment with common superscripts in the same column are not significantly different (Tukey's test, P > 0.05)

Table 5: Comparison of types and percentages of gonad spicules of F_2 hybrids, produced experimentally through backcrossing among F_1 conspecifics and the reciprocal F_1 hybrids of *Echinometra* sp. A (Ea) and *Echinometra* sp. C (Ec) one year after metamorphosis. A total of 20 adult individuals were measured for each treatment. All values represent (mean±SD) in percentage with ranges in parentheses

Crosses	Spindle	Spindle-like	Triradiate	Spindle-triradiate	Bihamate	Irregular
EaEa x EaEa	98.4±0.4^{a}**	0.00^{i}	0.00^{j}	0.00^{i}	0.6±0.2^{a}	1.0±0.2^{a}
	(97.8-99.4)				(0.4-1.1)	(0.9-1.3)
EaEa x EcEa	66.4±1.7^{d}	23.8±1.2^{d}	1.9±0.2^{i}	6.3±0.6^{g}	0.8±0.2^{a}	0.8±0.3^{a}
	(63.8-68.8)	(21.9-25.7)	(0.9-2.5)	(5.6-7.9)	(0.3-1.2)	(0.3-1.3)
EcEa x EaEa	58.4±2.1^{e}	27.8±1.6^{b}	2.8±0.4^{g}	9.1±0.6^{e}	0.8±0.4^{a}	1.1±0.3^{a}
	(54.7-61.1)	(25.8-29.9)	(1.9-3.7)	(8.2-10.2)	(0.0-1.1)	(0.5-1.6)
EaEa x EaEc	82.1±1.9^{b}	6.7±1.1^{h}	2.3±1.0^{h}	7.5±0.7^{f}	0.8±0.2^{a}	1.1±0.2^{a}
	(79.3-85.2)	(5.1-8.0)	(1.4-3.5)	(5.9-8.0)	(0.4-1.1)	(0.7-1.4)
EaEc x EaEa	75.4±1.7^{c}	8.6±1.2^{g}	3.9±0.9^{f}	10.1±1.1^{d}	1.0±0.4^{a}	1.0±0.3^{a}
	(73.4-79.1)	(5.9-10.6)	(3.0-4.6)	(8.5-12.5)	(0.0-1.5)	(0.5-1.5)
EcEc x EcEa	12.6±0.8^{i}	17.5±1.1^{f}	49.7±1.6^{b}	18.5±0.8^{a}	1.1±0.3^{a}	0.7±0.2^{a}
	(11.4-14.1)	(16.1-19.0)	(47.1-52.2)	(16.8-20.0)	(0.6-1.6)	(0.3-1.0)
EcEa x EcEc	16.5±1.0^{h}	21.8±1.6e	43.7±1.6^{c}	16.4±0.8^{b}	1.0±0.3^{a}	0.8±0.2^{a}
	(15.4-16.5)	(20.1-23.8)	(41.4-45.9)	(15.2-17.9)	(0.5-1.4)	(0.4-1.2)
EcEc x EaEc	19.3±1.0^{g}	25.8±1.3^{c}	40.7±1.6^{d}	12.6±0.9^{e}	0.8±0.3^{a}	1.0±0.3^{a}
	(18.1-20.5)	(21.7-28.1)	(38.1-44.2)	(11.5-14.6)	(0.0-1.3)	(0.5-1.5)
EaEc x EcEc	24.9±1.0^{f}	29.9±1.2^{a}	38.7±1.5^{e}	5.0±0.8^{h}	0.9±0.3^{a}	0.7±0.2^{a}
	(23.5-26.8)	(28.4-31.5)	(36.2-41.3)	(3.0-7.3)	(0.4-1.4)	(0.0-0.9)
EcEc x EcEc	4.9±0.7^{j}	0.00^{i}	94.0±0.9^{a}	0.00^{i}	1.1±0.2^{a}	0.0^{b}
	(3.8-6.1)		(92.6-95.1)		(0.8-1.6)	

*Mean values within each experiment with common superscripts in the same column are not significantly different ((Tukey's test, P > 0.05).

however, indicated the closer genetic affinity between Ea and Ec.

