Evolution, 57(10), 2003, pp. 2293–2302

ADAPTIVE EVOLUTION OF BINDIN IN THE GENUS *HELIOCIDARIS* IS CORRELATED WITH THE SHIFT TO DIRECT DEVELOPMENT

KIRK S. ZIGLER,[1,2,3] ELIZABETH C. RAFF,[4,5] ELLEN POPODI,[4,6] RUDOLF A. RAFF,[4,7] AND H. A. LESSIOS[1,8]
[1]*Smithsonian Tropical Research Institute, Box 2072, Balboa, Panama*
[2]*Department of Biology, Duke University, Durham, North Carolina 27708*
[4]*Department of Biology and Molecular Biology Institute, Indiana University, Bloomington, Indiana 47405*
[5]*E-mail: eraff@bio.indiana.edu*
[6]*E-mail: epopodi@bio.indiana.edu*
[7]*E-mail: rraff@bio.indiana.edu*
[8]*E-mail: lessiosh@naos.si.edu*

Abstract.—Sea urchins are widely used to study both fertilization and development. In this study we combine the two fields to examine the evolution of reproductive isolation in the genus *Heliocidaris*. *Heliocidaris tuberculata* develops indirectly via a feeding larva, whereas the only other species in the genus, *H. erythrogramma*, has evolved direct development through a nonfeeding larva. We estimated the time of divergence between *H. erythrogramma* and *H. tuberculata* from mitochondrial DNA divergence, quantified levels of gametic compatibility between the two species in cross-fertilization assays, and examined the mode of evolution of the sperm protein bindin by sequencing multiple alleles of the two species. Bindin is the major component of the sea urchin sperm acrosomal vesicle, and is involved in sperm-egg attachment and fusion. Based on our analyses, we conclude that: the two species of *Heliocidaris* diverged less than five million years ago, indicating that direct development can evolve rapidly in sea urchins; since their divergence, the two species have become gametically incompatible; *Heliocidaris* bindin has evolved under positive selection; and this positive selection is concentrated on the branch leading to *H. erythrogramma*. Three hypotheses can explain the observed pattern of selection on bindin: (1) it is a correlated response to the evolution of direct development in *H. erythrogramma;* (2) it is the result of an intraspecific process acting in *H. erythrogramma* but not in *H. tuberculata;* or (3) it is the product of reinforcement on the species that invests more energy into each egg to avoid hybridization.

Key words.—Evolution of development, fertilization, gamete recognition, sea urchin.

Received November 11, 2002. Accepted April 25, 2003.

Studies on sea urchins have contributed greatly to our understanding of developmental biology, gamete interactions, and the process of fertilization. The evolution of proteins involved in gamete interactions has also received considerable attention (review in Vacquier 1998). One of these proteins is the sea urchin sperm protein bindin, which is the major component of the sea urchin sperm acrosomal vesicle and is involved in sperm-egg attachment and fusion (Vacquier and Moy 1977; Ulrich et al. 1998). Changes in bindin can thus contribute to the evolution of reproductive isolation and speciation. Bindin evolves under positive selection in some (Metz and Palumbi 1996; Biermann 1998), but not all (Metz et al. 1998; Zigler and Lessios 2003a), genera of sea urchins. The selective forces acting on bindin are still unclear, but both avoidance of maladaptive hybridization (reinforcement) and various intraspecific mechanisms, such as male heterozygote advantage, nontransitive female preferences, or interlocus conflict evolution have been suggested (Metz et al. 1998; Palumbi 1999).

Some sea urchins develop indirectly from feeding larvae, whereas others develop directly from nonfeeding larvae. Because of extensive similarities between the sea urchin feeding larva and those of other echinoderms, development via a feeding larva is thought to be the ancestral mode of development in echinoids (Strathmann 1978). Although this ancestral pattern of development has been preserved in the majority of echinoid species, direct development has evolved at least 20 times in echinoids (Emlet 1990; Wray 1996). The two species of the genus *Heliocidaris* are the best-studied example of this transition. *Heliocidaris tuberculata* follows the ancestral mode of developing from small (95 μm diameter) eggs via a feeding larva, whereas *H. erythrogramma* has evolved direct development from large (430 μm) eggs, bypassing the planktonic feeding stage (Raff 1987). Correlated with the increase in *H. erythrogramma* egg size are radical differences in development between the two species, including cleavage pattern and cell lineage, axial specification, morphogenesis, and gene expression (e.g., Wray and Raff 1989; Henry and Raff 1990; Henry et al. 1991; Emlet 1995; Haag et al. 1999; Raff and Sly 2000).

In addition to the more than 100-fold difference in dry organic mass per egg (Hoegh-Guldberg and Emlet 1997), there are other important differences in gametogenesis between the two species of *Heliocidaris*. The small eggs of *H. tuberculata* are predominantly provisioned with vitellin protein (yolk) and triglyceride lipids, whereas the large eggs of *H. erythrogramma* show a profound decrease in yolk content and a great increase in wax ester content (Scott et al. 1990; Byrne et al. 1999; Villinski et al. 2002). Sperm size is also correlated with developmental mode; direct-developers generally have longer sperm heads than related indirect-developers (Eckelbarger et al. 1989; Raff et al. 1990). This holds true in *Heliocidaris*, where *H. tuberculata* sperm have heads 5.6 μm long and *H. erythrogramma* sperm heads 11 μm long (Raff et al. 1990).

The two *Heliocidaris* species coexist in intertidal and shal-

[3] Present address: Friday Harbor Laboratories, 620 University Road, Friday Harbor, Washington 98250; E-mail: ziglerk@u.washington.edu.

low subtidal rocky habitats on the southeast coast of Australia (Mortensen 1943; Keesing 2001). It is unclear how long ago these two species diverged, as estimates vary from five to 13 million years ago (Smith et al. 1990; McMillan et al. 1992). *Heliocidaris erythrogramma* and *H. tuberculata* have partially overlapping reproductive seasons (Laegdsgaard et al. 1991), and their gametes can be crossed in the laboratory (Raff et al. 1999), but the efficiency of cross fertilizations has not been determined.

In this study we present data on divergence time, gametic compatibility, and mode of evolution of bindin between the two species of *Heliocidaris*. To estimate the divergence time between *H. erythrogramma* and *H. tuberculata*, we sequenced part of the gene for cytochrome oxidase I (COI) from the two species. We quantified levels of gametic compatibility between the two species of *Heliocidaris* by fertilization efficiency assays. We sequenced part of the gene for bindin from the two species to examine its relation to reproductive isolation and the possibility that its evolution is related to developmental mode. Previous studies of gamete recognition protein evolution in marine invertebrates have involved species with planktonic development; this study is the first comparison of gamete recognition protein evolution between species with different developmental modes.

Materials and Methods

Samples

Heliocidaris erythrogramma and *H. tuberculata* were collected near Sydney, Australia. Additional *H. erythrogramma* individuals were collected from Hobart, Tasmania, Australia. Individual gonads were stored in 95% ethyl alcohol and refrigerated until use.

