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SEA URCHIN BINDIN DIVERGENCE PREDICTS GAMETE COMPATIBILITY

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Abstract.—Studies on the evolution of reproductive proteins have shown that they tend to evolve more rapidly than other proteins, frequently under positive selection. Progress on understanding the implications of these patterns is possible for marine invertebrates, where molecular evolution can be linked to gamete compatibility. In this study, we surveyed data from the literature from five genera of sea urchins for which there was information on gamete compatibility, divergence of the sperm-egg recognition protein bindin, and mitochondrial divergence. We draw three conclusions: (1) bindin divergence at nonsynonymous sites predicts gamete compatibility, whereas (2) bindin divergence at synonymous sites and mitochondrial DNA divergence do not, and (3) as few as 10 amino acid changes in bindin can lead to complete gamete incompatibility between species. Using mitochondrial divergence as a proxy for time, we find that complete gamete incompatibility can evolve in approximately one and a half million years, whereas sister species can maintain complete gamete compatibility for as long as five million years.

Key words.—Bindin, gamete compatibility, reproductive isolation, sea urchin, speciation.

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As molecules involved in sexual reproduction were identified, biologists examined how they evolve with the hope of gaining insight into the evolution of reproductive isolation and the process of speciation. Studies of reproductive protein evolution have been conducted in protozoans, algae, *Drosophila*, primates, plants, and a variety of marine invertebrates. This work shows that reproductive proteins evolve more rapidly than nonreproductive proteins, and that they often evolve under positive selection (reviewed in Swanson and Vacquier 2002a,b). Here, we synthesize data on reproductive protein divergence, mitochondrial divergence, and gamete compatibility in sea urchins to examine how gamete incompatibility evolves.

Echinoids (sea urchins and sand dollars) broadcast gametes that are fertilized externally. As a result, all of the proteins mediating gamete interactions are associated with the sperm or the egg. Central to sea urchin fertilization is the sperm protein bindin. Bindin coats the acrosomal process of the sperm and binds sperm to the vitelline envelope of the egg; bindin may also be involved in the fusion of sperm and egg membranes (Vacquier and Moy 1977; Ulrich et al. 1998). Failure of sperm binding to the egg vitelline envelope and blockage of the fusion of sperm and egg membranes are two steps in the fertilization process that commonly prevent gametes of different sea urchin species from cross-fertilizing (Metz et al. 1994). Thus, changes in bindin may contribute to the evolution of reproductive isolation between species.

Sea urchins have been used as model organisms for the study of fertilization and early development for a century. There is an extensive history of studies of cross-fertilization and development of hybrid sea urchins (reviewed in Harvey 1956). Recent studies have quantified gamete compatibility between pairs of sea urchin species, providing a unique dataset relating bindin evolution, mitochondrial divergence, and gamete compatibility. The evolution of bindin has been studied in six genera of sea urchins. Bindin and mitochondrial divergence information is available from almost 30 species within these genera and gamete compatibility information from crosses between 14 species pairs in five of these genera.

We use the combined sea urchin dataset to examine several questions: (1) How much change in bindin is required to lead to gamete incompatibility? (2) Is gamete compatibility predicted by bindin divergence or time since separation between species? (3) How rapidly can gamete incompatibility evolve? (4) How long can gamete compatibility be maintained after two species diverge?

MATERIALS AND METHODS

Bindin Evolution and Mitochondrial DNA Divergence

We compiled data from 14 species pairs for which there was information in the literature on bindin divergence, mitochondrial (mtDNA) divergence (from the cytochrome oxidase I [COI] gene) and gamete compatibility (Table 1). To estimate bindin and COI divergence we followed the methods of Zigler and Lessios (2003a). Briefly, mature bindin amino acid sequences within the same genus were aligned by eye. We made no attempt to align bindin sequences between genera. In some genera, glycine-rich repeat regions could not be aligned unambiguously; these regions were excluded from analysis. We then used MEGA (ver. 2.1; Kumar et al. 2001) to calculate mean nonsynonymous (d_N) and synonymous (d_S) bindin divergence for all pairwise comparisons between species by the Pamilo and Bianchi (1993) and Li (1993) method. Mean COI divergence (as calculated by the Kimura (1980) two-parameter method) for all pairwise comparisons between

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TABLE 1. Sea urchin bindin divergence, mitochondrial divergence, and gamete compatibility.