Larval, juvenile and adult performances: Despite, there were slight differences in the survival of matured larvae, the values did not differed significantly (Tukey's test, P > 0.05) among the F_2 conspecifcs and F_2 hybrids. Metamorphosis, recovery and adult survival were also followed the same trends as larval survival (Table 2). The mean live weight attained by the one year old adult EaEa x EcEa, EcEa x EaEa, EaEa x EaEc and EaEc x EaEa was non-significantly very closer to their F_2 parental EaEa x EaEa, but all of them were differed significantly from slow-growing EcEc x EcEc. Whereas, the same for EcEc x EcEa, EcEa x EcEc, EcEc x EaEc and EaEc x EcEc was closer to their F_2 conspecific EcEc x EcEc, though the values of these crosses were differed significantly (Tukey's test, P < 0.05) (Table 2). Similar trends were observed in test sizes (length, width and height) and spine length of adult F_2 hybrids and their conspecific controls (Table 3). Therefore, the F_2 hybrids in repect of all growth performances were tended to be intermediate but very closer to the maternal character of their F_2 conspecifics. Similar results were also obtained in the F_1 hybrids between *Echinometra* sp. A and *Echinometra* sp. C (Rahman *et al.*, 2000, 2001).

Table 6: Valve length (VL) of four types of pedicellariae in F_2 hybrids, produced experimentally through backcrossing among F_1 conspecifics and the reciprocal F_1 hybrids of *Echinometra* sp. A (Ea) and *Echinometra* sp. C (Ec) one year after metamorphosis. A total of twenty adult individuals were examined from each cross with 10 pedicellariae of each type from each individual. All values represent mean±SD in μm followed by the ranges in parentheses

Crosses	Tridentate	Globiferous	Ophiocephalous	Triphyllous
EaEa x EaEa	1050.7±18.3^{a*}	742.3±20.8^{a}	631.8±20.5^{a}	209.3±12.9^{a}
	(1000.0-1180.0)	(690.0-800.0)	(580.0-690.0)	(180.0-260.0)
EaEa x EcEa	1027.6±18.5^{a}	722.4±17.7^{a}	616.8±17.4^{a}	201.3±11.8^{a}
	(970.0-1150.0)	(660.0-780.0)	(560.0-680.0)	(170.0-250.0)
EcEa x EaEa	1022.6±19.2^{a}	718.4±18.1^{a}	611.9±17.9^{a}	198.2±12.3^{a}
	(970.0-1140.0)	(660.0-770.0)	(560.0-670.0)	(160.0-250.0)
EaEa x EaEc	1043.9±18.0^{a}	736.4±18.8^{a}	626.7±18.9^{a}	206.4±12.9^{a}
	(990.0-1170.0)	(680.0-790.0)	(590.0-680.0)	(170.0-260.0)
EaEc x EaEa	1032.7±19.4^{a}	727.6±17.6^{a}	620.7±19.4^{a}	203.7±12.7^{a}
	(980.0-1160.0)	(670.0-780.0)	(570.0-680.0)	(170.0-250.0)
EcEc x EcEa	894.8±18.7^{b}	655.0±18.4^{b}	549.8±16.7^{b}	146.7±10.9^{b}
	(840.0-950.0)	(610.0-710.0)	(510.0-600.0)	(110.0-180.0)
EcEa x EcEc	905.4±16.8^{b}	665.7±17.9^{b}	554.9±15.8^{b}	150.4±11.2^{b}
	(840.0-950.0)	(620.0-720.0)	(520.0-610.0)	(120.0-180.0)
EcEc x EaEc	900.7±17.7^{b}	659.6±18.5^{b}	550.4±15.9^{b}	148.4±10.0^{b}
	(840.0-940.0)	(610.0-710.0)	(510.0-610.0)	(110.0-180.0)
EaEc x EcEc	910.6±18.7^{b}	672.4±18.3^{b}	560.3±16.6^{b}	152.6±10.8^{b}
	(870.0-970.0)	(630.0-720.0)	(520.0-620.0)	(120.0-190.0)
EcEc x EcEc	882.7±18.6^{b}	650.4±16.3^{b}	541.5±15.4^{b}	144.3±10.8^{b}
	(840.0-940.0)	(600.0-690.0)	(490.0-590.0)	(110.0-180.0)

*Mean values within each experiment with common superscripts in the same column are not significantly different ((Tukey's test, $P > 0.05$).

