Adaptive Evolution of Sperm Bindin Tracks Egg Incompatibility in Neotropical Sea Urchins of the Genus *Echinometra*

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Bindin is a gamete recognition protein known to control species-specific sperm-egg adhesion and membrane fusion in sea urchins. Previous analyses have shown that diversifying selection on bindin amino acid sequence is found when gametically incompatible species are compared, but not when species are compatible. The present study analyzes bindin polymorphism and divergence in the three closely related species of *Echinometra* in Central America: *E. lucunter* and *E. viridis* from the Caribbean, and *E. vanbrunti* from the eastern Pacific. The eggs of *E. lucunter* have evolved a strong block to fertilization by sperm of its neotropical congeners, whereas those of the other two species have not. As in the Indo-West Pacific (IWP) *Echinometra*, the neotropical species show high intraspecific bindin polymorphism in the same gene regions as in the IWP species. Maximum likelihood analysis shows that many of the polymorphic codon sites are under mild positive selection. Of the fixed amino acid replacements, most have accumulated along the bindin lineage of *E. lucunter*. We analyzed the data with maximum likelihood models of variation in positive selection across lineages and codon sites, and with models that consider sites and lineages simultaneously. Our results show that positive selection is concentrated along the *E. lucunter* bindin lineage, and that codon sites with amino acid replacements fixed in this species show by far the highest signal of positive selection. Lineage-specific positive selection paralleling egg incompatibility provides support that adaptive evolution of sperm proteins acts to maintain recognition of bindin by changing egg receptors. Because both egg incompatibility and bindin divergence are greater between allopatric species than between sympatric species, the hypothesis of selection against hybridization (reinforcement) cannot explain why adaptive evolution has been confined to a single lineage in the American *Echinometra*. Instead, processes acting to varying degrees within species (e.g., sperm competition, sexual selection, and sexual conflict) are more promising explanations for lineage-specific positive selection on bindin.

Introduction

Proteins involved in sexual reproduction often show rapid evolutionary divergence promoted by selection for amino acid sequence diversification (reviewed in Swanson and Vacquier 2002). Of these, gamete recognition proteins—those that control binding and fusion between gametes—are of particular interest because their evolution may contribute to the emergence of reproductive isolation between broadcast spawning marine species. High dispersal of larvae allowing genetic exchange between huge populations that may range over ocean basins, and absence of geographic barriers within basins, are factors thought to slow genomic divergence and to retard marine speciation (Palumbi 1992, 1994). Hence the divergence of a few critical genes mediating gamete recognition may facilitate the evolution of reproductive isolation between marine taxa.

Evidence for an important role of divergence of gamete recognition proteins in reproductive isolation exists for some marine invertebrates. In abalone and in teguline gastropods, adaptive evolution by positive selection promotes rapid divergence of the protein lysin (Lee and Vacquier 1992; Lee, Ota, and Vacquier 1995; Swanson and Vacquier 1997; Hellberg and Vacquier 1999), which dissolves glycoprotein vitelline envelopes to permit fertilization (Lewis, Talbot, and Vacquier 1982; Vacquier, Carner, and Stout 1990). In all cross-species combinations of gastropods so far examined, vitelline envelope dissolution is much reduced compared to within-species controls (Vacquier, Carner, and Stout 1990; Shaw et al. 1994; Hellberg and Vacquier 1999). Positive selection in lysin appears to be acting over the entire length of the protein (Lee, Ota, and Vacquier 1995; Swanson and Vacquier 1997; Hellberg and Vacquier 1999).

In sea urchins, adhesion of sea urchin sperm to eggs and fusion between sperm and egg membranes is controlled by the sperm protein bindin (Vacquier and Moy 1977; Moy and Vacquier 1979; Metz et al. 1994; Ulrich et al. 1998). Rapid adaptive divergence of bindin is observed, but in not all cases. Indo-West Pacific (IWP) species of *Echinometra* (Metz and Palumbi 1996) and species of *Strongylocentrotus* from temperate seas (Biermann 1998) show high bindin sequence divergence within and between species. In a third sea urchin genus, *Heliocidaris*, there is high bindin divergence proceeding under strong selection between species, but low intraspecific polymorphism (Zigler et al. 2003). In contrast to lysin, positive selection in bindin appears to be operating on specific regions of the molecule (putative recognition domains). In all three genera, there are strong barriers to cross-fertilization in species combinations that have been studied (Biermann 1998; Metz and Palumbi 1996; Zigler et al. 2003). In two other sea urchin genera, *Arbacia* (Metz, Gomez-Gutierrez, and Vacquier 1998) and *Tripneustes* (Zigler and Lessios 2003), bindin divergence between species and polymorphism within species are much lower, and selection on amino acid sequences is purifying rather than diversifying. In the single interspecific *Arbacia* combination tested thus far, *A. inscisa* and

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Abbreviations: IWP, Indo-West Pacific; PAML, Phylogenetic Analysis by Maximum Likelihood; ML, maximum likelihood; LRT, likelihood ratio test.

Key words: gamete recognition, sea urchin, bindin, speciation, *Echinometra*.

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Mol. Biol. Evol. 21(4):732–745. 2004
DOI:10.1093/molbev/msh071
Advance Access publication February 12, 2004
Molecular Biology and Evolution vol. 21 no. 4
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A. punctulata are gametically compatible (Metz, Gomez-Gutierrez, and Vacquier 1998).

