

## GAMETIC INCOMPATIBILITY BETWEEN SPECIES OF THE SEA URCHIN *ECHINOMETRA* ON THE TWO SIDES OF THE ISTHMUS OF PANAMA

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**Abstract.**—The Pliocene rise of the Central American Isthmus has resulted in numerous “geminate pairs,” i.e., closely related species, one on each coast. Such species pairs can provide information on the evolution of isolating mechanisms in allopatry and on the relationship between genetic divergence and reproductive isolation in populations separated at a known time. The sea urchin genus *Echinometra* has one species, *E. vanbrunti*, in the eastern Pacific, and two, *E. lucunter* and *E. viridis*, in the Caribbean. *E. viridis* is morphologically distinct from the other two species, leading to the conclusion that *E. lucunter* and *E. vanbrunti* constitute a geminate pair. Allozyme data, on the other hand, place the speciation event of the two currently sympatric species after the rise of the Isthmus. We report fertilization experiments between the gametes of the three species performed to determine degree of reproductive isolation. Crosses between *E. viridis* and *E. vanbrunti* produce rates of fertilization almost equal to those manifested in homogamic crosses. Sperm of *E. lucunter* can fertilize eggs of the other two species, but few of its eggs permit fertilization by heterospecific sperm. Contrary to the predictions of the “speciation by reinforcement” hypothesis, degree of incompatibility between the allopatric *E. lucunter* and *E. vanbrunti* is higher than between the sympatric *E. lucunter* and *E. viridis*. Despite the incomplete and unidirectional nature of their gametic isolation, *E. lucunter* and *E. viridis* maintain their genetic integrities. Consideration of the likely phylogenetic relationships between the three species suggests that incompatibility of *E. lucunter* eggs with heterospecific sperm has evolved in the last 3.5 million years, after the rise of the Isthmus. There is no correlation between genetic divergence and strength of reproductive isolation, either within *Echinometra*, or among the geminate species pairs of echinoids that have been studied to date. Because recognition between echinoid gametes depends on the chemical compositions of a sperm protein and an egg glycoprotein, the appearance of gametic isolation would require only the fixation of a few mutations in each population on either side of a geographic barrier and could be independent of any other kind of genetic divergence. Thus, in animals with external fertilization, speciation need not be accompanied by major genomic reorganization.

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The emergence of reproductive isolation converts geographically separated populations into different species, yet we know little about how and when isolating mechanisms arise in the speciation process. We can study isolating mechanisms between species as they exist today, but without information about the order of splitting of species lineages, the time of their separation, and the nature and efficacy of the geographic barrier that allowed these mechanisms to evolve, we can only guess as to the causes of their speciation. One set of species for which we know the time and effectiveness of the geographic barrier that interrupted gene flow between populations, and for which we can infer phylogenetic relationships with some accuracy, is composed of the so-called “geminate” species on the

two sides of the Central American Isthmus. Connections between the tropical Atlantic and Pacific Oceans were severed during the Pliocene 3.1 to 3.5 million years ago (Saito, 1976; Keigwin, 1978, 1982). The concomitant cessation of gene flow between previously continuous marine populations has resulted in numerous pairs of closely related species, one on each side of Central America (Jordan, 1908; Ekman, 1953; Mayr, 1954).

Sea urchins are one of the many taxa containing an array of geminate species (Mayr, 1954; Chesher, 1972). Among these, three pairs, belonging to the genera *Euclidaris*, *Diadema*, and *Echinometra*, are the best studied with respect to their transisthmian divergence (Lessios, 1979, 1981a, 1984). Pacific and Atlantic species of *Echinometra* display more morphological and allozymic divergence than geminate species of *Euclidaris* and *Diadema* (Lessios, 1981a). Differences in the timing of reproduction that

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TABLE 1. Characteristics of the three neotropical species of *Echinometra*, relevant to reproductive isolation among their populations.