Table 7: Gamete sizes of F_2 hybrids, produced experimentally through backcrossing among F_1 conspecifics and the reciprocal F_1 hybrids of *Echinometra* sp. A (Ea) and *Echinometra* sp. C (Ec) one year after metamorphosis. Twenty adult individuals were examined from each cross with 25 eggs and 25 sperm from each individual. All values represent mean±SD in μm followed by the ranges in parentheses

Crosses	Egg diameter	Sperm-head length
EaEa x EaEa	66.98±1.11^{b*} (65.00-67.50)	4.07±0.52^{d} (3.10-4.96)
EaEa x EcEa	67.20±1.12^{b} (65.00-67.50)	4.70±0.75^{c} (3.72-5.58)
EcEa x EaEa	67.52±1.08^{b} (66.25-68.75)	4.86±0.76^{c} (4.34-5.58)
EaEa x EaEc	67.37±1.21^{b} (65.00-68.75)	4.77±0.71^{c} (3.72-5.58)
EaEc x EaEa	67.71±1.19^{b} (66.25-68.75)	4.94±0.74^{c} (4.34-5.58)
EcEc x EcEa	71.58±1.06^{a} (70.00-73.75)	5.77±0.72^{b} (4.96-6.82)
EcEa x EcEc	71.17±1.10^{a} (68.75-72.50)	5.66±0.70^{b} (4.96-6.82)
EcEc x EaEc	71.85±1.14^{a} (70.00-75.00)	5.80±0.68^{b} (4.96-6.82)
EaEc x EcEc	71.35±1.17^{a} (70.00-73.75)	5.72±0.68^{b} (4.96-6.82)
EcEc x EcEc	72.68±1.30^{a} (71.25-75.00)	6.53±0.77^{a} (5.58-7.44)

*Mean values within each experiment with common superscripts in the same column are not significantly different ((Tukey's test, $P > 0.05$)

Table 8: Percentage of eggs fertilized in backcrosses among laboratory-reared F_2 generation of conspecifics and hybrids of *Echinometra* sp. A (Ea) and *Echinometra* sp. C (Ec) at limited sperm concentration (10^{-5} dilution). Counts made 1.5 hours after gamete mixing of number of eggs out of 100 that had reached 2-4 cell stage (fertilized). Each value represents 9 replicate crosses with gametes from new individuals in each replicate. All values represent mean in percentage with ranges in parentheses

	Sperm from									
Egg from	aa x aa	aa x ca	ca x aa	aa x ac	ac x aa	cc x ca	ca x cc	ac x cc	cc x ac	cc x cc
aa x aa	89.9	89.1	85.9	90.3	85.89	22.1	23.2	23.8	21.9	0
	(88-92)	(87-91)	(85-89)	(89-91)	(85-87)	(21-23)	(22-25)	(22-25)	(20-23)	
aa x ca	88.8	98.1	97.3	98.5	97.6	55.3	53.6	53.2	56.3	46.2
	(87-91)	(97-99)	(96-99)	(97-100)	(96-98)	(53-57)	(53-55)	(52-55)	(54-58)	(45-48)
ca x aa	88.3	97.0	99.8	97.1	95.9	53.1	50.4	51.1	54.4	43.4
	(86-90)	(96-98)	(98-100)	(96-98)	(95-97)	(52-54)	(49-52)	(48-52)	(52-56)	(43-44)
aa x ac	87.7	94.9	95.9	99.0	98.4	51.6	48.1	47.9	52.6	45.2
	(86-89)	(94-96)	(95-97)	(98-100)	(97-100)	(50-53)	(47-49)	(46-50)	(50-55)	(44-48)
ac x aa	86.9	96.2	95.0	98.2	97.9	49.3	46.0	45.9	49.6	43.1
	(86-88)	(95-98)	(94-96)	(98-100)	(97-99)	(48-51)	(45-47)	(45-47)	(46-53)	(42-44)
cc x ca	87.3	90.2	91.2	90.8	90.2	99.3	99.3	98.4	98.7	99.9
	(86-88)	(86-92)	(89-92)	(90-92)	(88-92)	(98-100)	(98-100)	(97-100)	(97-100)	(99-100)
ca x cc	87.9	89.0	88.2	90.0	88.0	98.0	98.3	97.0	98.0	98.6
	(87-89)	(88-91)	(87-90)	(99-92)	(87-89)	(97-100)	(98-100)	(96-98)	(97-100)	(97-100)
ac x cc	88.5	90.7	89.9	91.6	91.3	95.8	95.1	99.2	95.3	100
	(87-90)	(89-92)	(88-92)	(90-92)	(90-92)	(95-96)	(94-96)	(98-100)	(93-97)	
cc x ac	86.9	88.3	88.4	89.9	89.6	96.9	96.8	95.4	99.2	98.3
	(86-88)	(87-90)	(97-90)	(89-92)	(88-71)	(96-98)	(96-98)	(95-97)	(98-100)	(97-100)
cc x cc	83.0	85.0	86.6	83.8	86.4	98.4	97.3	96.3	98.6	100
	(82-85)	(84-86)	(86-88	(82-85)	(86-87)	(98-100)	(97-98)	(95-97)	(97-100)	