	Species		Bindin divergence[1]			COI divergence[2]		Gamete compatibility[3]			
Genus	A	B	d_N	d_S	Source	K_2	Source	A × B	B × A	Mean	Source
Arbacia	*punctulata*	*incisa*	0.003	0.088	Metz et al. 1998	0.139	Metz et al. 1998	1.00	1.00	1.00	Metz et al. 1998
Echinometra	sp. A	sp. B (*mathaei*)	0.028	0.051	Metz and Palumbi 1996	0.034	Landry et al. 2003	0.00	0.00	0.00	Uehara et al. 1990
								0.00	0.01	0.01	Metz et al. 1994
	sp. A	sp. D (*oblonga*)	0.024	0.076	Metz and Palumbi 1996	0.040	Landry et al. 2003	0.00	0.52	0.26	Uehara et al. 1990
								0.02	0.40	0.21	Metz et al. 1994
	sp. B (*mathaei*)	sp. D (*oblonga*)	0.021	0.054	Metz and Palumbi 1996	0.026	Landry et al. 2003	0.00	0.22	0.11	Uehara et al. 1990
								0.02	0.07	0.05	Metz et al. 1994
								0.01	0.72	0.37	Rahman et al. 2004
	lucunter	*viridis*	0.022	0.047	McCartney and Lessios 2004	0.050	McCartney et al. 2000	0.19	0.97	0.58	Lessios and Cunningham 1990
								0.24	0.88	0.56	McCartney and Lessios 2002
	lucunter	*vanbrunti*	0.026	0.046	McCartney and Lessios 2004	0.102	McCartney et al. 2000	0.09	1.00	0.55	Lessios and Cunningham 1990
								0.12	0.89	0.50	McCartney and Lessios 2002
	viridis	*vanbrunti*	0.014	0.083	McCartney and Lessios 2004	0.126	McCartney et al. 2000	0.92	1.00	0.96	Lessios and Cunningham 1990
								0.48	1.00	0.74	McCartney and Lessios 2002
Heliocidaris	*erythrogramma*	*tuberculata*	0.069	0.149	Zigler et al. 2003	0.147	Zigler et al. 2003	0.00	0.82	0.41	Zigler et al. 2003
Lytechinus	*pictus*	*variegatus*	0.013	0.105	Zigler and Lessios 2004	0.135	Zigler and Lessios 2004	0.72	1.00	0.86	Minor et al. 1991
	variegatus	*williamsi*	0.006	0.022	Zigler and Lessios 2004	0.017	Zigler and Lessios 2004	1.00	1.00	1.00	K. Zigler and H. Lessios, unpubl. ms.
Strongylocentrotus	*droebachiensis*	*franciscanus*	0.111	0.183	Biermann 1998	0.121	Biermann et al. 2003	0.02	0.00	0.01	Levitan 2002a
	droebachiensis	*pallidus*	0.018	0.019	Biermann 1998	0.038	Biermann et al. 2003	0.82	0.07	0.45	Strathmann 1981
	droebachiensis	*purpuratus*	0.022	0.075	Biermann 1998	0.072	Biermann et al. 2003	0.75	0.00	0.37	Levitan 2002a
	franciscanus	*purpuratus*	0.112	0.238	Biermann 1998	0.131	Biermann et al. 2003	0.01	0.00	0.01	Minor et al. 1991
								0.00	0.00	0.00	Levitan 2002a

[1] Nonsynonymous (d_N) and synonymous (d_S) bindin divergences were calculated in MEGA (ver. 2.1; Kumar et al. 2001) by the Pamilo and Bianchi (1993) and Li (1993) method.
[2] Mitochondrial cytochrome oxidase I (COI) divergence was calculated by the Kimura (1980) two-parameter (K_2) method.
[3] Gamete compatibility calculations are described in Materials and Methods. Results are presented as egg species × sperm species.

species were either taken from the literature or calculated from sequences available in GenBank.

Gamete Compatibility

Data from 22 measurements of gamete compatibility between species were collected (for species and sources see Table 1). Most studies of gamete compatibility present fertilization percentages over a range of conspecific and heterospecific sperm concentrations. To calculate a metric of gamete compatibility from each cross, we identified the lowest sperm concentration that fertilized >90% of the eggs in conspecific crosses, and calculated the ratio of (percent fertilization by heterospecific sperm)/(percent fertilization by conspecific sperm) at that sperm concentration. If the heterospecific cross was more successful than the conspecific cross, the value was set to one. We repeated this process for the reciprocal cross and then took the mean of the ratios from the two reciprocal crosses for each comparison. By this calculation, two species that have complete gamete compatibility in both directions have a value of one, whereas two species that are completely incompatible for both reciprocal crosses have a value of zero.