	<i>E. lucunter</i>	<i>E. viridis</i>	<i>E. vanbrunti</i>
Geographic range	Both sides of tropical Atlantic	Caribbean only	Tropical eastern Pacific
Bathymetric range	Intertidal	Intertidal to 15 m	Intertidal
Preferred habitat	Reef flat	Live coral reef	Rocky shores
Isozyme similarity	Similar to <i>E. viridis</i>	Similar to <i>E. lucunter</i>	Different from other two species
Morphologic similarity	Similar to <i>E. vanbrunti</i>	Different from other two species	Similar to <i>E. lucunter</i>
Annual reproductive cycle	Ill-defined	Well-defined, overlaps with <i>E. vanbrunti</i>	Well-defined, overlaps with <i>E. viridis</i>
Lunar reproductive cycle	Ill-defined	Ill-defined	?

could serve as mechanisms of reproductive isolation were found between the Pacific *Diadema mexicanum* and the Atlantic *D. antillarum* (Lessios, 1984). It is of interest to know whether the species of *Echinometra* in the two oceans are also reproductively isolated.

*Echinometra* has one species, *E. vanbrunti*, in the eastern Pacific and two, *E. lucunter* and *E. viridis*, in the Caribbean. Table 1 summarizes aspects of their biology that are relevant to the evolution of reproductive isolation. Despite geographic and bathymetric overlap, introgression between *E. lucunter* and *E. viridis* does not appear to be occurring to any appreciable extent, because there are loci in which the two species do not share alleles (Lessios, 1979). Temporal reproductive isolation through asynchronous annual reproductive cycles does not exist among the three species of *Echinometra*; annual periodicity in *E. lucunter* is very ill-defined, and the reproductive seasons of *E. viridis* and *E. vanbrunti* overlap (Lessios, 1981*b*, 1985*a*). Potential reproductive isolation between the geminate species of *Diadema* is attained through non-overlapping lunar cycles (Lessios, 1984), but there is no lunar periodicity in the spawning of either *E. viridis* or *E. lucunter* (Lessios, unpubl.). For animals such as echinoids, which free-spawn gametes into the water column, another possible mode of prezygotic reproductive isolation is incompatibility between gametes. 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) have been found in the egg membrane of echinoids. Though the effectiveness of these barriers in relation to phylogenetic affinity between echinoid species has not yet been determined, Strathmann (1981) has found unidirectional gametic incompatibility between the closely related, sympatric echinoid species *Strongylocentrotus droebachiensis* and *S. pallidus*.

This paper addresses the following questions: (1) Is there gametic incompatibility between the neotropical species of *Echinometra*? (2) If so, how and when did gametic incompatibility evolve? (3) What are the implications of prezygotic reproductive isolation in sympatric and allopatric populations of this genus for our current view of speciation?

#### MATERIALS AND METHODS

*Echinometra lucunter* and *E. viridis* were collected at Isla Margarita, next to the Atlantic entrance to the Panama Canal, and *E. vanbrunti* was collected at Punta Paitilla, off Panama City, between April and September, a period of reproductive activity for all three species (Lessios, 1981*b*). Gametes were obtained from each sea urchin with the injection of 0.3 ml of 0.5 M KCl solution. Eggs were passed through gauze to clear debris, washed twice with filtered sea water, and kept for a maximum of two hours until fertilization. Sperm was pipetted off the genital pores and kept in concentrated form for no more than an hour. A drop of semen was diluted in sea water and examined micro-

scopically for sperm motility. If the sperm appeared sluggish, the batch was discarded. The concentration of eggs was determined by counting their number in a 100  $\mu$ l aliquot in a capillary tube (Hinegardner, 1975), and then adjusted to 3,000 eggs per milliliter with the addition of the appropriate amount of sea water.

Sperm and egg from each of the three species were crossed in all possible combinations. Each replicate cross between two species involved four individuals, i.e., a male and a female of each species. A drop of concentrated sperm from each species was added to one dish containing 10 ml of conspecific egg suspension and another drop to a dish with 10 ml of heterospecific egg suspension. Sperm was allowed to remain with the eggs for ten minutes, then was removed with two consecutive washes with filtered sea water. Peak homospecific sperm binding in *Echinometra* occurs approximately one minute after the addition of sperm; additional time does not increase the degree of heterospecific sperm penetration (Summers and Hylander, 1976). The eggs were then layered on the bottom of Petri dishes and incubated at 30°C for 2 hours. Preliminary experiments had shown that in this time fertilized eggs reach the four-cell stage, which allows unambiguous identification of dividing eggs regardless of the angle of observation. Aliquots from each dish were then placed on microscope slides, which were examined in equally spaced transects run from one end of the cover-slip to the other. The first 100 eggs encountered were classified as "cleaving" if they had reached the four-cell stage, "fertilized" if they had an obvious fertilization membrane regardless of whether they showed any cell divisions, and "unfertilized" if no cell division or fertilization membrane was evident. Cultures were allowed to continue developing. In all cases (including heterogamic crosses) they produced apparently normal plutei in approximately 32 hrs.