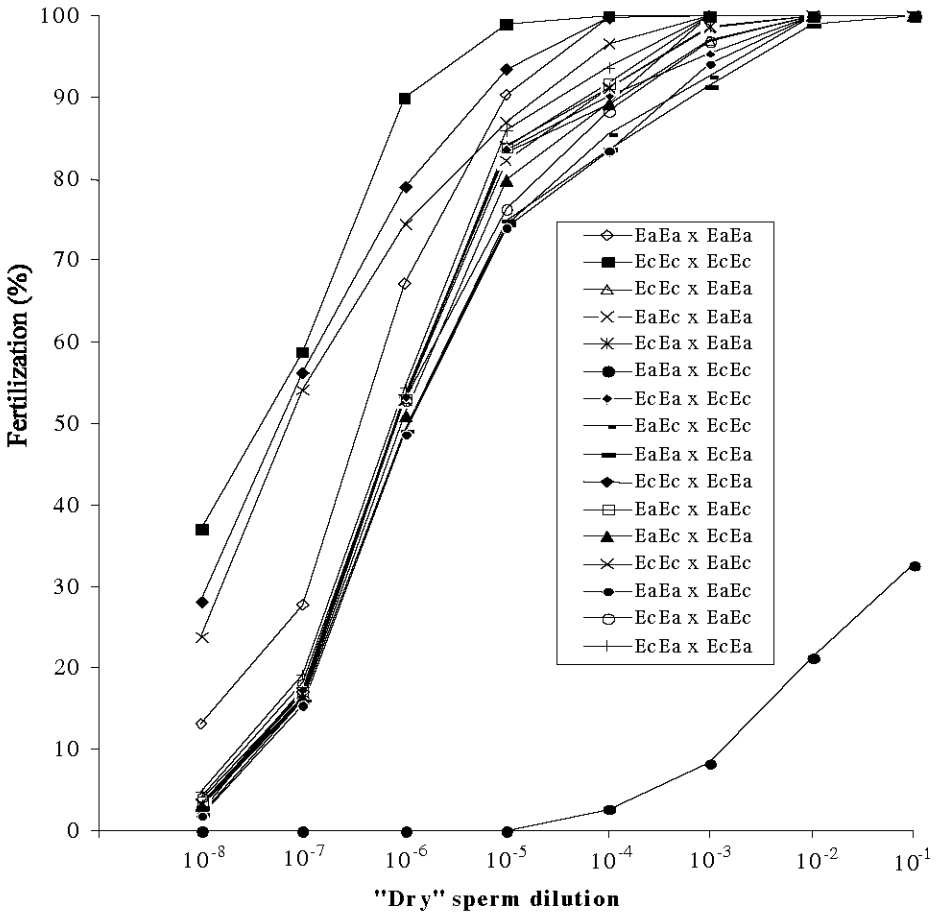

Fig. 1: Mean percentages of fertilization in F_1 backcrosses using the gametes from laboratory cultured Ea x Ea, Ec x Ec and their reciprocal F_1 hybrids under a series of sperm concentrations ("dry" sperm dilution). A total of 18 replicate crosses were done for each combination using the gametes from new individuals in each time. Standard deviations were not included, as these values were usually smaller than the symbols