Two studies (Levitan 2002a; McCartney and Lessios 2002) reported the sperm concentration at which 50% of the eggs were fertilized (F_{50} values). We used regression equations derived from the raw data to calculate conspecific and heterospecific F_{90} values and their ratio. In seven cases crosses between the same two species were examined by more than one study (Table 1). In these cases, we averaged the mean gamete compatibility calculated from each study to obtain a final value. We excluded three studies (Aslan and Uehara 1997; Rahman et al. 2000, 2001) that reported conspecific and heterospecific cross efficiency at a single sperm concentration that gave very high levels of conspecific fertilization (>98%) because of the possibility that high sperm concentrations may have inflated heterospecific fertilization rates.

Not all comparisons were statistically independent, because many involved a comparison of an outgroup to two sister species. Shared phylogenetic history between pairwise comparisons can lead to biased results (Felsenstein 1985). To correct for this we derived a phylogenetically corrected set of 10 independent comparisons from the original 14 crosses by averaging the values of reproductive compatibility and of divergence from an outgroup to two sister species (after Coyne and Orr 1989). To determine phylogenetic relationships, we relied on mtDNA phylogenies of Metz et al. (1998) for *Arbacia*, of Landry et al. (2003) for Indo-West Pacific *Echinometra*, of McCartney et al. (2000) for Neotropical *Echinometra*, of Zigler and Lessios (2004) for *Lytechinus*, and of Biermann et al. (2003) and Lee (2003) for *Strongylocentrotus*. We refer to the original set of 14 comparisons as uncorrected and the derived set of 10 comparisons as corrected. We calculated correlations between nonsynonymous bindin divergence, synonymous bindin divergence, mitochondrial divergence, and gamete compatibility using JMP (ver. 5.1; SAS Institute Inc., Cary, NC).

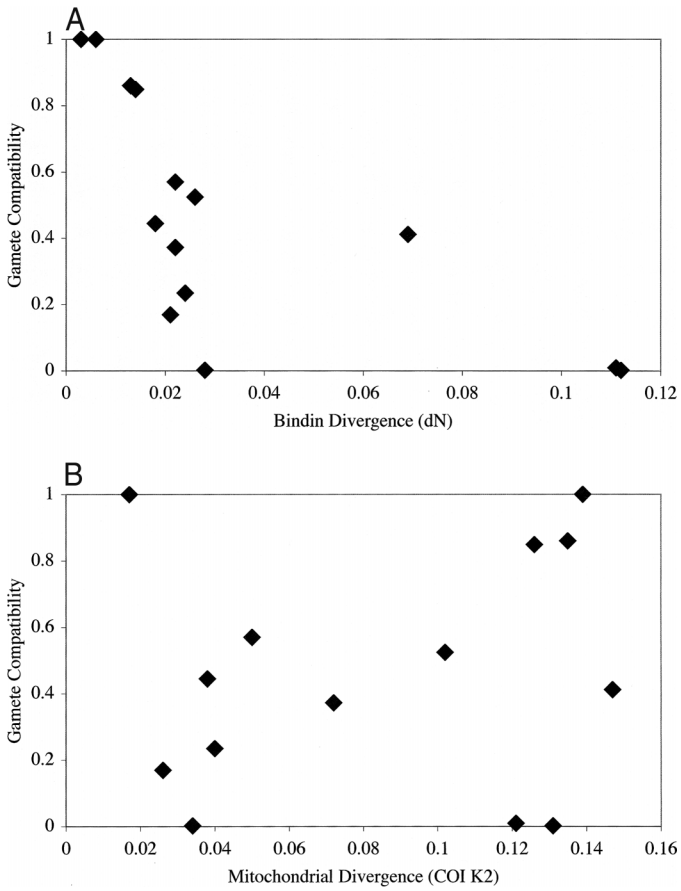

FIG. 1. Correlation between gametic compatibility and (A) divergence of bindin at nonsynonymous sites and (B) COI divergence for 14 interspecific (uncorrected for phylogenetic and statistical interdependence) comparisons. Nonsynonymous bindin divergence (d_N) was calculated in MEGA (ver. 2.1; Kumar et al. 2001) by the Pamilo and Bianchi (1993) and Li (1993) method. Mitochondrial cytochrome oxidase I (COI) divergence was calculated by the Kimura (1980) two-parameter (K_2) method.