Cytochrome Oxidase I Sequencing, Phylogeny, and Genetic Distances

DNA was extracted as described Lessios et al. (1996). A 640-bp section of the mitochondrial COI gene was amplified and sequenced from seven individuals of *H. erythrogramma* and four of *H. tuberculata*, using primers COIa (5′-AGTATAAGCGTCTGGGTAGTC-3′) and COIf (5′-CCTGCA GGAGGAGGAGAYCC-3′). We rooted the *Heliocidaris* COI sequences with four randomly chosen COI sequences from each of the seven *Echinometra* species for which COI data were available in GenBank. Four of these species are from the Indo-West Pacific (*E. type A*, *E. mathaei*, *E. type C*, and *E. oblonga*; Palumbi et al. 1997) and three from the Americas (*E. viridis*, *E. lucunter*, and *E. vanbrunti*; McCartney et al. 2000). *Heliocidaris* and *Echinometra* are both members of the subfamily Echinometrinae (Smith 1988). We used Modeltest version 3.06 (Posada and Crandall 1998) to identify the simplest model of DNA evolution that best describes the data under maximum likelihood (ML). The model identified was that of Tamura and Nei (1993). Inclusion of site-specific rates in place of a gamma correction or invariable sites produced a much larger likelihood value, so these rates were included. We used PAUP* version 4.0b10 (Swofford 2001) to construct a neighbor-joining tree based on the estimated ML parameters (transversions = 1, A-G = 7.6447, C-T = 13.0757; relative rates: first position = 0.1547, second position = 0.0405, third position = 2.7962) . The tree was bootstrapped in 1000 iterations. We tested for variation in substitution rates between taxa by comparing the likelihoods of the best ML tree with and without a molecular clock enforced (Felsenstein 1981). *Heliocidaris* COI sequences have been deposited into GenBank (accession numbers AF529574–AF529584).

Fertilization Efficiency Assays

Shed eggs were washed, and a subset was test-fertilized with homospecific sperm to ensure that they exhibited normal fertilization. *Heliocidaris erythrogramma* eggs have a thick jelly coat; for fertilization tests of dejellied *H. erythrogramma* eggs, the eggs were dejellied after washing by brief treatment with pH 5 seawater as previously described (Raff et al. 1999). Fresh sperm were diluted in 10-fold serial dilutions in filtered seawater; sperm concentration was determined from paraformaldehyde-fixed samples of sperm suspensions by counting on a hemocytometer slide. Washed eggs were aliquoted in 2 ml of filtered seawater in each well of 24-well plastic microtiter dishes. The large, lipid-rich *H. erythrogramma* eggs float, whereas the small *H. tuberculata* eggs sink, as is typical for eggs from indirect-developing sea urchins. Because the size and buoyancy of eggs from the two species is so different, no attempt was made to use the same concentration of eggs in the fertilization experiments. However, the egg suspensions of both species were sufficiently dilute for sperm to be in vast excess, even at the lowest sperm concentration.

For the experiments, sperm at selected concentrations were added to the eggs in the microtiter wells, and the eggs and sperm were mixed by stirring. In different experiments, final concentrations of sperm in the egg suspensions ranged from approximately 5×10^2 to 5×10^7 sperm/ml. To determine the percent of fertilized eggs, the zygotes were fixed by adding a small amount of paraformaldehyde. Fertilization was scored either by fixing after 12 min and counting the number of eggs with raised fertilization envelopes, or by fixing after 2 h and counting the number of eggs that had cleaved (2-cell or 4-cell stage). Both methods gave comparable results. One hundred or more eggs were counted for each fertilization test.

Characterization of Bindin

Genomic DNA was extracted as described by Lessios et al. (1996) from gonad tissue preserved in ethanol. Mature bindin alleles were amplified from genomic DNA with primers HeF1 (5′-ATTAATGGTGCCCACCAACAG-3′) and HeR1 (5′-GCGTGGCTGCTCCCTTTC-3′) based on a previously determined *H. erythrogramma* bindin sequence (GenBank accession number AF530406; Zigler and Lessios 2003b) and cloned as described previously (Zigler and Lessios 2003a). One to four clones were sequenced per individual. *Heliocidaris erythrogramma* alleles were sequenced using primers HeF1, HeR1, Heout5 (5′-GGCGTAGTAAGAGTCCAAGATCG-3′), Heout51 (5′-CAAACCTGATGGGTTGCGTA-3′), HeF4 (5′-TTGACCATTAGGTCTAAGCCG-3′), and MB1130+ (5′-TGCTSGGTGCSACSAAGATT-

GA-3′). *Heliocidaris tuberculata* alleles were sequenced using primers HeR1, HeF1, Heout5, Htout52 (5′-CAAGGGGTCATCGTGGTCAT-3′), HeR4 (5′-TTCAATCCCATCAATCACTTG-3′), and MB1130+. This combination of primers sequenced both strands of the mature bindin and its intron. Sequencing was performed on an ABI 377 automated sequencer (Applied Biosystems, Inc., Foster City, CA) and edited using Sequencher 3.1 (Gene Codes Corp., Ann Arbor, MI). Sequences have been deposited in GenBank (accession numbers AF530401–AF530443). We sequenced 13 mature bindin alleles from *H. erythrogramma* and 15 from *H. tuberculata*.

A total of 20 mutations unique to a single allele (singletons) were observed among 28 mature bindin and intron sequences with a combined length of 40,500 bp. Singleton mutations may represent true differences, or they may arise from polymerase error during amplification or cloning. Thus, the conservative upper limit of sequencing error in this study is 0.05%.

Mature bindin sequences were aligned by eye in Se-Al (ver. 1.0, written by A. Rambaut, Dept. of Zoology, University of Oxford, Oxford, U.K.). In the glycine-rich region 3′ of the core, 24 codons could not be unambiguously aligned between the two *Heliocidaris* species and were excluded from further analysis. Ignoring gaps introduced for alignment with the outgroup, there are 205 alignable amino acids in the mature bindins of the two *Heliocidaris* species.

Bindin Gene Genealogy

Two sequences from each of the three species of *Echinometra* for which mature bindin sequences are available (Metz and Palumbi 1996) were used to root the *Heliocidaris* bindin gene genealogy. Some amino acids were unalignable between *Echinometra* and *Heliocidaris*: 38 amino acids 5′ of the core and 33 amino acids 3′ of the core were excluded from further analysis for this reason. Modeltest identified the model of Tamura and Nei (1993) with a gamma correction as the model with the highest likelihood. We fixed the parameters to their ML estimates (transversions = 1, A-G = 1.8616, C-T = 3.8848; $\alpha = 0.9223$) and used PAUP* to reconstruct the bindin gene genealogy and to determine bootstrap support (1000 iterations) by the neighbor-joining method.

Tests for Selection and Episodic Evolution

For analysis, the bindin molecule was divided into three regions: 33 amino acids of the hotspot region of rapid evolution identified in *Echinometra* by Metz and Palumbi (1996) and in *Strongylocentrotus* by Biermann (1998); 55 amino acids of the core (i.e., the conserved area in all previously studied bindins; Vacquier et al. 1995; Zigler and Lessios 2003b); and 117 amino acids from the rest of the molecule. For each of these three regions, we used MEGA version 2.1 (Kumar et al. 2001) to calculate the proportion of differences per synonymous (d_S) and per nonsynonymous site (d_N) between the two species of *Heliocidaris* by the Pamilo and Bianchi (1993) and Li (1993) method. We tested for evidence of positive selection in each of the three regions of the molecule, using Fisher's exact tests on all pairwise comparisons between sequences (Zhang et al. 1997), under Nei and Gojobori's (1986) model of evolution. Additionally, we tested for selection on the *Heliocidaris* mature bindin sequences using the McDonald and Kreitman (1991) test, comparing the ratio of amino acid replacement to silent substitutions that are polymorphic within species to the same ratio of substitutions that are fixed between species.