Thus, high polymorphism and interspecific divergence promoted by positive selection characterizes sea urchin bindin evolution in roughly half the genera so far examined. This suggests two questions: (1) Is bindin divergence correlated with the strength of fertilization barriers? So far, most correlations have been made across genera, and only the recent study of Geyer and Palumbi (2003) has addressed the relationship between gametic incompatibility and bindin divergence in pairwise combinations of species of the same genus. (2) What specific factors promote divergence in bindin in concert with incompatibility between gametes? Metz, Gomez-Gutierrez, and Vacquier (1998) and Zigler and Lessios (2003*a*) suggest that, when genera are compared, diversification of bindin may be correlated with degree of geographic overlap between species. Genera with sympatric species show greater bindin divergence than genera in which all species are allopatric. According to Zigler and Lessios, this pattern is less likely to be the product of selection to avoid hybridization (reinforcement), and more likely to be a secondary effect of the ability of species with incompatible gametes to persist in sympatry. Clearly evidence from comparisons across genera that do and do not contain allopatric species needs to be augmented by studies of congeneric species that have and have not evolved in sympatry with each other.

The three neotropical species of *Echinometra* offer an opportunity to characterize bindin evolution within a genus containing species with different degrees of gametic compatibility and different patterns of geographic overlap. This genus contains one species, *E. vanbrunti*, in the eastern Pacific and two species, *E. lucunter* and *E. viridis*, in the Caribbean. Studies of in vitro fertilization (Lessios and Cunningham 1990; McCartney and Lessios 2002) have demonstrated a pronounced asymmetry in gamete compatibility of one species towards the other two. All heterospecific crosses involving *E. lucunter* eggs showed a much lower rate of fertilization compared to homospecific controls. In contrast, fertilization in both of the reciprocal crosses between eggs of the other two species and *E. lucunter* sperm, and in all *E. viridis* × *E. vanbrunti* crosses, is much closer to values seen in homospecific crosses. If the evolution of sperm bindin tracks evolution in egg bindin receptors, then bindin in *E. lucunter*, the species with incompatible eggs, should show a signal of diversifying selection confined to this lineage.

The phylogeny of these species and the timing of their speciation relative to the closure of the Isthmus have been studied through comparisons of isozymes (Lessios 1979, 1981; Bermingham and Lessios 1983), morphology (Lessios 1981) and mitochondrial DNA (Bermingham and Lessios 1993; McCartney, Keller, and Lessios 2000). Hence, we can determine the relationships between bindin divergence and time since separation, geographic distribution, and gametic compatibility.

Here we report that, like their Indo-West Pacific congeners (Metz and Palumbi 1996; Landry et al. 2003), the neotropical species of *Echinometra* show extensive bindin polymorphism resulting from amino acid replacements and from length variation in repetitive regions. We also demonstrate a striking amount of interspecific amino acid sequence evolution concentrated along the lineage leading to one species, *E. lucunter*. By testing maximum likelihood (ML) models of variable ratios of nonsynonymous to synonymous nucleotide substitution, among lineages and across codon sites, we identify residues evolving under positive selection and confirm that selection has been almost entirely confined to the *E. lucunter* lineage.

Materials and Methods

cDNA Library Construction and Screening

We isolated our first full-length bindin sequence by screening an *E. lucunter* cDNA library. One gram of testis tissue was pulverized in liquid N_2. RNA was extracted using the Poly(A) Pure kit (Ambion, Austin Tex.), and twice selected over the oligo d(T) resin provided in the kit. First and second cDNA strand synthesis, cDNA size selection, and ligation into the lambda Uni-ZAP XR vector followed the manufacturer's protocol (Stratagene, La Jolla Calif.). A total of 5.71×10^6 primary clones were pooled, and they were not amplified prior to being plated for screening.

As a probe for bindin, we first obtained the 3′ end of the *E. lucunter* mature protein (including the 3′ portion of the highly conserved core region [Vacquier, Swanson, and Hellberg 1995]) using the 3′ rapid amplification of cDNA ends procedure (RACE: [Frohman, Dush, and Martin 1988]), modified as follows: First-strand cDNA synthesis from 1 μg *E. lucunter* testis total RNA used 2.5 pmol of the oligo d (T) adapter-primer designed by Frohman, Dush, and Martin (1988). Template plus primer were heated to 70°C for 10 min, then placed on ice. We then added 200 units Superscript II Reverse Transcriptase and 1 μl RNase inhibitor (Life Technologies), plus the following components at the given final concentration: 1× 1st strand buffer, 10 mM DTT, and 0.5 mM of each dNTP. The reactants were incubated at 42°C for 50 min; then the reaction was stopped by heating at 70°C for 15 min.