RESULTS

Bindin Divergence and Gamete Compatibility

Nonsynonymous bindin divergence was negatively correlated with gamete compatibility (uncorrected: $n = 14$, Spearman $\rho = -0.83$, $P = 0.0003$; corrected: $n = 10$, Spearman $\rho = -0.86$, $P = 0.0014$; Fig. 1A). Of the 14 crosses, three are incompatible in both directions, two are compatible in both directions, and the remaining nine exhibit varying degrees of asymmetric incompatibility (i.e., stronger when crossing A females × B males than in the reciprocal cross; Table 1). Asymmetric incompatibility has been noted in a number of heterospecific crosses between sea urchins (Strathmann 1981; Lessios and Cunningham 1990; Levitan 2002a; Zigler et al. 2003). Correlations between gamete compatibility and synonymous bindin divergence were not significant (uncorrected: $n = 14$, Spearman $\rho = -0.27$, $P = 0.34$; corrected: $n = 10$, Spearman $\rho = -0.27$, $P = 0.44$).

Mitochondrial Divergence and Gamete Compatibility

Correlations between mitochondrial divergence and gamete compatibility were not significant (uncorrected: $n = 14$,

Spearman $\rho = 0.18$, $P = 0.54$; corrected: $n = 10$, Spearman $\rho = 0.11$, $P = 0.76$; Fig. 1B). Correlations between nonsynonymous bindin divergence and mitochondrial divergence were also not significant (uncorrected: $n = 14$, Spearman $\rho = 0.13$, $P = 0.65$; corrected: $n = 10$, Spearman $\rho = 0.08$, $P = 0.83$). Mitochondrial divergence, however, was significantly correlated with synonymous bindin divergence (uncorrected: $n = 14$, Spearman $\rho = 0.76$, $P = 0.0015$; corrected: $n = 10$, Spearman $\rho = 0.82$, $P = 0.0038$).

Rate of Evolution of Gametic Incompatibility

The mean rate of echinoid COI sequence divergence, based on comparisons between Pacific and Atlantic representatives of sea urchin genera separated by the Isthmus of Panama, is 2.6% per million years between lineages (Lessios et al. 2001). Assuming this rate of COI divergence for the genera studied here, we can calculate the minimum time it takes for complete gamete incompatibility to evolve. The minimum time is represented by the comparison of *Echinometra* sp. A to *E.* sp. B (3.4% COI divergence), which diverged around 1.5 million years ago (Fig. 1B). In contrast, as the comparison of *Arbacia incisa* to *A. punctulata* indicates (13.9% COI divergence), complete gamete compatibility can be maintained for at least five million years.

Discussion

Gamete Compatibility Is Correlated with Bindin Nonsynonymous Divergence, but Not with Time Since Separation

Given bindin's central role in sea urchin fertilization, it is not surprising that nonsynonymous bindin divergence is correlated with gamete incompatibility. Our analysis, however, permits an estimate of how much bindin sequence divergence is required for incompatibility to develop between pairs of species. Species with bindin sequences that differ at less than 1% of nonsynonymous sites appear to be completely compatible. The bindins involved in this study range in size from 200 to 300 amino acids, so 1% divergence at nonsynonymous sites (approximately two-thirds of all nucleotides) corresponds to four to six amino acid differences. Species whose bindins differ by more than 1% at nonsynonymous sites tend to be partially or completely gametically incompatible. Absolute prezygotic reproductive isolation between two incipient species could be achieved by just eight to 10 amino acid changes in bindin (and, presumably, coordinated changes in the egg receptor for bindin).

Bindin has been sequenced in a range of echinoids including sea urchins, sand dollars, heart urchins, and pencil urchins (Zigler and Lessios 2003b). The general structure of the bindin molecule is the same in each of these groups: it contains a 55- amino-acid central region (the core) that has been conserved in both length and composition for at least 250 million years, back to the common ancestor of all extant echinoids. This core region is thought to function in sperm-egg membrane fusion (Ulrich et al. 1998). Flanking the core are 5′ and 3′ regions that vary in length and amino acid composition. These regions of the bindin molecule are thought to be involved in sperm-egg attachment, and the great majority of amino acid changes seen within and between genera are found in the flanking regions. Further analyses of the distribution of amino acid changes in bindin are limited by the great variability observed between genera in the flanking regions. Although it is feasible to align bindin sequences within genera, it is impossible to construct a reliable bindin alignment between distantly related genera (Zigler and Lessios 2003b).