To test for episodic adaptive evolution, we used a series of ML models implemented in the program PAML (ver. 3.0, Yang 2000). To simplify the analysis, we used only two sequences from each species of *Heliocidaris* and *Echinometra* for a total of 10 sequences. Within each *Heliocidaris* species, two divergent alleles were chosen to cover the small range of intraspecific variability present. We used the same alignment as for the reconstruction of the bindin gene genealogy. In addition to the regions already excluded as unalignable, we also excluded the core because of its slow rate of evolution (Vacquier et al. 1995; Zigler and Lessios 2003b). We also excluded all sites that had an indel in one or more sequences because of difficulties in reconstructing ancestral states at such sites (Yang 2000). The final dataset for this analysis included 116 amino acids.

To determine the single best tree for the 10 sequences included in the analysis, we reconstructed the phylogeny by ML in PAUP* using the best model of DNA evolution identified by Modeltest. For this smaller set of data, the best model was Kimura (1980) two-parameter distance with a gamma correction ($\kappa = 1.9044$, $\alpha = 0.9074$). Using this model, a ML branch-and-bound search identified the tree with the highest likelihood. On this tree, we compared the ratio of nonsynonymous to synonymous substitution rates ($\omega = d_N/d_S$) on the branch leading to *H. erythrogramma* (ω_E) to the ratio on the branch leading to *H. tuberculata* (ω_T). Using likelihood ratio tests in PAML, we tested whether ω_E or ω_T was equal to the background rate of all other branches in the bindin gene genealogy (ω_B); whether ω_E equaled ω_T; and whether ω_E was significantly larger than one, a highly conservative indication of positive selection (Yang 1998; Yang and Bielawski 2000).

RESULTS

Cytochrome Oxidase I Phylogeny and Timing of Divergence between the Species of Heliocidaris

The COI phylogenetic reconstruction clearly put mitochondrial DNA sequences of the two *Heliocidaris* species in separate clades (Fig. 1). Comparison of a tree constrained by the molecular clock and one in which rates were free to vary indicated that there was no significant variation in substitution rates in the COI phylogeny ($2\Delta\ln L = 46.4$, df = 37, $P > 0.10$). We used a general echinoid and a specific echinometrid calibration of COI divergence to date the split of the two species of *Heliocidaris*. COI Kimura two-parameter distances between congeners on opposite sides of the Isthmus of Panama have been determined in eight genera of sea urchins, including *Echinometra* (Lessios et al. 2001). Six of these are in the range of 9.0–13.0%. (the other two values are smaller and assumed to be due to more recent separation). Assuming these six pairs of species were split by the completion of the Isthmus of Panama 3.1 million years ago

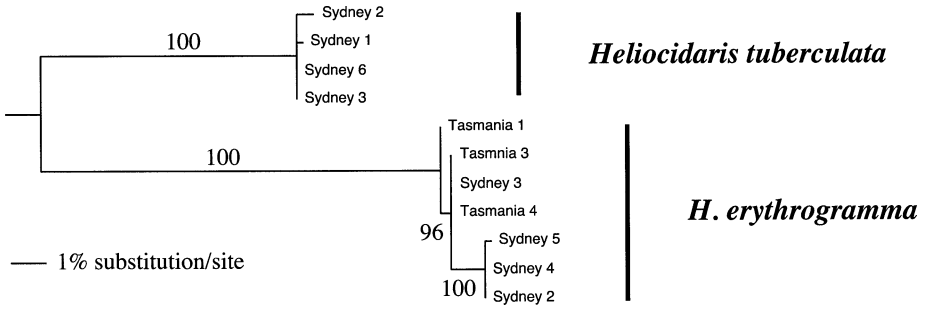

FIG. 1. Maximum-likelihood tree of cytochrome oxidase I of *Heliocidaris*, from a heuristic search based on the Tamura and Nei (1993) model with rates specific to codon position. The tree is rooted with four sequences each from *Echinometra mathaei*, *E. oblonga*, *Echinometra* type C and type A (Palumbi et al. 1997) and *E. vanbrunti*, *E. lucunter* and *E. viridis* (McCartney et al. 2000). Bootstrap values on branches supported by more than 70% from a neighbor-joining bootstrap (1000 replicates) are indicated.

(Coates and Obando 1996) produces an estimate of approximately 3.5% divergence per million years for this region of COI. Dividing the mean COI Kimura two-parameter divergence between the two species of *Heliocidaris* (14.7%) by this estimated rate suggests that *H. erythrogramma* and *H. tuberculata* were separated approximately 4.2 million years ago. Mean COI divergence between neotropical species of *Echinometra* also produces an estimated rate of 3.5% per million years, with a potential range (based on branch length variation in the genus) of 24% of the mean (McCartney et al. 2000). Thus, the range of estimates of time of splitting between the species of *Heliocidaris* would be 3.7–4.7 million years ago. McMillan et al. (1992) determined mitochondrial DNA divergence between *H. erythrogramma* and *H. tuberculata* through restriction fragment length polymorphisms (RFLP) to be 7.9%. Calibrated with Bermingham and Lessios's (1993) estimated mitochondrial DNA RFLP divergence of 1.6–2.1% per million years for sea urchins, this also produces an estimated time of 3.8–4.9 million years ago for *Heliocidaris*.

Heliocidaris Gametic Compatibility

Eggs of each species of *Heliocidaris* could be fertilized by sperm of the other, but neither of these crosses was efficient.

TABLE 1. Fertilization efficiency of homologous and heterologous crosses between *Heliocidaris erythrogramma* (*H. e.*) and *H. tuberculata* (*H. t.*) gametes at different sperm concentrations. Results from representative experiments are shown, illustrating the range in sperm concentrations required to give effective fertilization rates in different homologous and heterologous crosses.

Egg species × sperm species	% fertilization	Sperm concentration (per ml)
H. e. × *H. e.*	98	3×10^5
	63	3×10^3
H. e. × *H. t.*	2	5×10^7
	0	5×10^6
Dejellied *H. e.* × *H. e.*	98	5×10^6
	25	5×10^4
Dejellied *H. e.* × *H. t.*	87	5×10^7
	25	5×10^4
H. t. × *H. t.*	97	4×10^5
	22	4×10^2
H. t. × *H. e.*	80	4×10^6
	12	4×10^4

Obtaining equivalent fertilization percentages of *H. tuberculata* eggs required more than an order of magnitude more *H. erythrogramma* sperm than *H. tuberculata* sperm (Table 1). Inefficiency in the reciprocal cross was much more severe; a *H. erythrogramma* sperm concentration of 3×10^5 per milliliter fertilized 98% of *H. erythrogramma* eggs, whereas 150 times more *H. tuberculata* sperm (5×10^7 per milliliter) only fertilized 2% of *H. erythrogramma* eggs.