Second strand synthesis and first round RACE polymerase chain reaction (PCR) used the 3′ OUT2 primer (5′ GATCCGTCGACATCGATACG 3′) paired with a degenerate primer (MB 1130+: 5′ TGCT-SGGTGCSACSAAGATTGA 3′), designed by M.A.M. to anneal to the bindin conserved core. Second round RACE paired the "internal" 3′ primer IN2 (5′ CGATAA-TACGACTCACTATAGG 3′) with primer EL 1207+ (5′ AGGCATCACTCCAATCTCCTGGC 3′). The multiple bands obtained on agarose gels were cloned into pMOS T-vector (Life Technologies) and sequenced. The inferred amino acid sequence of a portion of one clone was very similar to the highly conserved core region of published bindin cDNAs. This clone was grown, and its plasmid DNA was prepared using Wizard kits (Promega, Madison Wis.). The bindin RACE product was excised by digestion with *EcoR*I and *Pst*I, then radiolabeled with a random-prime labeling kit (Boehringer) to a specific activity of $\sim 1 \times 10^9$ cpm/μg DNA and used as a probe for screening the library.

Approximately 400,000 clones from the lambda ZAP library were plated. Plaque lifts and hybridization screening followed standard methods (Sambrook, Fritsch, and Maniatis 1989). One positive clone (pBS B231) was confirmed through sequencing to correspond to a full length bindin cDNA of approximately 1.9 kb.

Isolation of Bindin Alleles

Reproductively ripe individuals of *Echinometra vanbrunti* were collected from Isla Taboguilla off the Pacific coast of Panamá. *E. lucunter* and *E. viridis* were collected from the Caribbean coast of Panamá, some from near Portobelo and others from the San Blas Islands, Panamá. Gonad tissue was placed in liquid nitrogen, and then transferred to 80°C for long-term storage.

Multiple bindin alleles from each of the three *Echinometra* species were obtained either by reverse-transcriptase PCR (RT-PCR) from RNA, or through PCR from genomic DNA. Primers for RT-PCR were designed (from pBS B231) to amplify the full-length bindin, including the preprobindin region. PCRs of genomic DNA were designed to amplify only the mature bindin, including its intron.

For RT-PCR, we used total RNA as template. RNA was extracted from frozen testis of several males of each of the three species using the TotallyRNA kit (Ambion). First strand cDNA synthesis followed the protocol used for RACE above, except that 3PRTA- (5′-CTGCAACC-CAAGGAAATCAATTC 3′), a primer that anneals to the bindin 3′ untranslated region, was used to prime synthesis, and that the first strand reaction was incubated for 2 h. One μl of cDNA product was used in each 50 μl PCR reaction containing 1× PCR buffer (PerkinElmer/Applied Biosystems), 2 mM of each dNTP, 1.5 mM $MgCl_2$, 0.5 μM primer PPB A+ (5′-AGYATCATGGGTKTCCAYCAAAT), 0.5 μM primer 3PRTB- (5′GGTGGTATCTCTCTATTTYTACCC), and 0.5 μl AmpliTaq (PerkinElmer/Applied Biosystems). Thermocycling conditions were 95°C for 1 min, followed by 40 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 3 min, followed by 72°C for 10 min. Products were cloned into pMOS T-vector or blunt-end vector. Lysates of positive clones were PCR-amplified using T7 and U19 vector primers to obtain insert DNAs. The products were separated on agarose gels, and those containing the expected insert size (~1.8 kb) were cleaned over QIAquick PCR Purification columns (QIAGEN), then sequenced using either FS or d-Rhodamine Ready Reaction Dye Terminator cycle sequencing kits (Perkin-Elmer/Applied Biosystems).

Genomic DNA extractions were obtained by "back-extraction" of RNA extracts (Ambion: http://www.ambion.com/techlib/tb/tb_161.html). The PCR cocktails were made as described above, substituting primers BGENF2 (5′ AACTACCCCCAAGCCATGAATC 3′) and BGENR2 (5′ TTTCTACCCTTGCCGATAACCC 3′) to amplify the mature bindin. For genomic amplifications, "hot start" PCR was carried out as follows: Template, primers, and a drop of "hot start" wax were combined, centrifuged, then chilled to solidify the wax. The remaining PCR reagents were then added. Tubes were thermocycled under the following conditions: 95°C for 2 min, followed by 40 cycles of 94°C for 30 s, 52°C for 45 s, and 72°C for 3 min, followed by 72°C for 10 min. Products were cloned into T-vector or blunt-end vector, then inserts were recovered, purified, and sequenced as above. We obtained 34 mature bindin alleles from *E. lucunter* (6 from cDNA, 28 from genomic DNA), 31 alleles from *E. viridis* (13 from cDNA, 18 from genomic DNA), and 17 alleles from *E. vanbrunti* (11 from cDNA, 6 from genomic DNA) (Supplementary Data available online at www.mbe.oupjournals.org); GenBank Accession numbers AY451242–AY451323). The sequences of 13 full-length bindin alleles from the IWP species of *Echinometra* (Metz and Palumbi 1996) were obtained from GenBank to be used as outgroups in the analyses.

Initial Sequence Analysis

Raw electropherogram data were edited, and contigs were assembled using Sequencher 3.1 (Gene Codes Corp., Ann Arbor Mich.). Sequences were translated and rechecked for premature stop codons. In questionable cases cloned alleles were re-sequenced. A single allele cloned from each individual was chosen at random for sequencing. Initial sequencing of five clones from one sea urchin per species revealed an average of 1.5 base pair substitutions per mature bindin sequence. Some of these differences may be due to allelic differences, but if they all represent cloning and PCR artifacts, then there is a maximum error of 0.16%. In addition, 1 of the 15 clones contained a single-codon insertion that must have resulted from PCR error. The sequences were aligned using ClustalW (Thompson, Higgins, and Gibson 1994), and minor adjustments to the alignment were made manually.