Over the time scale studied here (less than 10 million years) we see no correlation between time since divergence and gamete incompatibility. This differs from the pattern observed in *Drosophila*, fish, and snapping shrimp (Coyne and Orr 1989, 1997; Knowlton et al. 1993; Mendelson 2003a), where prezygotic isolation increases with genetic distance. This conclusion must be qualified with two caveats: (1) a larger sample size might have revealed a trend, and (2) our comparisons were limited to congeneric species, due to the availability of compatibility information and alignable bindin sequences. This result may be explained by the fact that we studied only one aspect of prezygotic isolation (gamete compatibility), whereas the studies on *Drosophila*, fish, and snapping shrimp quantified behavioral isolation, and in *Drosophila* included the potential for both mechanical isolation and interactions between dozens of accessory proteins in the ejaculate and the female reproductive tract (Swanson et al. 2001). The small number of molecules involved in gamete interactions of externally fertilizing organisms such as sea urchins may increase the stochasticity of the evolution of gamete incompatibility compared to the more complex mating systems of internally fertilizing organisms.

Asymmetric Gamete Incompatibility

Asymmetric prezygotic isolation has been observed in a variety of taxa, including *Drosophila*, salamanders, fish, and sea urchins (e.g., Kaneshiro, 1976, 1980; Watanabe and Kawanishi 1979; Strathmann 1981; Lessios and Cunningham 1990; Arnold et al. 1996; Levitan 2002a; Mendelson 2003b; Zigler et al. 2003). Even though a number of models have been advanced to explain this pattern, causes of asymmetric prezygotic isolation remain unclear (Kaneshiro, 1976, 1980; Watanabe and Kawanishi 1979; Arnold et al. 1996; Levitan 2002b). Asymmetric gamete incompatibility is evident in a number of crosses in the sea urchin dataset (Table 1). In several cases, this asymmetric compatibility may be attributable to rapid bindin (and presumably bindin receptor) evolution.

Two cases of highly asymmetrical prezygotic isolation are associated with the episodic adaptive evolution of bindin. In the genus *Heliocidaris*, bindin has evolved rapidly in the lineage leading to *H. erythrogramma* (Zigler et al. 2003) and in *Echinometra* in the lineage leading to *E. lucunter* (McCartney and Lessios 2004). In *Heliocidaris*, the eggs of *H. erythrogramma* are more protected from heterospecific sperm than are eggs in the reciprocal cross (Table 1). Similarly, eggs of *E. lucunter* are more protected from heterospecific sperm than are eggs in the reciprocal crosses (Table 1). In both cases, eggs of species in which males carry a ''derived'' bindin discriminate against sperm from species with bindin sequences closer to the ''ancestral'' form, whereas the effect

in the other direction is much weaker. If one assumes that changes in bindin are tracking changes in the conspecific egg receptor, then the species with the most changes in bindin is also the species in which eggs are more discriminating against heterospecifics. We predict that the more isolated member of a species pair will show more derived gamete recognition proteins, and look forward to tests of this hypothesis in other organisms that exhibit asymmetric prezygotic isolation.

Conclusions

Sea urchins fertilize externally, and only molecules associated with the sperm and egg mediate gamete interactions. All other things being equal, a close correspondence between gamete recognition protein divergence and gamete incompatibility in externally fertilizing organisms is to be expected. However, such a correlation may not be always obtained, because different species may also be isolated by shifts in reproductive timing (Levitan et al. 2004), spawning sites (Pernet 1999), or chemical communication of conspecific individuals. It is also possible that gametic compatibility is conferred not by overall bindin divergence but by amino acid replacements in specific key sites of the molecule. It remains to be seen whether such a close association between divergence in a key reproductive protein and gametic compatibility will occur in internally fertilizing organisms, where behavioral and mechanical isolation may affect the likelihood of contact between gametes and where complex interactions involving accessory proteins in the male ejaculate and the female reproductive tract influence gamete usage. Further comparative analyses of bindin evolution and gamete compatibility will contribute to the emerging picture of speciation in sea urchins. In addition, studies of the evolution of the recently sequenced bindin receptor on the egg (Kamei and Glabe 2003) should help shed light on the evolutionary mechanisms underlying the evolution of gamete incompatibility.

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