A substantial extent of fertilization of *H. erythrogramma* eggs by *H. tuberculata* sperm could be achieved only after removal of the jelly coat from the eggs (Table 1). At the same sperm concentration, *H. tuberculata* sperm fertilized 2% of jelly-coat-intact *H. erythrogramma* eggs and 87% of dejellied *H. erythrogramma* eggs. We suspect that the small percent of fertilization of jelly-coat-intact *H. erythrogramma* eggs by *H. tuberculata* sperm reflects fertilization of eggs with compromised jelly coats, as we observed that in highly concentrated sperm suspensions the egg jelly coats became weakened, perhaps reflecting normal digestive processes of sperm. Whereas dejellying greatly increased the ability of *H. tuberculata* sperm to fertilize *H. erythrogramma* eggs, dejellying resulted in a 10-fold decrease in the normal fertilization efficiency of *H. erythrogramma* eggs by homospecific sperm.

Structure of the Heliocidaris Bindin Molecule

Heliocidaris erythrogramma bindin was described by Zigler and Lessios (2003b), and *H. tuberculata* bindin is similar (Fig. 2). Both contain the bindin core, the characteristic bindin intron, and have glycine-rich regions both 5′ and 3′ of the core. *Heliocidaris erythrogramma* mature bindins are either 206 or 210 amino acids long, whereas *H. tuberculata* bindins range from 223 to 226 amino acids in length. The *H. erythrogramma* intron is 715 nucleotides (nt) long. We amplified mature bindin and its intron in *H. tuberculata* but were unable to sequence through the middle of the intron. Partial intron sequences from *H. tuberculata* are 600 nt in length.

Mode of Bindin Evolution in Heliocidaris

Little intraspecific amino acid variation is observed among the *Heliocidaris* mature bindin alleles (Table 2). Seven of 13 *H. erythrogramma* alleles have identical amino acid sequences, and only six of 210 amino acid sites are polymorphic.

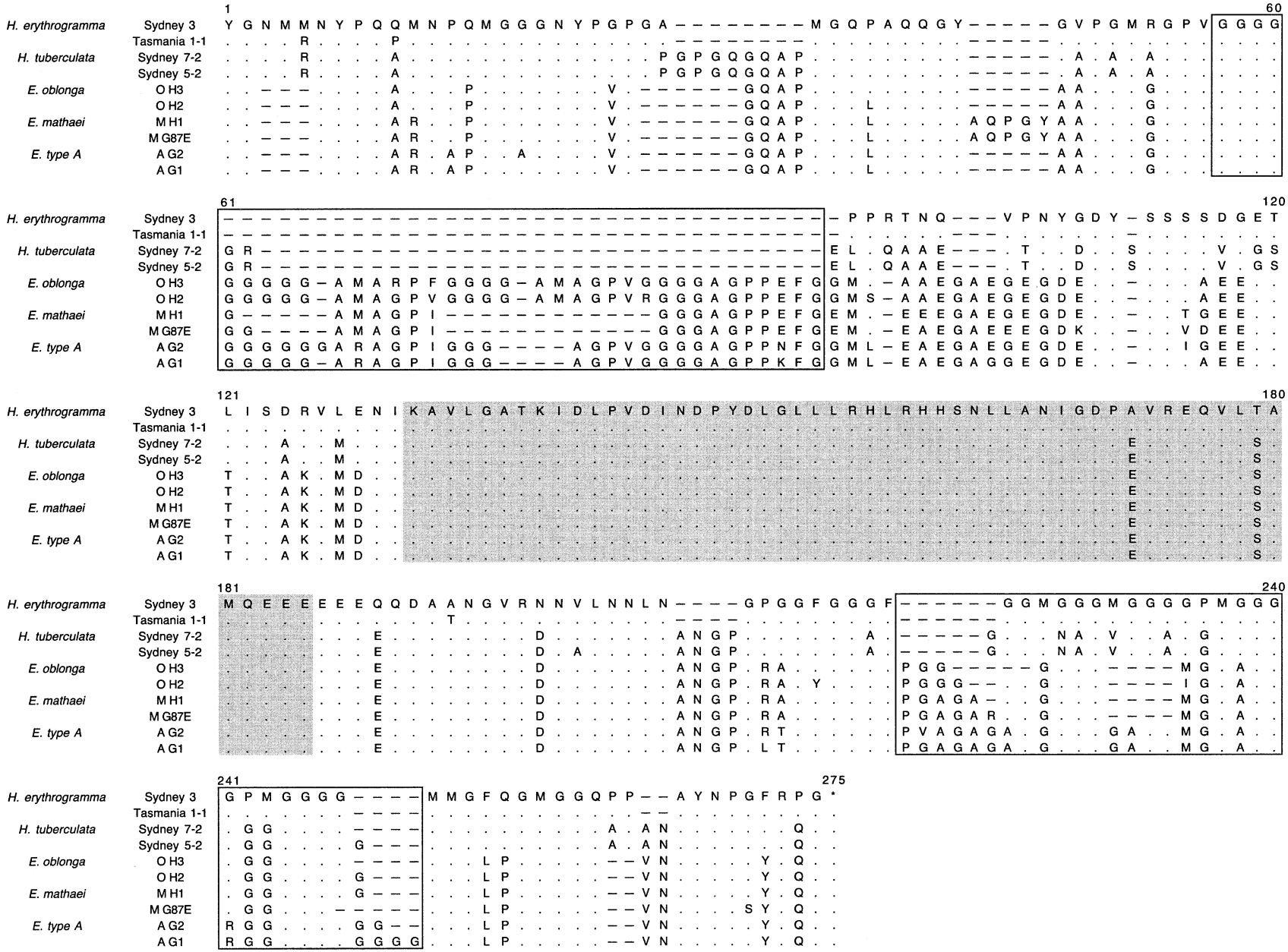

FIG. 2. Amino acid alignment of two mature bindin alleles per species in *Heliocidaris* and Indo-West Pacific *Echinometra*. *Echinometra* sequences are from Metz and Palumbi (1996). Dots indicate identity to the first sequence, gaps are indicated by dashes. The conserved core region is shaded. Unalignable regions excluded from analysis are boxed.

TABLE 2. Replacement (d_N) and silent (d_S) substitutions per site in three regions of *Heliocidaris erythrogramma* (*H. e.*) and *H. tuberculata* (*H. t.*) mature bindin and their ratio ($\omega = d_N/d_S$). We show means of pairwise comparisons calculated by the Pamilo and Bianchi (1993) and Li (1993) methods. The hotspot and core are composed of amino acids 95–130 and 131–185 in Figure 2, respectively. The ω-value for the hotspot is greater than the neutral expectation for all pairwise comparisons by Fisher's exact test using the Nei and Gojobori (1986) model of evolution ($0.015 < P < 0.038$).