Phylogenetic Analysis

The best-fit model of molecular evolution with the least variance was obtained by calculating hierarchical likelihood ratio tests (LRTs) with ModelTest 3.06 (Posada and Crandall 1998). ModelTest was run on a data set containing a haphazardly selected subset of alleles (three from each of the neotropical species, one from each of the IWP species), and the simplest model with significantly highest LRT was determined to be F81 (Felsenstein 1981) with a γ correction ($\alpha = 1.067$). Model parameters were entered into PAUP* 4.0b10 (Swofford 2002), which was used for all phylogenetic analyses. A Neighbor-Joining (NJ) tree was constructed using F81, with 1,000 Neighbor-Joining bootstrap replications used to assess support. Minimum evolution (ME) trees were also constructed based on F81. A ME heuristic search was run to completion. Maximum-parsimony (MP) trees were constructed using transection-branch reconstruction (TBR) branch-swapping and treating gaps as missing data. In a separate analysis, gaps were alternatively coded as "5th-state" characters to determine whether the numerous bindin insertion/deletion mutations (indels) were phylogenetically informative. Maximum likelihood (ML) trees were constructed as follows: to reduce computation time,

5 alleles from each of the neotropical species were haphazardly selected for analysis. The Neighbor-Joining tree with F81 distances was used as the starting tree for a ML heuristic search, with TBR branch swapping. This resulted in a single tree, which was next used as a starting tree for a second heuristic search, this time using nearest-neighbor interchange (NNI) branch swapping. One hundred bootstrap replications, obtained with the fast stepwise addition option in PAUP*, were used to ascertain support for nodes in ME, MP, and ML trees.

Analysis of Bindin Nucleotide Substitutions

Measures of bindin polymorphism and divergence and the McDonald-Kreitman (1991) test for adaptive divergence were calculated using DnaSP 3.0 (Rozas and Rozas 1999). Analyses of pairwise estimates of the ratio of nonsynonymous to synonymous nucleotide substitution (d_N/d_S) were performed according to the methods of Pamilo and Bianchi (1985) and Li (1993) as implemented in MEGA 2.1 (Kumar et al. 2001).

To obtain ML estimates of d_N/d_S on different branches of the bindin genealogy and at different codon positions, we applied codon-based models (Goldman and Yang 1994) using the program codeml contained in the PAML 3.0 package (Yang 2000). To reduce computing time, we used one haphazardly selected *E. oblonga* sequence (GenBank Accession Number U39503) as an outgroup. For this analysis, we generated a new alignment using ClustalW. The tree topology used in all models in PAML was obtained from the NJ search above. We also tested two alternative tree topologies—one with the branching order between species found for mtDNA (McCartney and Lessios 2002), which disagrees with the bindin tree (see below), and a second where the split between the three neotropical species was a tritomy. The two topologies produced very similar findings in the ML analysis. Hierarchical tests for d_N/d_S variation across lineages were performed as described in Yang (1998). Because our genealogy contains >150 branches, many of them short, the "free ratio" model (Yang 1998) was not attempted because of low power and poor precision from simultaneous estimation of parameters on a large number of branches (Yang 1998, 2000). We performed three analyses based on a "two ratio" model, in which one d_N/d_S ratio was estimated for the branch of the species being examined, and another for all other branches. Then we tested whether the likelihood of each two-ratio model was significantly higher than that of the one-ratio model, in which d_N/d_S was equal on all branches.

We applied a subset of Yang's (2000) models for surveying d_N/d_S variation across amino acid sites. These were the neutral model, the selection model, one discrete model with $k = 2$ and the other with $k = 3$ (where $k =$ the number of site classes, each defined by its value of $\omega = d_N/d_S$), the *beta* model and the (*beta* + ω) model (where a continuous distribution of ω values are modeled using the beta distribution). All models were evaluated using LRTs as described in Yang et al. (2000). We also used the more recently developed "branches-sites" models (Yang and Nielsen 2002) to simultaneously survey variation in selection across amino acid sites and across lineages. We tested each of the three neotropical species separately as a "foreground" lineage, using both the "Model A" and the "Model B" approach, as described in Yang and Nielsen (2002).

Results

Structure of Bindin in the Neotropical *Echinometra*

The cDNA clone pBS B231 contained a 5′ region of 254 inferred amino acid residues that showed strong similarity to other published preprobindins. The DDDVSKRAS motif near residue 175 (numbers refer to our preprobindin alignment, not shown) was identical to that in *Strongylocentrotus*, and the 15-codon block starting at residue 35 was very similar to the homologous region of moderate conservation across distantly related sea urchins (Vacquier, Swanson, and Hellberg 1995). A motif of basic amino acids, RQKR, followed the preprobindin. This is the presumed site, well conserved across echinoids (Zigler and Lessios 2003*b*), of proteolytic cleavage of the translated protein to release the mature bindin. The remainder of the clone contained 855 bp (285 codons)m clearly recognizable as a mature bindin sequence, followed by a 236-bp 3′ UTR (untranslated region). The sequence of the clone is deposited in GenBank (Accession Number AY452683).