Comparison of phenotypic characteristics: Major phenotypic color patterns of the F_2 hybrids and their conspecific parents were examined at the end of the experiment. From aboral view, the spine and test of EaEa x EaEa were brownish dark and clear white ring at the base of each white tipped spine, whereas EcEc x EcEc were uniformly green without white tip but each spine had fadded white basal ring and the test color was dominated by deep green. EaEa x EcEa, EaEa x EaEc, EaEc x EaEa and EaEc x EcEc hybrids were more similar to EaEa x EaEa in having brownish dark test and white tipped spines with a clear white basal ring. On the other hand, EcEc x EcEa, EcEa x EcEc, EcEa x EaEa and EcEc x EaEc hybrids were more closer to EcEc x EcEc, having uniformly deep green test and spines and each spine had a barely detectable white tips and basal white rings. At oral coloration, EaEa x EaEa had white tipped spines around the mouth and brownish dark test, whereas EcEc x EcEc had yellowish green spines around the mouth and a greenish test color. EaEa x EcEa, EaEa x EaEc, EaEc x EaEa and EaEc x EcEc hybrids were more similar to EaEa x EaEa, whereas EcEc x EcEa, EcEa x EcEc, EcEa x EaEa and EcEc x EaEc hybrids were more similar to EcEc x EcEc. Therefore, majority of the F_2 hybrid urchins showed the coloration patterns more closer to their maternal species. This may be due to the fact that maternal genomes are prefentially expressed in their F_2 progenies. A few of them also experienced intermediate colors between their parental progenies, may be because of the combine effects of their parental genomes. Similar color patterns were also observed in the F_1 hybrids between *Echinometra* sp. A and *Echinometra* sp. C (*Rahman et al.*, 2001) and between *Echinometra* sp. A and *Echinometra oblonga* (Aslan, 1995).

Tubefoot spicules in EaEa x EaEa were always bihamate (100%), whereas EcEc x EcEc were triradiate (100%) (Table 4). Although tubefoot spicules of EaEa x EcEa, EcEa x EaEa, EaEa x EaEc and EaEc x EaEa hybrids were bihamate (65.73-82.62%), bihamate-like (6.25-29.08%), triradiate-bihamate (7.42-13.69%) and triradiate (1.01-3.12%) but they were dominated by bihamate type

(Table 4), whereas those of EcEc x EcEa, EcEa x EcEc, EcEc x EaEc and EaEc x EcEc were bihamate (13.09-25.44%), bihamate-like (15.82-27.57%), triradiate-bihamate (8.07-19.59%) and triradiate (38.92-51.50%) but dominated by triradiate type (Table 4). The F_2 hybrids and their conspecific F_2 parents differed significantly (Tukey's test, $P < 0.05$) in respect of corresponding similar types of spicules and no intermediate types were found in conspecifics (Table 4). Therefore, the tubefoot spicule morphologies of hybrids were intermediate and tended towards maternal affinities.

The spicules of gonad in EaEa x EaEa were almost all spindle-shaped (98.4%); other spicules seen: bihamete (0.6%), Irregular (1.0%), no triradiates were found (Table 5). In contrast, those in Ec x Ec gonads were nearly all triradiate (94.0%); other spicules seen: spindle (4.9%) and bihamete (1.1%) (Table 5). Gonads in EaEa x EcEa, EcEa x EaEa, EaEa x EaEc and EaEc x EaEa hybrids had high proportions of spindle-shaped spicules (58.4-82.1%) with spindle-like (6.7-27.8%), spindle-triradiate (6.3-10.1%), triradiate (1.9-3.9%) and bihamate (0.8-1.0%) and irregular (0.8-1.1%) in smaller proportions whereas, those in EcEc x EcEa, EcEa x EcEc, EcEc x EaEc and EaEc x EcEc hybrids had high proportion of triradiate-shaped spicules (38.7-49.7%) followed by spindle-like (17.5-29.9%), spindle (12.6-24.9%) and spindle-triradiate (5.0-18.5%) with a very small proportion of bihamate (0.8-1.1%) and irregular (0.7-1.0%) types. The very fewer proportions of bihamate and irregular types were not differed significantly (Tukey's test, $P > 0.05$) among the conspecific and hybrid groups (Table 5). Although, both the hybrids showed various proportions of major types of spicules, the values of each types were significantly different (Tukey's test, $P > 0.05$). Though, some significant differences were recognized among the spicule types and proportions, hybrids of both directions seemed to be more closer to those of their maternal types (Table 5).