Region	Comparison	d_N	d_S	ω
Hotspot (33 codons)	between *H. e.* and *H. t.*	0.222	0	>1
	within *H. e.* and *H. t.*	0.002	0	
Core (55 codons)	between *H. e.* and *H. t.*	0.016	0.197	0.08
	within *H. e.* and *H. t.*	0.001	0.002	
Rest of molecule (117 codons)	between *H. e.* and *H. t.*	0.058	0.149	0.39
	within *H. e.* and *H. t.*	0.003	0.009	
Total (205 codons)	between *H. e.* and *H. t.*	0.070	0.139	0.50
	within *H. e.* and *H. t.*	0.003	0.006	

One indel of four residues is shared by three alleles of *H. erythrogramma*. Among 15 *H. tuberculata* alleles there are five singleton amino acid changes and four small (one or two residue) indels. All the observed indels are located in the glycine-rich regions.

The bindin gene genealogy separates the *H. erythrogramma* and *H. tuberculata* alleles (Fig. 3). As in bindins of other sea urchin genera (Palumbi and Metz 1996; Metz et al. 1998; Biermann 1998; Zigler and Lessios 2003a), nonsynonymous changes are accumulating slowly in the core of *Heliocidaris* bindin and more rapidly in the hotspot just 5′ of the core

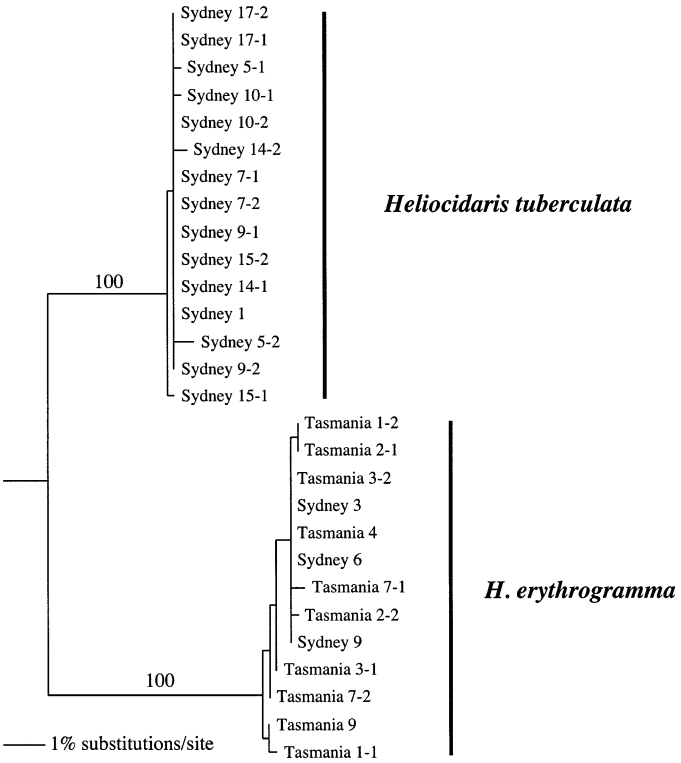

FIG. 3. Maximum-likelihood tree of *Heliocidaris* mature bindin, from a heuristic search based on the Tamura and Nei (1993) model with a gamma correction. The tree is rooted with the six *Echinometra* sequences shown in Figure 2. Bootstrap values on branches supported by more than 70% from a neighbor-joining bootstrap (1000 replicates) are indicated. We indicate when both alleles were sequenced from a single individual (e.g., Sydney 15-1 and Sydney 15-2).

(Table 2). In *Heliocidaris*, there is greater than a 10-fold difference in the rate of nonsynonymous change between these two adjacent regions of the molecule. Rates of synonymous change follow the opposite pattern: there are no synonymous changes observed in the hotspot, whereas nearly 20% of the synonymous sites are substituted in the core region between the two species. In the hotspot, there is a significant excess of nonsynonymous to synonymous changes (12–14 to 0, depending on the specific pairwise comparison).

McDonald-Kreitman (1991) tests for the entire mature bindin did not reveal any evidence of selection in *Heliocidaris*. Twenty-six replacement and 17 silent differences were fixed between the species, and nine replacement and seven silent differences were polymorphic within the species (Fisher's exact test, $P = 0.50$). When the core region (amino acids 131–185 in Fig. 2) was excluded, there were 24 replacement and eight silent fixed differences, as compared to seven replacement and five silent polymorphic differences (Fisher's exact test, $P = 0.24$). The small number of intraspecific differences within each species (Table 2) makes it difficult to identify selection using this statistical test.

Amino Acid Replacements along the Lineages of Heliocidaris erythrogramma and H. tuberculata

We tested for shifts in the ratio of amino acid replacement to silent substitutions (ω) on the branches leading to *H. erythrogramma* and *H. tuberculata* compared to the background rate (ω_B) and to each other. We estimated the log likelihood of six models on the 10-taxon tree composed of two sequences from each of the species of *Heliocidaris* and from each of the species of *Echinometra* sequenced by Metz and Palumbi (1996; see Fig. 4). The number of estimated parameters in each model ranged from 18 to 20, including 16 branch lengths, one transition/transversion ratio, and one to three ω-values (Table 3). We then compared various nested models to test four hypotheses (Table 4). The ratio of nonsynonymous to synonymous substitution on the branch leading to *H. erythrogramma* (ω_E) is significantly greater than the background ratio (ω_B), whereas the ratio on the branch leading to *H. tuberculata* (ω_T) is not. Additionally, ω_E is significantly greater than ω_T. Finally, ω_E is significantly greater than one. When ω_E, ω_T, and ω_B are all allowed to vary (model C in Table 3) the estimated ω-values are ∞, 0.32, and 0.30, respectively. Under this model, 16.6 nonsynonymous changes

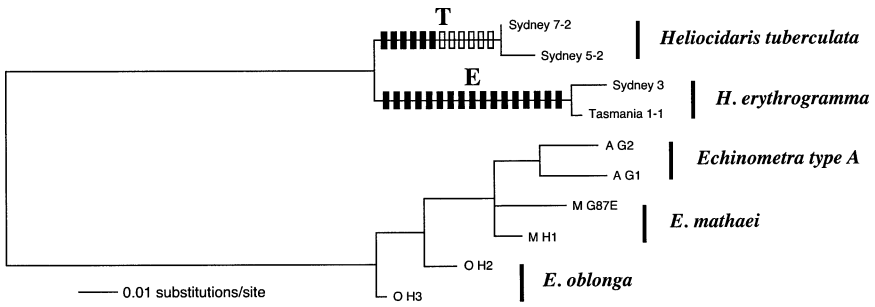

FIG. 4. Maximum-likelihood assignment of nucleotide changes along the branches of the *Heliocidaris* bindin tree, using *Echinometra* as an outgroup. T marks the branch leading to *H. tuberculata*, E marks the branch leading to *H. erythrogramma*. Reconstructed nonsynonymous changes on branches E and T under model C (see Table 3) are indicated by filled boxes, synonymous changes by unfilled boxes.

occurred on the branch of *H. erythrogramma* (and 0.1 synonymous change), whereas on the branch of *H. tuberculata* 6.0 nonsynonymous changes (and 6.4 synonymous changes) have occurred (Fig. 4). Two lines of evidence suggest these results are not influenced by the choice of *Echinometra* as the outgroup for this analysis. First, the accuracy of the inferred ancestral amino acid sequences is calculated by PAML to be 99.4% for all the ancestral nodes and 98.6% for the common ancestor of the two species of *Heliocidaris*. Second, similar patterns of statistical significance were obtained when the long branch connecting the *Heliocidaris* and *Echinometra* sequences was allowed to have its own ω value in each of the models in Table 3.