In the three neotropical *Echinometra*, indels produced considerable length variation, both within and between species, which is more dramatic than the variation described in either *Strongylocentrotus* (Biermann 1998) or IWP *Echinometra* (Metz and Palumbi 1996), and much more dramatic than in *Arbacia* (Metz, Gomez-Gutierrez, and Vacquier 1998) or *Tripneustes* (Zigler and Lessios 2003*a*). The neotropical *Echinometra* bindins feature multiple tandem copies of a 10-residue glycine-rich repeat, most commonly of the motif AMA(G/R)PVGGGG, with substantial between-species and within-species variation in motif length and amino acid composition (Supplementary Data available online at www.mbe.oupjournals.org). The insertion of five copies of this repeat, arranged in tandem between codons 73 and 124 (in our alignment), which is absent in the IWP *Echinometra* (Supplementary Data), accounted for the greater length of bindin in the neotropical species (270 to 292 residues) than in the IWP *Echinometra* (236–252 residues: Metz and Palumbi [1996]).

The 64-residue core region (residues 167–230) of greatest amino acid conservation across genera (Vacquier, Swanson, and Hellberg 1995) lacked length variation and showed no fixed substitutions between any of the three species of neotropical *Echinometra*. Somewhat surprisingly, we found a few amino acid substitutions in the core to be segregating within species. Although some of these were present in only a single sequence and could therefore represent PCR error, there remained a few substitutions (e.g., positions 188 and 192) that we found in multiple individuals (Appendix). As in other bindins (Biermann 1998; Metz and Palumbi 1996), there was a region 3′ of the core that contained multiple repeats of codons for glycine. The number of repeats in this polyglycine region (residues 250–292) varied considerably both within and between species.

A bindin hypervariable domain was previously identified from the IWP species of *Echinometra* (Metz and Palumbi 1996). This region features a cluster of excess replacement substitutions segregating within IWP *Echinometra* species (Metz and Palumbi 1996), and excess nonsynonymous replacements between *Strongylocentrotus* sea urchins (Biermann 1998). The homologous region in the neotropical *Echinometra* contained 10 amino acid replacements fixed between species, 18 replacements polymorphic within species, and numerous indels, including the ~50-bp insertion described above. This region is clearly a hot spot for amino acid sequence variation in these species of *Echinometra* as well.

Bindin Polymorphism and Divergence

Echinometra lucunter displayed much less bindin polymorphism than the other two neotropical species. The 34 *E. lucunter* mature bindins (22 unique haplotypes) showed an average number of pairwise nucleotide differences (k) of 2.85. In comparison, k was 7.03 for the 31 *E. viridis* bindins (28 haplotypes), and 8.94 for the 17 *E. vanbrunti* bindins (17 haplotypes). *Echinometra lucunter* also showed dramatically reduced variation within most of the amino acid repeat arrays. For instance, repeats starting at residues 37, 52, 62, 73, 84, 251, and 284 were identical in all 34 *E. lucunter* alleles, but they varied in length and/or amino composition of each of the other two species.

Among the neotropical species of *Echinometra*, amino acid sequence divergence was greatest between *E. lucunter* and *E. vanbrunti*, followed by *E. lucunter*/*E. viridis*, then by *E. vanbrunti*/*E. viridis* (table 1). This matches the rank-order of gametic incompatibility between species (McCartney and Lessios 2002). *Echinometra viridis* bindin amino acid sequences were more similar to those of the IWP species than were the bindins of either of the other two neotropical species (table 1). The mtDNA phylogeny shows the neotropical *Echinometra* as a clade distinct from IWP species, with *E. vanbrunti* as having split from the other two neotropical species before they separated from each other (McCartney, Keller, and Lessios 2000). Thus it appears that there have been convergent amino acid substitutions in bindins of *E. viridis* and the IWP species.

Bindin Genealogies

A minimum evolution (ME) search of >50 million rearrangements produced 266 shortest trees containing the same major clades and only minor differences in terminal clades within the species. Figure 1 shows one of these trees. Maximum parsimony (MP) and maximum likelihood (ML) searches also produced trees in which major clades agreed. According to all three analyses, bindins from each of the neotropical species of *Echinometra* formed monophyletic groups. Alleles of this gamete-recognition protein have completely sorted within species boundaries. The bindin gene tree did not entirely agree with the mtDNA tree (McCartney, Keller, and Lessios 2000). While the mtDNA tree clearly unites *E. lucunter* as sister to its sympatric *E. viridis*, the bindin tree groups *E. lucunter* more closely with the allopatric *E. vanbrunti*. Bootstrap support for the node uniting *E. lucunter* with *E. vanbrunti*, however, was low (64%) on the ME bindin tree. Maximum likelihood and MP reconstructions yielded an *E. lucunter*/*E. vanbrunti* bindin clade, with higher bootstrap support (86% and 82%, respectively). Maximum-likelihood tests revealed no significant difference ($P = 0.149$ by 2-tailed test [Kishino and Hasegawa 1989]; $P = 0.087$ by one-tailed test [Shimodaira and Hasegawa 1999]) between the topology uniting bindins of *E. lucunter* and *E. vanbrunti* ($-ln$ L = 4018.92) and the topology uniting bindins of *E. lucunter* and *E. viridis* ($-ln$ L = 4026.14).