Replicate crosses between species were performed with gametes from new individuals each time. Data from experiments in which either of the homogamic crosses produced less than 70% cleaving eggs were discarded because of the possibility that the gametes of one of the species were defective.

Five out of 32 crosses between *Echinometra vanbrunti* and *E. viridis*, 11 out of 33 crosses between *E. lucunter* and *E. viridis* and 10 out of 36 crosses between *E. lucunter* and *E. vanbrunti* were excluded for this reason. The percentage of fertilized and cleaving eggs from each heterogamic cross was compared to each of the two homogamic crosses with Wilcoxon's paired sample tests. One-tailed tests were used, because the question posed to the data was whether success of fertilization might be lower when gametes of different species were brought in contact. However, the probability levels were adjusted with the Bonferroni method (Holm, 1979; Rice, 1989) to take into account the use of the same data in multiple comparisons.

## RESULTS

In both the homogamic and the heterogamic crosses few eggs that had fertilization membranes failed to cleave (Table 2). Conclusions about reproductive incompatibility thus remain the same whether percent of fertilization or percent of cleaving is used as an index. The fraction of fertilized eggs that did not cleave at the normal rate was not higher in heterogamic crosses than it was in homogamic ones, which suggests that any barrier to future fusion between the species is prezygotic. Some reduction in the number of successful fertilizations was evident in most heterogamic crosses, as indicated by significant Wilcoxon's paired sample tests. Though this reduction suggests that gametes of each species can be sufficiently distinct to be recognized by allospecific gametes, it does not signify that *Echinometra viridis* and *E. vanbrunti* exhibit potential reproductive isolation, because the proportion of fertilized and cleaving eggs in these crosses is still close to unity. Crosses of *E. lucunter* with either its sympatric *E. viridis* or its presumed geminate *E. vanbrunti* showed a distinct asymmetry. Percent of successful heterogamic fertilization was high when sperm of *E. lucunter* was involved; yet few *E. lucunter* eggs were fertilized by either *E. viridis* or *E. vanbrunti* sperm. Thus the egg of *E. lucunter* appears to have evolved a modification that prevents primary binding by heterospecific sperm. The alternative hypothesis, that *E.*

TABLE 2. Crosses between *Echinometra lucunter* (Atlantic), *E. viridis* (Atlantic) and *E. vanbrunti* (Pacific). Number of eggs out of 100 examined two hours after gamete mixing that had visible fertilization membranes (FERTILIZED), or had reached the four-cell stage (CLEAVING). *N*: number of replicates; gametes from different individuals were used in each replicate. Dual significance levels next to heterogamic crosses refer to Wilcoxon's paired sample tests between that cross and each of the homogamic crosses. One-tailed probabilities corrected for multiple comparisons with the Bonferroni method are shown. NS:  $P > 0.05$ , \*:  $0.05 > P > 0.01$ , \*\*:  $0.01 > P > 0.001$ , \*\*\*:  $P < 0.001$ , -: comparison not performed, because median of heterogamic crosses was larger than that of the homogamic ones.

	<i>E. lucunter</i> (A) × <i>E. viridis</i> (B) <i>N</i> = 22		<i>E. lucunter</i> (A) × <i>E. vanbrunti</i> (B) <i>N</i> = 26	
	FERTILIZED Median (range)	CLEAVING Median (range)	FERTILIZED Median (range)	CLEAVING Median (range)
A♂ × A♀	99.0 (84-100)	90.5 (70-100)	98.5 (82-100)	94.0 (70-100)
B♂ × B♀	96.5 (80-100)	91.0 (71-99)	99.0 (90-100)	97.0 (80-100)
A♂ × B♀	95.5 (43-100)*/NS	88.0 (30-98)*/NS	98.0 (52-100) NS/NS	95.5 (45-100) -/NS
A♀ × B♂	27.0 (2-59)***/**	17.5 (2-59)***/**	9.0 (0-37)***/**	9.0 (0-37)***/**

*viridis* and *E. vanbrunti* sperm have evolved the ability to discriminate against eggs of *E. lucunter*, is less likely because in the experiments numerous heterospecific sperm were observed surrounding *E. lucunter* eggs, and because of the promiscuity exhibited in crosses between *E. viridis* and *E. vanbrunti*. The degree of the protection of *E. lucunter* eggs against heterospecific sperm is not the same towards both species. Significantly more *E. lucunter* eggs in each replicate were fertilized (Mann-Whitney  $U = 124$ ,  $P = 0.0008$ ) and ended up cleaving (Mann-Whitney  $U = 143$ ,  $P = 0.0032$ ) when exposed to *E. viridis* sperm than to *E. vanbrunti* sperm. Thus, eggs of *E. lucunter* appear to discriminate better against sperm from its allopatric congener than from the sympatric congener.