The pedicellaria found in both F_2 conspecifics and their hybrids were tridentate, globiferous, ophiocephalous and triphyllus. Only the valve length (VL) of all four types were measured and compared among conspecifics and their hybrid groups (Table 6). As shown in Table 6, all four types of pedicellaria VL of EaEa x EaEa were significantly (Tukey's test, $P < 0.05$) larger than those of their corresponding types of EcEc x EcEc. All the hybrid groups experienced intermediate sizes, but non-significantly closer to their maternal controls (Table 6).

Egg diameters of EcEc x EcEc control were significantly (Tukey's test, $P < 0.05$) larger than the EaEa x EaEa eggs. Hybrids contained intermediate sized eggs which were non-significantly closer to their maternal conspecifics (Table 7). The size of sperm heads were also smallest in EaEa x EaEa and were significantly different (Tukey's test, $P < 0.05$) from all the crosses. Though, the values differed among the hybrid groups, all were intermediate between values of Ea x Ea (smallest) and Ec x Ec (largest) (Table 7).

Existence of natural hybrids: Five hundred individuals with more or less similar coloration to the laboratory reared F_2 hybrids were collected from intertidal zone of the sea where their parental Ea and Ec inhabit at closer proximity. However, detailed comparisons of the above morphological characters revealed that none of these individuals actually had common character combinations to the experimentally obtained F_2 hybrids. Similarly, Aslan and Uehara (1997) and Rahman *et al.* (2001) did not find any natural hybrids between Ea and Ed and between Ea and Ec in the field even though they were able to produce sexually matured hybrids of these species in the laboratory.

Fertilization rates in F_2 backcrosses: Fertilization rates in F_2 backcrosses using the gametes of F_2 hybrids and their conspecific F_2 parental species were also conducted under various sperm concentrations. Only the fertilization rates at limited sperm concentration (10^{-5} dilution of "dry" sperm) are shown in Table 8. At this concentration of "dry" sperm, ova from all F_2 hybrid groups (aa x ca, ca x aa, aa x ac, ac x aa, cc x ca, ca x cc, ac x cc and cc x ac) exhibited similar high rates of fertilization (86.9-88.8%) with the sperm from F_2 conspecific aa x aa, whereas ova of aa x ca, ca x aa, aa x ac and ac x aa hybrids produced very low percentage of fertilization (43.1-46.2%) than the ova of cc x ca, ca x cc, ac x cc and cc x ac (98.3-100%) with the same sperm from F_2 conspecific cc x cc (Table 8). Consequently, ova from F_2 conspecific cc x cc produced higher percentages of fertilization (83.8-100%) with the sperm from each of all hybrid groups than the percent fertilization exhibited by the ova from conspecific aa x aa (21.9-89.1%) with the sperm from each groups of F_2 hybrids (Table 8). However, the sperm of F_2 hybrids appeared to be more discriminating with ova of F_2 conspecific aa x aa than with F_2 conspecific cc x cc. Thus, the differences in fertilization rates of the gametes among the F_2 conspecifics and their F_2 hybrids clearly indicates the sequence differences in their gamete recognition alleles. Similarly, the discrimination of fertilization between and among the hybrid groups also indicates the presence of polymorphic gamete recognition genes in their binding alleles. However, the F_2 hybrids were completely fertile, indicating that Ea and Ec are genetically very close.

Discussion

Hybridization was asymmetrical however and fertilization rate was generally lower in some backcrosses. This

reduction, at least among some heterogametic crosses, indicates the presence of a gamete recognition protein binding system, as reported by Metz *et al.* (1994), Metz and Palumbi (1996) and Palumbi (1998) which might eventually lead to gametic incompatibility and reproductive isolation. However, the high fertilization rates in many of the heterogametic crosses suggested that gametic incompatibility among these species and their hybrids is unlikely to provide a mechanism for maintaining species integrity in these species (Metz and Palumbi, 1996; Aslan and Uehara, 1997; Rahman, 1997; Rahman; 2000 and Rahman *et al.*, 2001). The compatibility of the gametes of the F_1 and F_2 hybrids of *E.* sp. A and *E.* sp. C demonstrates that if gamete recognition molecules are involved in fertilization in these species, they are not strongly species specific (Byrne and Anderson, 1994). Moreover, the similarly higher larval survival, metamorphosis and recovery rates of F_2 hybrids in laboratory conditions eliminate the possibilities that hybrid inviability, sterility or breakdown is the postygotic mechanism of reproductive isolation.