There is also evidence for a concentration of change in bindin on the branch leading to *H. erythrogramma* in the core and the indels that were not included in the previous analysis. In the core there are two amino acid differences between *H. erythrogramma* and *H. tuberculata* (at residues 172 and 179 in Fig. 2). In both cases *H. tuberculata* has the same residue as all of the outgroup sequences, which suggests that both changes occurred on the branch leading to *H. erythrogramma*. This is the first case of amino acid variation between congeners. In the four genera of echinoids for which bindin sequences have been published (Metz and Palumbi 1996; Biermann 1998; Metz et al. 1998; Zigler and Lessios 2003a) core amino acids remain the same within a genus. Additionally, several small indels have arisen on the branch leading to *H. erythrogramma* (at residues 30–33, 206–209, and 264–265 in Fig. 2).

TABLE 3. Maximum-likelihood models estimating the ratio of nonsynonymous to synonymous substitution (ω) of bindin on the lineages leading to *Heliocidaris erythrogramma* (E) and *H. tuberculata* (T). Branches E and T are indicated in Figure 4; B is the background rate for all other branches.

		Estimates of ω			
Model	Free ω	ω_B	ω_E	ω_T	−ln L
A	1	0.40	$=\omega_B$	$=\omega_B$	1081.23
B	2	0.30	1.19	$=\omega_E$	1077.29
C	3	0.30	∞	0.32	1074.36
D	1	0.31	=1	$=\omega_B$	1076.87
E	2	0.31	∞	$=\omega_B$	1074.37
F	2	0.39	$=\omega_B$	0.52	1081.16

DISCUSSION

We found that the two species of *Heliocidaris* diverged less than five million years ago. Our estimated time of divergence is considerably shorter than the 10–13 million years ago estimated by DNA-DNA hybridization methods (Smith et al. 1990) but more in line with the five to eight million years ago derived from mitochondrial RFLP divergence by McMillan et al. (1992). These results suggest that the evolution of nonfeeding development and the differences in amino acid sequence of bindin in *Heliocidaris* have occurred within the past 5 million years. There are only two species of *Heliocidaris*, but a subspecies, *H. erythrogramma armigera*, is found on the south and west coast of Australia (Mortensen 1943). McMillan et al. (1992) estimate that, based on RFLP divergence, *H. erythrogramma armigera* diverged from *H. erythrogramma erythrogramma* 1 million years ago. Because *H. erythrogramma armigera* individuals also develop directly from large eggs (R. A. Raff, pers. obs.) , this suggests that the transition from indirect to direct development in *H. erythrogramma* occurred between five million years ago and one million years ago.

Cross-fertilization experiments revealed that there is a small likelihood of hybrid production between the two *Heliocidaris* species under normal environmental conditions, even though there is a part of the year during which both species produce gametes (Laegdsgaard et al. 1991). The major barrier to cross-fertilization between the two species is gametic incompatibility, with especially strong incompatibility between *H. erythrogramma* eggs and *H. tuberculata* sperm. Another factor that may contribute to isolation between the species is the different buoyancy of the eggs. Eggs of *H. tuberculata* females, like those of other indirect-de-

TABLE 4. Testing hypotheses regarding the ratio of nonsynonymous to synonymous substitution (ω) on branches E and T by likelihood-ratio test. Branches E and T are indicated in Figure 4, B is the background rate for all other branches. Null (H_0) and alternative (H_1) model details and log likelihoods are in Table 3.

Test	H_0	H_1	$2(\ln L_1 - \ln L_0)$	df	$P(\chi^2)$
$\omega_E = \omega_B$	A	E	13.72	1	0.000
$\omega_T = \omega_B$	A	F	0.14	1	0.708
$\omega_E = \omega_T$	B	C	5.86	1	0.015
$\omega_E = 1$	D	E	5.00	1	0.025

veloping sea urchin species, sink as they are extruded. In still water, *H. tuberculata* eggs remain layered over the female or sink to the substrate. In contrast, eggs of *H. erythrogramma* females immediately spiral upward toward the water surface as they are shed. The buoyancy and behavior of *Heliocidaris* sperm have not been characterized, but the difference in egg buoyancy implies that *H. erythrogramma* sperm in the wild might have to float or swim upward to achieve most efficient fertilization, whereas upward movement by *H. tuberculata* sperm might decrease fertilization efficiency.

Postzygotic isolation further reduces the possibility of gene flow between the two species. Embryos derived from the cross between *H. tuberculata* eggs and *H. erythrogramma* sperm arrest at gastrulation (Raff et al. 1999). Successful fertilization in the reciprocal cross would be most likely for *H. erythrogramma* eggs with damaged or missing jelly coats. Embryos from dejellied *H. erythrogramma* eggs are more fragile than normal embryos. Of those that survive past metamorphosis, no hybrids lived past the juvenile adult stage, even though we can routinely raise nonhybrid individuals past this stage.

The evolution of bindin in *Heliocidaris* is characterized by positive selection in the hotspot region, low intraspecific variability, and a burst of selection on the branch leading to *H. erythrogramma*. The evidence for positive selection in the hotspot region is due to the combination of an increased rate of nonsynonymous substitution and a decreased rate of synonymous substitution relative to the rest of the molecule. A similar pattern is seen in the bindins of *Echinometra* and *Strongylocentrotus*, where there is evidence of positive selection, and in *Tripneustes*, where there is not (Metz and Palumbi 1996; Biermann 1998; Zigler and Lessios 2003a). The cause of this pattern is unclear. There is no evidence of codon usage bias in the bindins of any of these genera, so it is unlikely that indirect selection on synonymous substitutions is contributing to this effect (Zigler and Lessios 2003a,b).

Three hypotheses could explain the observation of adaptive evolution of bindin along the branch leading to *H. erythrogramma*: (1) a correlated response to the evolution of direct development in *H. erythrogramma;* (2) an intraspecific process acting within *H. erythrogramma* but not *H. tuberculata;* and (3) selection against hybridization (reinforcement).

That adaptive evolution of bindin has occurred along the *H. erythrogramma* lineage suggests that it may be correlated with the evolution of direct development. The associated changes in gametogenesis leading to large egg and large sperm may have required correlated changes in *H. erythrogramma* bindin. Although bindin is the first molecule found to be under episodic selection along a lineage where direct development has evolved, it is undoubtedly just one of many proteins that have changed rapidly in concert with this developmental transition. There is, however, no obvious connection between the evolution of a gamete recognition protein and the mode of development. Determining whether the selection on bindin is a result of the shift to direct development will require examining how bindin has evolved along other lineages where direct development has arisen.

A second hypothesis is that some intraspecific process that promotes evolution under positive selection is occurring in *H. erythrogramma* but not *H. tuberculata*. Palumbi (1999) suggested that an intraspecific process such as male heterozygote advantage, nontransitive female preferences, or interlocus conflict evolution (Rice and Holland 1997) may explain the high intraspecific diversity observed in *Echinometra*. Given the low intraspecific diversity in *Heliocidaris*, male heterozygote advantage or nontransitive female preferences are unlikely to apply, but interlocus conflict evolution could still account for the selection on *H. erythrogramma* bindin. It is not clear, however, why this sort of selection should be present in one, but not the other, species.