Table 1
Uncorrected Mean Percent Amino Acid Sequence Divergence between Bindins of Neotropical and IWP Species of *Echinometra*

	E. lucunter	*E. viridis*	*E. vanbrunti*	*E. mathaei*	*E. oblonga*	*E.* Type A
E. lucunter	0.72					
E. viridis	6.74	1.26				
E. vanbrunti	8.45	6.30	1.74			
E. mathaei	10.54	9.05	9.42	1.08		
E. oblonga	10.71	7.66	8.38	4.53	1.92	
E. type A	12.89	11.92	12.04	6.41	6.64	2.42

Either short internodes causing poor resolution of branching order or lineage-specific rates of bindin evolution (see below) could cause discrepancies between gene and species trees. If so, we might expect a phylogeny based on the intron of bindin to resemble the mtDNA tree. To test this prediction, we constructed a phylogeny based on 54 intron alleles, 30 from *E. lucunter*, 18 from *E. viridis*, and 6 from *E. vanbrunti*. We rooted this tree by using two intron sequences of *E. oblonga* (provided by E. Metz) as outgroups. Alignment of the intron sequences revealed many indels, as well as numerous point substitutions, both within and between species. A MP heuristic search was performed, ignoring the indels. The completed search of >3 million rearrangements identified 62 shortest trees, each of 178 steps. All of these trees united the bindin intron of *E. lucunter* with *E. viridis* in a clade exclusive of *E. vanbrunti*. Bootstrap support for this arrangement in the strict consensus tree was moderately high (~80%). Although support was lower for ME and ML reconstructions (the latter performed on data sets reduced as described for exons), all methods joined the introns of *E. lucunter* and *E. viridis* in the same clade. Thus, the genealogy of the bindin introns matches the mtDNA tree, which suggests that they estimate the species tree, and that the discordant topology of the bindin exon tree is caused by lineage-specific evolution in this molecule.

When parsimony was used to assign amino acid substitutions to the branches of the bindin exon tree, a striking result emerged (fig. 1, table 2). Twelve of the 20 amino acid replacements within the neotropical clade were found to have occurred along the *E. lucunter*, 6 along the *E. vanbrunti*, and only 2 along the *E. viridis* branch. The excess of amino acid substitutions along the *E. lucunter* branch differed greatly from expectations based on branch length (table 2). This excess remained when the bindin exon tree was constrained to the topology of the mtDNA