#### DISCUSSION

Ideally, we would like to know the point in the history of the genus at which the incompatibility between eggs of *E. lucunter* and sperm of *E. viridis* and *E. vanbrunti* arose. A reasonable assumption to be used in this reconstruction is that compatibility between eggs and sperm of two populations, once lost in evolution, is unlikely to be regained. Primary binding between sea urchin egg and sperm involves a reaction between "bindin," a protein on the acrosome process of the sperm and a "bindin receptor," a glycoprotein on the vitelline layer of the egg. The affinity between the two molecules is generally species-specific (Glabe and Vacquier, 1977, 1978; Glabe and Lennarz, 1979;

Vacquier, 1980); this specificity may arise from a small number of differences in the chemical composition of molecules from different species (Bellet et al., 1977). It seems improbable that the egg membrane molecules of a derived species will accidentally revert to an ancestral state once they have been modified in the course of evolution. Thus, it is unlikely that compatibility between the gametes of *E. viridis* and *E. vanbrunti* was secondarily acquired. A single evolutionary change, isolating *E. lucunter* reproductively from the other two species, is a much more likely hypothesis. In addition to the development of discrimination by *E. lucunter* eggs, the change would also require some interspecific variation in sperm so that *E. lucunter* eggs can differentiate between homospecific and heterospecific sperm.

How does the appearance of gametic isolation fit in the history of the genus, and how is it related to the rise of the Central American Isthmus? The four possible tree topologies for the neotropical species of *Echinometra* in relation to the separation of the oceans are shown in Figure 1. All possibilities are not equally likely. An ancient origin of *E. lucunter* (Fig. 1A) is suggested by the data on gamete incompatibility. However, such an early split of this species is supported by none of the existing data on proteins or morphology (Mortensen, 1943 p. 372; Mayr, 1954; Chesher, 1972; Lessios, 1979, 1981a); on both levels *E. viridis* and *E. vanbrunti* are the most divergent pair of species. A more credible hy-

TABLE 2. Extended.

<i>E. viridis</i> (A) × <i>E. vanbrunti</i> (B) N = 27	
FERTILIZED Median (range)	CLEAVING Median (range)
97.0 (74–100)	91.0 (71–100)
99.0 (80–100)	97.0 (74–100)
98.0 (87–100) —/NS	92.0 (61–99) —/NS
95.0 (52–100)*/**	89.0 (33–100) NS/**

pothesis is that *E. vanbrunti* was separated from Caribbean stock by the rise of the Isthmus, and that the two Atlantic species were formed at some point in the last 3.5 million years (Fig. 1B). This tree topology is suggested by isozyme data, which show that the genetic distance between *E. lucunter* and *E. viridis* is much smaller than the distance between either of them to *E. vanbrunti* (Lessios, 1979, 1981a). Under this scheme, the asymmetric reproductive isolation between *E. lucunter* and *E. viridis* could have been the product of selection against hybridization. The bathymetric range of *E. lucunter* is very restricted, extending from the intertidal to 1 m. Individuals of *E. viridis* can be found in the *E. lucunter* zone, but the majority of *E. viridis* populations live subtidally (McPherson, 1969; Lessios et al., 1984, their Table 4). Thus, *E. lucunter* eggs are in greater danger of being fertilized by *E. viridis* sperm, and this species might have been under more intense selection to protect its eggs than *E. viridis*. Though this hypothesis seems plausible, it would not provide unequivocal evidence for reinforcement of prezygotic isolation (Dobzhansky, 1940), because "speciation by reinforcement" would expect reproductive isolation to be stronger between sympatric than between allopatric species. *E. lucunter* eggs, however, show a lower percent of fertilization when exposed to sperm of *E. vanbrunti* than of *E. viridis*. Another possible tree topology is that the common stock of neotropical *Echinometra* trifurcated into the clades leading to the existing species at the time of the Isthmus