The growth patterns of all the ten combinations showed similar trends in triplicate aquaria. It was observed that one year old adult of EaEa x EcEa, EcEa x EaEa, EaEa x EaEc and EaEc x EaEa while being very closer to their F_2 parental EaEa x EaEa, registered similar faster growth than the other groups. Whereas, the same for EcEc x EcEa, EcEa x EcEc, EcEc x EaEc and EaEc x EcEc was slower but close to their F_2 conspecific EcEc x EcEc, the slowest. The growth of F_2 hybrids, produced through backcrossing among the ova of F1 hybrids and sperm of their F_1 conspecifics were almost the similar during the culture period of one year. Subsequently, the growth of EaEa x EcEa, EcEa x EaEa, EaEa x EaEc and EaEc x EaEa was surpassed by EcEc x EcEa, EcEa x EcEc, EcEc x EaEc and EaEc x EcEc, mostly appears to be intermediate between that of their parental species. The first-growing F_1 parents partially transmitting this trait to their F_2 hybrids. The growth and survival data however, confirmed that hybridization among F_1 generation of *E.* sp. A and *E.* sp. C has been successful in laboratory-rearing conditions and the resulting F_2 hybrids are as viable as conspecifics and showed evidence of parental heterosis. Rahman *et al.* (2000, 2001) also observed the similar phenomena in the F_1 hybrids between *Echinometra* sp. A and *Echinometra* sp. C.

Although the expression of an intermediate phenotypes by the laboratory-reared F_1 (previous Study) and F_2 hybrids (present study) has assisted to find out hybrid genotyes in the field (Aslan and Uehara, 1997; Rahman, 1997 and Rahman *et al.*, 2001). The coloration patterns of both the F_1 and F_2 hybrids tended to be maternal. Other remarkable characters such as test sizes, growth performances, spine lengths, gonad and tubefoot spicules, gamete sizes tended to be intermediate. In our previous experiment (Rahman, 1997), we had done an intensive investigation to discover the F_1 hybrids in the field by using these morphological characters, but we were failed to find any evidence of natural hybridization. This may be due to the fact that there might be some sorts of isolating mechanisms (such as gametic incompatibility, habitat segregassion and gamete competition) prevent these two species from hybridizing in the field and so that no introgression takes place despite their sympatric existence (Rahman, 2000; Rahamn *et al.*, 2001). Though we were not able to find any hybrids in the field, the higher fertilization and survival rates of the F_1 and F_2 hybrids could open the door to do further investigations by using molecular and genetic markers. If genetic analyses, using allozymes and DNA markers also fails to find evidence of hybrids (F_1 or F_2) in the field, there must be some effective isolating mechanisms that separates these two congeners into distinct species.

A good result regarding the fertilization rates through backcrossing between the hybrid and parental progeny suggests that these two species may share the same gene pool with their hybrid progeny. In other words, these species are genetically very close to each other and perhaps no effective introgression takes place. The recent hybridization experiment conducted by Lessios and Pearse (1996) also revealed that gene introgression among the three tropical Indo-Pacific species of the genus *Diadema* (*D. paucispinum, D. savignyi* and *D. setosum*) in Okinawa is limited.

Whether the *Echinometra* sp. A and *Echinometra* sp. C are distinct species or not, the results from our hybridization experiments (F_1 and F_2) indicate that the two species are very close in respect to their genetic similarity and phylogenetic relationships and it seems almost certain that the two species are the recent derivatives from one ancestral species and also sufficient for their speciation as suggested by Matsuoka and Hatanaka (1991) and Palumbi and Metz (1991). Whether incipient gametic incompatibility plays an important role in speciation of these species as suggested by Metz *et al.* (1994), Metz and Palumbi (1996) and Aslan and Uehara, (1997) or if some other mechanism (s) is involved deserve further study. We believe that the genetic integrity maintained between *Echinometra* sp. A and *Echinometra* sp. C warrants their recognition as distinct evolutionary species despite the viable F_1 and F_2 hybrids were produced experimentally through cross-fertilization in the laboratory.

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