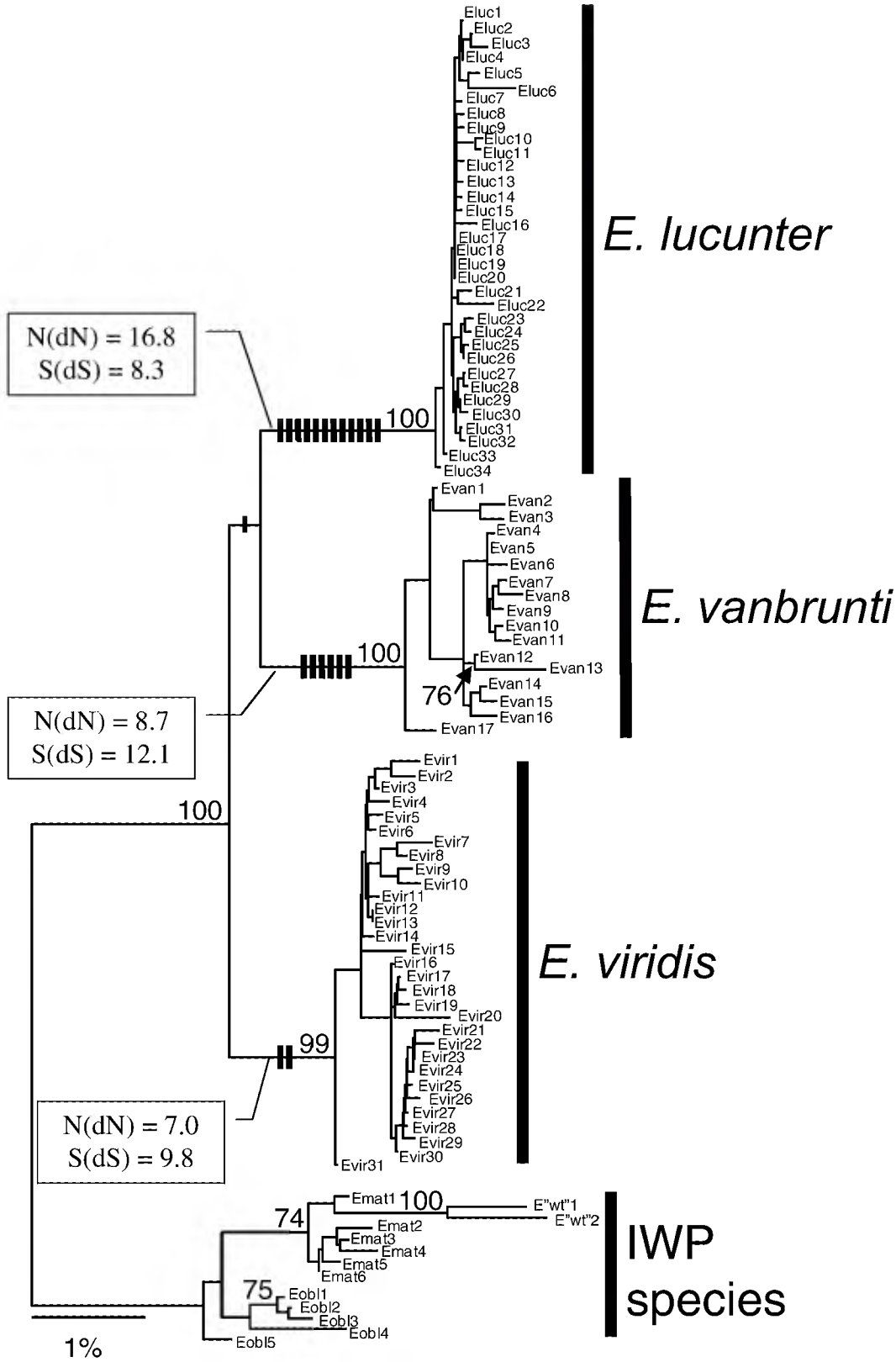

FIG. 1.—Genealogy of mature bindin alleles in neotropical *Echinometra*. The phylogeny shows one of 266 equal-length trees produced from a minimum-evolution search run to completion. Genetic distances are based on Felsenstein's (1981) model. Bootstrap support values (100 replications) >70% are shown. Numbers of nonsynonymous and synonymous nucleotide substitutions (N(d_N) and S(d_S), respectively) are taken from the best-fit two-ratio model for d_N/d_S variation across lineages (model B2, table 3). Each hatch mark represents one amino acid substitution fixed along branches as determined from parsimony. IWP: Indo-West Pacific species of *Echinometra* (data from Metz and Palumbi 1996).

Table 2
Proportion of Bindin Amino Acid Replacements Across Lineages

Lineage	Branch Length[a]	Proportional Branch Length	Expected No. Substitutions[b]	Observed No. Substitutions[c]	G_{adj}[d]	P
Bindin topology[e]						
E. lucunter	.016	.16	3.36	12	12.657	< 0.001
E. viridis	.033	.33	6.93	2		
E. vanbrunti	.042	.42	8.82	6		
internode	.009	.09	1.89	1		
mtDNA topology[e]						
E. lucunter	.024	.056	0.84	11	48.113	< 0.001
E. viridis	.086	.201	3.01	2		
E. vanbrunti	.162	.378	5.67	2		
internode	.156	.364	5.46	0		

[a] Branch lengths were estimated from the corresponding trees, using distances at third codon positions corrected using the appropriate model of molecular evolution.

[b] Expected number of substitutions is the product of the total number of bindin replacements and the proportional branch lengths.

[c] Observed number of substitutions along each branch was estimated from parsimony.

[d] G_{adj} = G-statistic, adjusted using the Williams correction (Sokal and Rohlf 1981).

[e] Bindin topology refers to the branching order on the bindin genealogy, mtDNA topology refers to the branching order inferred from the mitochondrial *cytochrome oxidase I* phylogeny (McCartney, Keller, and Lessios 2000).

(and bindin intron) trees, and when mtDNA divergence (instead of bindin divergence) was used to generate the expected numbers of bindin replacements across lineages (table 2). Of the 20 fixed substitutions, 11 do not conserve the class of amino acid (as defined by Dickerson and Geis [1983]), and 6 lead to a change in charge. Thus, contrary to most comparisons between orthologous proteins of closely related species, those between the bindins of the neotropical *Echinometra* show replacements of amino acids with different physical properties.

d_N/d_S Variation Among Lineages

We calculated the amino acid replacement rate (d_N) and silent substitution rate (d_S) first in pairwise comparisons between bindin alleles of the neotropical and IWP species (Li 1993; Pamilo and Bianchi 1993). All mean interspecific d_N/d_S values were considerably less than one, but those involving *E. lucunter* showed the highest ratios (not shown). Explicit ML tests for d_N/d_S ratio (ω) variation among branches on the bindin genealogy showed clear evidence of lineage-dependent selection. Model B2, in which ω along the *E. lucunter* lineage was permitted to differ from ω for all other clades, showed a highly significant increase in likelihood ratio ($2\Delta lnL = 16.384$, $P < 0.001$) over Model B1, in which ω was equal in all lineages. The estimate for ω along the *E. lucunter* lineage was 1.052, indicating relaxed selection compared to the *E. viridis* lineage ($\omega = 0.245$) and the *E. vanbrunti* lineage ($\omega = 0.283$) in both of which, purifying selection was indicated. The two-ratio model that tested ω in the *E. viridis* lineage against the other two was statistically indistinguishable from the "background" model of no lineage variation (Model B1), and the two-ratio model testing ω in the *E. vanbrunti* lineage actually showed stronger purifying selection along this branch than along background branches. We used the highest-likelihood model (Model B2) to estimate the number of replacement and silent substitutions along branches of the bindin genealogy. Only the *E. lucunter* lineage showed more replacements than silent substitutions (fig. 1).

d_N/d_S Variation Across Sites and Lineages

Variable selection across lineages is incompatible with a neutral model of molecular evolution, but it is not definitive proof for positive selection (Yang 1998). The estimated average ratio of near 1 for the entire gene can be the result of positive selection on some gene regions and negative selection on others. We therefore examined d_N/d_S variation in different regions of bindin and across branches of the genealogy simultaneously, using ML.