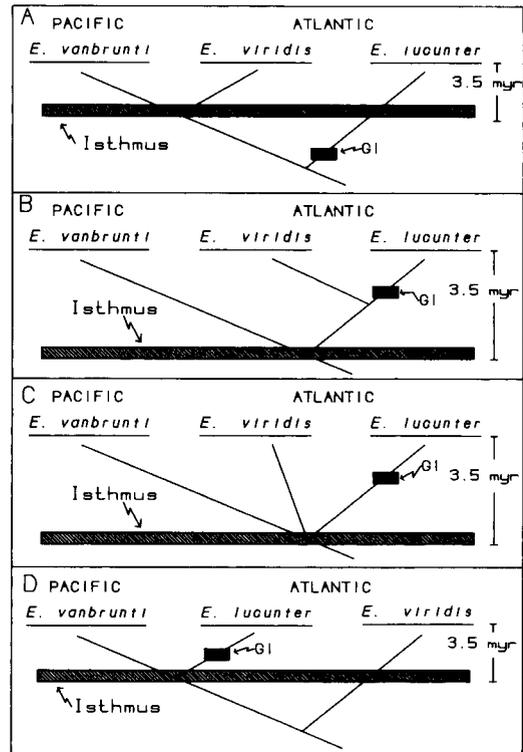


FIG. 1. The four possible phylogenetic tree topologies of the neotropical species of *Echinometra* in relation to the rise of the Central American Isthmus. GI: emergence of reproductive isolation under the assumption that compatibility between gametes, once lost in evolution, is unlikely to be regained.

emergence (Fig. 1C). The implications of this hypothesis with regards to the evolution of reproductive isolation are identical to those of the previous one (Fig. 1B). If avoidance of hybridization between the sympatric *E. viridis* and *E. lucunter* were the driving force in the evolutionary development of reproductive isolation, the eggs of the latter should have been better protected against fertilization by the former, than by the allopatric *E. vanbrunti*. The final phylogenetic hypothesis is that *E. viridis* split from stock that would eventually result in the two geminate species, *E. lucunter* and *E. vanbrunti*, before the rise of the Isthmus (Fig. 1D). This tree topology is supported by morphological data, whether they are analyzed phenetically (Lessios, 1981a), or by the tenets of traditional echinoid systematics (Mortensen, 1943 p. 372; Mayr, 1954; Cheshier, 1972). If this postulate is correct,

and if gamete compatibility between species is unlikely to be secondarily acquired, then *E. lucunter* eggs must have evolved their selectivity in the last 3.5 million years. Otherwise, the propensity to avoid fertilization by *E. viridis* sperm would have been present in *E. vanbrunti* as well as *E. lucunter* eggs.

If an early split of *Echinometra lucunter* from the neotropical *Echinometra* stock is unlikely, and if the hypothesis of reinforcement of isolating mechanisms by selection against hybridization does not fit the data, then why did *E. lucunter*, rather than either of the other two species, evolve reproductive isolation? It is possible that *E. lucunter* may have undergone a population bottleneck, which the other two species somehow escaped. Such bottlenecks have been postulated as possible causes of reproductive isolation in general (Mayr, 1963; Carson, 1975; Templeton, 1980, 1981; Carson and Templeton, 1984), and asymmetric mating preferences in particular (Kaneshiro, 1976; Watanabe and Kawanishi, 1979; Giddings and Templeton, 1983). However, heterozygosity of *E. lucunter* is as high as that of six other species of neotropical sea urchins (Lessios, 1979, 1985b), while its Nei's distance from *E. viridis* is small (Lessios, 1981a); this would indicate that an excessively restricted bottleneck (the effects of which would be manifested both in divergence and genetic variability) did not occur more recently than  $10^7$  generations ago (Chakraborty and Nei, 1977). Thus, there is no reason to reject the hypothesis that each of the three *Echinometra* lineages had an equal chance of having developed gametic incompatibility towards the other two, and that *E. lucunter* simply happened by accident to have modified the chemical composition of its binding receptors to a larger extent than the other two species.