A third potential explanation for the selection on *Heliocidaris* bindin is the avoidance of maladaptive hybridization (Dobzhansky 1940). The evolution of bindin has been studied in five genera of sea urchins. Selection on bindin has been reported in *Echinometra* (Metz and Palumbi 1996) and *Strongylocentrotus* (Biermann 1998), which have multiple sympatric species, and now *Heliocidaris*, whose two species have partially overlapping ranges. No positive selection has been observed in the bindins of *Arbacia* (Metz et al. 1998) or *Tripneustes* (Zigler and Lessios 2003a), in which extant species are all allopatric. The observed positive selection on bindin of *H. erythrogramma* may track changes in the bindin receptors of the egg necessary to prevent fertilization by sperm of *H. tuberculata*. Consistent with this idea, we observed stronger gametic incompatibility between *H. erythrogramma* eggs and *H. tuberculata* sperm than in the reciprocal cross. *H. erythrogramma* females produce far fewer large eggs than *H. tuberculata*, so each egg lost to hybridization is more costly to a female of *H. erythrogramma* than to a female of *H. tuberculata*. The low intraspecific diversity of bindin in *Heliocidaris* is also consistent with directional selection caused by reinforcement. This is a pattern different than that of *Echinometra* or *Strongylocentrotus*, in which intraspecific variability in bindin is high and in which there are high d_N/d_S ratios between alleles of the same species, which is not consistent with expectations of reinforcement (Zigler and Lessios 2003a).

The partially overlapping geographic distributions of the *Heliocidaris* species permit a test of the importance of reinforcement in the evolution of bindin (Noor 1997). Their ranges overlap on the southeast Australian coast, but *H. erythrogramma* is spread along the south coast to southwest Australia, whereas *H. tuberculata* is distributed up the east coast of Australia and off northern New Zealand (Mortensen 1943). There is extensive differentiation between local populations of *H. erythrogramma*, presumably due to the limited dispersal abilities of their direct-developing larvae (McMillan et al. 1992). If the selection on *H. erythrogramma* bindin arises from the need to avoid cross-fertilization, and if gene flow is sufficiently restricted to keep conspecific populations from becoming homogeneous, there should be fewer changes in *H. erythrogramma* bindins in west and south Australia, where they do not have to contend with *H. tuberculata*. Fertilization assays should also show gametes of *H. erythrogramma* from south and west Australia to be more compatible with gametes from *H. tuberculata* than those of *H. erythrogramma* from eastern Australia. The two alternative hypotheses (shift in developmental mode and interlocus conflict evolution) should operate regardless of patterns of allopatry

or sympatry. Thus, there is a need to study bindin in *H. erythrogramma* populations from south and west Australia, particularly populations of the subspecies *H. erythrogramma armigera*, which has presumably remained free of gene flow from the area of overlap for one million years (McMillan et al. 1992).

Acknowledgments

We thank H. Pedersen and K. Wilson for collecting tissue samples of *Heliocidaris* and A. Calderón, L. Calderón, and E. Archie for assistance in the laboratory. The manuscript was improved by comments from R. Collin, C. Cunningham, D. McClay, G. Wray, an anonymous reviewer, and the associate editor. This work was supported by National Science Foundation and Smithsonian predoctoral fellowships to KSZ and the Duke University Department of Zoology, the Smithsonian Molecular Evolution Program, and National Science Foundation and National Institutes of Health grants to RAR.

Literature Cited

Bermingham, E., and H. A. Lessios. 1993. Rate variation of protein and mitochondrial DNA evolution as revealed by sea urchins separated by the Isthmus of Panama. Proc. Nat. Acad. Sci. USA 90:2734–2738.

Biermann, C. H. 1998. The molecular evolution of sperm bindin in six species of sea urchins (Echinoida: Strongylocentrotidae). Mol. Biol. Evol. 15(12):1761–1771.

Byrne, M., J. T. Villinski, P. Cisternas, E. Popodi, and R. A. Raff. 1999. Maternal factors and the evolution of developmental mode: evolution of oogenesis in *Heliocidaris erythrogramma*. Dev. Genes Evol. 209:275–283.

Coates, A. G., and J. A. Obando. 1996. The geologic evolution of the Central American Isthmus. Pp. 21–56 *in* J. B. C. Jackson, A. G. Coates, and A. Budd, eds. Evolution and environment in tropical America. Univ. of Chicago Press, Chicago.

Dobzhansky, T. 1940. Speciation as a stage of evolutionary divergence. Am. Nat. 74:312–321.

Eckelbarger, K. J., C. M. Young, and J. L. Cameron. 1989. Modified sperm in echinoderms from the bathyal and abyssal zones in the deep sea. Pp. 67–74 *in* J. S. Ryland and P. A. Tyler, eds. Reproduction, genetics and distributions of marine organisms. Olsen and Olsen, Fredensborg, Denmark.

Emlet, R. B. 1990. World patterns of developmental mode in echinoid echinoderms. Pp. 329–335 *in* M. Hoshi and O. Yamashita, eds. Advances in invertebrate reproduction. Vol. 5, Elsevier, New York.

———. 1995. Larval spicules, cilia, and symmetry as remnants of indirect development in the direct developing sea urchin *Heliocidaris erythrogramma*. Dev. Biol. 167:405–415.

Felsenstein, J. 1981. Evolutionary trees and DNA sequences: a maximum likelihood approach. J. Mol. Evol. 17:368–376.

Haag, E. H., B. J. Sly, and R. A. Raff. 1999. Apextrin, a novel extracellular protein involved in adaptive evolution of larval ectoderm in the direct-developing sea urchin *Heliocidaris erythrogramma*. Dev. Biol. 211:77–87.

Henry, J. J., and R. A. Raff. 1990. Evolutionary change in the process of dorsoventral axis determination in the direct developing sea urchin, *Heliocidaris erythrogramma*. Dev. Biol. 141: 55–69.

Henry, J. J., G. A. Wray, and R. A. Raff. 1991. Mechanism of an alternate type of echinoderm blastula formation: the wrinkled blastula of the sea urchin *Heliocidaris erythrogramma*. Dev. Growth Differ. 33:317–328.

Hoegh-Guldberg, O., and R. B. Emlet. 1997. Energy use during the development of a lecithotrophic and a planktonic echinoid. Biol. Bull. 192:27–40.

Keesing, J. K. 2001. The ecology of *Heliocidaris erythrogramma*. Pp. 261–270 *in* J. M. Lawrence, ed. Edible sea urchins: biology and ecology. Elsevier, Amsterdam.

Kimura, M. 1980. A simple method for estimating evolutionary rates of base substitution through comparative studies of nucleotide sequences. J. Mol. Evol. 16:111–120.

Kumar, S., K. Tamura, I. B. Jakobsen, and M. Nei. 2001. MEGA 2: molecular evolutionary genetics analysis software. Bioinformatics 17(12):1244–1245.

Laegdsgaard, P., M. Byrne, and D. T. Anderson. 1991. Reproduction of sympatric populations of *Heliocidaris erythrogramma* and *H. tuberculata* (Echinoidea) in New South Wales. Mar. Biol. 110: 359–374.