The most likely hypotheses about the phylogeny of *Echinometra* suggest that the evolutionary emergence of reproductive incompatibility between at least two species is the accidental byproduct of events occurring in different oceans. Even hypotheses that invoke natural selection against hybridization to explain prezygotic reproductive isolation between sympatric species cannot explain the unidirectional incompatibility between *E. vanbrunti* and *E. lu-*

*cunter* as anything but an accident in relation to their speciation. Furthermore, *E. viridis* and *E. vanbrunti* though most distant in morphology and allozymes, have gametes that are capable of fertilizing each other, while *E. lucunter*, morphologically similar to *E. vanbrunti* and electrophoretically similar to *E. viridis*, has evolved a partial isolating mechanism towards both of these species. Thus, there is no correlation between reproductive isolation and existing measures of genetic divergence within the genus. Similarly, the geminate species of *Diadema*, though very closely related to each other in morphology and proteins, have evolved a mechanism of potential reproductive isolation that appears complete and bidirectional (Lessios, 1984), while *E. lucunter* and *E. vanbrunti*, morphologically and electrophoretically more distant from each other, are (as far as we know) still incompletely and unidirectionally isolated. Data from geminate species would, therefore, suggest that the emergence of reproductive isolation is independent of genomic reorganization in the populations involved.

Most mechanisms of reproductive isolation, whether pre- or post-zygotic, involve complex traits such as behavioral repertoires, reproductive seasons, habitat segregation, or reduced hybrid viability or fecundity. It is a common belief that the genetic bases of such traits involve various levels of epistatic and pleiotropic interactions, and that many genes must change before reproductive isolation is accomplished (e.g., Mayr, 1963; Barton and Charlesworth, 1984; Paterson, 1985). It is, therefore, not surprising that most hypotheses about the mode of allopatric speciation (Mayr, 1963; Carson, 1975; Templeton, 1980) are in fact models of radical genetic reorganization, rather than postulates regarding the emergence of reproductive isolation per se. Nor is it surprising that morphological differences between populations are the primary evidence used by systematists in recognizing specific status, and thus in inferring the existence of reproductive isolation. Even advocates of the view that a few genetic changes are sufficient for speciation (e.g., Lewontin, 1974; Templeton, 1981; Bush and Howard, 1986) often suggest that these changes may occur in loci with critical de-

velopmental roles. However, on the level of recognition between egg and sperm, the necessary genetic changes for the appearance of reproductive isolation may be the fixation of one or a few mutations that alter nothing more than the molecules involved in primary binding between the acrosome process of the sperm and the vitelline layer of the egg. Geographic isolation, along with stochastic introduction and spreading into a small, separated population are necessary for the initial establishment of such mutations, because they would otherwise be opposed by selection while their frequency is low (Moore, 1979, 1981; Spencer et al., 1986). However, destabilizing of the entire genome through genetic revolutions (Mayr, 1963, genetic transilience (Templeton, 1981), or founder-flush cycles (Carson, 1975), and transitions to new adaptive peaks (Barton and Charlesworth, 1984; Charlesworth and Rouhani, 1988) would not be required. Complete speciation between two populations requires bidirectional isolation. When gametic isolation is involved, three requirements must be met before two populations are converted into distinct species: (1) Sperm in at least one population must acquire characteristics that identify them as distinct to the eggs. The variation necessary for this differentiation may be present in the ancestral stock, maintained through pleiotropy or linkage (Moore, 1979; Lande, 1980, 1981, 1982). (2) Eggs from the same population must evolve the ability to preferentially bind with this modified sperm. (3) Eggs from the other population must evolve similar discriminating abilities. The data from *Echinometra* suggest that the evolution of discrimination by the eggs proceeds independently in each population. The need of two independent events, one in each population, lowers the probability of speciation through gametic isolation alone. However, modifications of eggs in two demes would convert them into new species, regardless of the degree of overall genetic divergence. Even if the new species were initially very similar, they would subsequently diverge genetically, thus making it difficult to ascertain the frequency of this mode of speciation by studying present-day patterns. The group of animals most likely to contain species in which gamete incompatibility

constitutes the only mechanism of reproductive isolation is marine invertebrates. Free-spawning of gametes that are fertilized in the water column with no apparent courtship between the sexes (other than the possible exchange of chemical cues) is a common mode of reproduction in coelenterates, polychaetes, molluscs, and echinoderms. In the Australian Great Barrier Reef, 133 species of corals, many of them closely related congeners, spawn once a year during the same few nights (Willis et al., 1985). In organisms with this mode of reproduction, decoupling of the emergence of reproductive isolation from genetic divergence may be common.

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