Lessios, H. A., B. D. Kessing, G. M. Wellington, and A. Graybeal. 1996. Indo-Pacific echinoids in the tropical east Pacific. Coral Reefs 15:133–142.

Lessios, H. A., B. D. Kessing, and J. S. Pearse. 2001. Population structure and speciation in tropical seas: phylogeography of the sea urchin *Diadema*. Evolution 55(5):955–975.

Li, W.-H. 1993. Unbiased estimation of the rates of synonymous and nonsynonymous substitution. J. Mol. Evol. 36:96–99.

McCartney, M. A., G. Keller, and H. A. Lessios. 2000. Dispersal barriers in tropical oceans and speciation in Atlantic and eastern Pacific sea urchins of the genus *Echinometra*. Mol. Ecol. 9: 1391–1400.

McDonald, J. H., and M. Kreitman. 1991. Adaptive protein evolution at the Adh locus in *Drosophila*. Nature 351:652–654.

McMillan, W. O., R. A. Raff, and S. R. Palumbi. 1992. Population genetic consequences of developmental evolution in sea urchins (genus *Heliocidaris*). Evolution 46(5):1299–1312.

Metz, E. C., and S. R. Palumbi. 1996. Positive selection and sequence rearrangements generate extensive polymorphism in the gamete recognition protein bindin. Mol. Biol. Evol. 13(2): 397–406.

Metz, E. C., G. Gomez-Gutierez, and V. D. Vacquier. 1998. Mitochondrial DNA and bindin gene sequence evolution among allopatric species of the sea urchin genus *Arbacia*. Mol. Biol. Evol. 15(2):185–195.

Mortensen, T. 1943. A monograph of the Echinoidea v. III.3 Camarodonta II. Echinidae, Strongylocentrotidae, Parasaleniidae, Echinometridae. C. A. Reitzel, Copenhagen.

Nei, M., and T. Gojobori. 1986. Simple methods for estimating the numbers of synonymous and nonsynonymous nucleotide substitutions. Mol. Biol. Evol. 3(5):418–426.

Noor, M. A. F. 1997. How often does sympatry affect sexual isolation in *Drosophila*? Am. Nat. 149(6):1156–1163.

Palumbi, S. R. 1999. All males are not created equal: fertility differences depend on gamete recognition polymorphisms in sea urchins. Proc. Nat. Acad. Sci. USA 96(22):12632–12637.

Palumbi, S. R., G. Grabowsky, T. Duda, L. Geyer, and N. Tachino. 1997. Speciation and population genetic structure in tropical Pacific sea urchins. Evolution 51(5):1506–1517.

Pamilo, P., and N. O. Bianchi. 1993. Evolution of the Zfx and Zfy genes: rates and interdependence between the genes. Mol. Biol. Evol. 10:271–281.

Posada, D., and K. A. Crandall. 1998. Modeltest: testing the model of DNA substitution. Bioinformatics 14(9):817–818.

Raff, E. C., E. M. Popodi, B. J. Sly, F. R. Turner, J. T. Villinski, and R. A. Raff. 1999. A novel ontogenetic pathway in hybrid embryos between species with different modes of development. Development 126:1937–1945.

Raff, R. A. 1987. Constraint, flexibility, and phylogenetic history in the evolution of direct development in sea urchins. Dev. Biol. 119:6–19.

Raff, R. A., and B. J. Sly. 2000. Modularity and dissociation in the evolution of gene expression territories in development. Evol. Dev. 2:102–113.

Raff, R. A., L. Herlands, V. B. Morris, and J. Healy. 1990. Evolutionary modification of echinoid sperm correlates with developmental mode. Dev. Growth. Differ. 32(3):283–291.

Rice, W. R., and B. Holland. 1997. The enemies within: intergenomic conflict, interlocus contest evolution (ICE) and the intraspecific red queen. Behav. Ecol. Sociobol. 41:1–10.

Scott, L. B., W. J. Lennarz, R. A. Raff, and G. A. Wray. 1990. The

''lecithotrophic'' sea urchin *Heliocidaris erythrogramma* lacks typical yolk platelets and yolk glycoproteins. Dev. Biol. 138: 188–193.
Smith, A. B. 1988. Phylogenetic relationship, divergence times, and rates of molecular evolution for Camarodont sea urchins. Mol. Biol. Evol. 5(4):345–365.
Smith, M. J., J. D. G. Boom, and R. A. Raff. 1990. Single copy DNA distance between two congeneric sea urchin species exhibiting radically different modes of development. Mol. Biol. Evol. 7(4):315–326.
Strathmann, R. R. 1978. The evolution and loss of feeding larval stages of marine invertebrates. Evolution 32:894–906.
Swofford, D. L. 2001. PAUP*: phylogenetic analysis using parsimony (* and other methods). Ver. 4. Sinauer Associates, Sunderland, MA.
Tamura, K., and M. Nei. 1993. Estimating the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol. Biol. Evol. 10:512–526.
Ulrich, A. S., M. Otter, C. G. Glabe, and D. Hoekstra. 1998. Membrane fusion is induced by a distinct peptide sequence of the sea urchin fertilization protein bindin. J. Biol. Chem. 273(27): 16748–16755.
Vacquier, V. D. 1998. Evolution of gamete recognition proteins. Science 281:1995–1998.
Vacquier, V. D., and G. W. Moy. 1977. Isolation of bindin: the protein responsible for adhesion of sperm to sea urchin eggs. Proc. Nat. Acad. Sci. USA 74(6):2456–2460.
Vacquier, V. D., W. J. Swanson, and M. E. Hellberg. 1995. What have we learned about sea urchin sperm bindin? Develop. Growth Differ. 37:1–10.
Villinski, J. T., J. C. Villinski, M. Byrne, and R. A. Raff. 2002. Convergent maternal provisioning and life-history evolution in echinoderms. Evolution 56(9):1764–1775.
Wray, G. A. 1996. Parallel evolution of nonfeeding larvae in echinoids. Syst. Biol. 45(3):308–322.
Wray, G. A., and R. A. Raff. 1989. Evolutionary modification of cell lineage in the direct-developing sea urchin *Heliocidaris erythrogramma*. Dev. Biol. 132:458–470.
Yang, Z. 1998. Likelihood ratio tests for detecting positive selection and application to primate lysozyme evolution. Mol. Biol. Evol. 15(5):568–573.
———. 2000. Phylogenetic analysis by maximum likelihood (PAML). Ver. 3.0. Univ. College London, London.
Yang, Z., and J. P. Bielawski. 2000. Statistical methods for detecting molecular adaptation. Trends in Ecol. Evol. 15(12):496–503.
Zhang, J., S. Kumar, and M. Nei. 1997. Small-sample tests of episodic evolution: a case study of primate lysozymes. Mol. Biol. Evol. 14(12):1335–1338.
Zigler, K. S., and H. A. Lessios. 2003a. Evolution of bindin in the pantropical sea urchin *Tripneustes*: comparisons to bindin of other genera. Mol. Biol. Evol. 20(2):220–231.
———. 2003b. 250 million years of bindin evolution. Biol. Bull. 205:8–15.

Corresponding Editor: G. Wallis