Yang and Nielsen's (2002) "branches-sites" models allow one to determine whether positive selection is operating on certain codon sites along specific lineages. We were interested in determining whether positive selection in bindin might be concentrated along the *E. lucunter* branch. Branch-site model A fixes two classes of codon sites, one with $\omega = 0$ and the second with $\omega = 1$, and estimates ω for a third class of sites along a "foreground" lineage. Positive selection is not allowed along background lineages (Yang and Nielsen 2002). The difference in likelihood of this model (B/S1 Model A, table 3) over the neutral sites-only model was highly significant (table 4); 3.8% of codon sites were estimated to be under very strong diversifying selection ($\hat{\omega}_2 = 27.8$) along the *E. lucunter* lineage (table 3). Eight positively selected sites were identified on the *E. lucunter* lineage (table 3). All of these sites contain fixed replacements in this species (Supplementary Data) and are thus likely to be involved in gamete recognition between *E. lucunter* and the other two species.

Branches-sites model B estimates ω along background lineages as well as along the foreground lineages, instead of fixing background values (Yang and Nielsen 2002). When we applied B/S Model B with *E. lucunter* as the foreground lineage, we found a highly significant increase in fit to the data in comparison to the appropriate sites-only model with two site classes (table 4). Like model

Table 3
Models of Variable d_N/d_S Across Codon Sites and Lineages Combined—Parameter Estimates and Log-Likelihoods

Model	P	Parameters	$-lnL$	Count of Positively Selected Sites
E. lucunter lineage				
B/S1 Model A	3	Background $\hat{p}_0 = 0.645$, $\hat{\omega}_0 = 0$ $\hat{p}_1 = 0.316$, $\hat{\omega}_1 = 1$	2947.08	Background —
		Foreground $(\hat{p}_2 + \hat{p}_3) = 0.038$, $\hat{\omega}_2 = 27.79$		Foreground 6, **1**, ***1***—
B/S1 Model B	5	Background $\hat{p}_0 = 0.698$, $\hat{\omega}_0 = 0.001$ $\hat{p}_1 = 0.264$, $\hat{\omega}_1 = 1.595$	2937.56	Background 4, **53**, ***1***
		Foreground $(\hat{p}_2 + \hat{p}_3) = 0.038$, $\hat{\omega}_2 = 28.32$		Foreground 6, **2**
E. viridis lineage				
B/S2 Model A	3	Background $\hat{p}_0 = 0.640$, $\hat{\omega}_0 = 0$ $\hat{p}_1 = 0.350$, $\hat{\omega}_1 = 1$	2947.91	Background —
		Foreground $(\hat{p}_2 + \hat{p}_3) = 0.01$, $\hat{\omega}_2 = 3.956$		Foreground None
B/S2 Model B	5	Background $\hat{p}_0 = 0.696$, $\hat{\omega}_0 = 0.001$ $\hat{p}_1 = 0.299$, $\hat{\omega}_1 = 1.568$	2944.32	Background 4, **61**, ***1***
		Foreground $(\hat{p}_2 + \hat{p}_3) = 0.005$, $\hat{\omega}_2 = 2.699$		Foreground None
E. vanbrunti lineage				
B/S3 Model A	3	Background $\hat{p}_0 = 0.627$, $\hat{\omega}_0 = 0$ $\hat{p}_1 = 0.333$, $\hat{\omega}_1 = 1$	2953.55	Background —
		Foreground $(\hat{p}_2 + \hat{p}_3) = 0.038$, $\hat{\omega}_2 = 2.574$		Foreground None
B/S3 Model B	5	Background $\hat{p}_0 = 0.701$, $\hat{\omega}_0 = 0.001$ $\hat{p}_1 = 0.293$, $\hat{\omega}_1 = 1.584$	2950.41	Background 4, **61**, ***1***
		Foreground $(\hat{p}_2 + \hat{p}_3) = 0.004$, $\hat{\omega}_2 = 69.96$		Foreground None

NOTE.—Foreground refers to lineage of interest; background, to all other lineages. Posterior probabilities for sites are as follows: normal text, $0.60 < P < 0.95$; bold text, $0.95 < P < 0.99$; bold and italics, $P \geq 0.99$.

A, branch-site model B suggested very strong positive selection ($\hat{\omega}_2 = 28.3$) on a small number (3.8%) of sites along the lineage leading to *E. lucunter* (table 3). Along background branches, 69.8% of codons showed strong purifying selection ($\hat{\omega}_0 = 0.001$), and 26.4% showed weak positive selection ($\hat{\omega}_1 = 1.59$). Like model A, Model B identified the same 8 *E. lucunter*-specific sites as evolving under positive selection. The remaining 58 positively selected sites identified by model B were the same as those detected in the sites-only models (not shown). In effect, branch-site model B partitioned positively selected sites that were also detected in the sites-only models into two categories—those on background branches under weak positive selection and those along the *E. lucunter* branch under strong positive selection.

The branches-sites models showed no evidence for lineage-specific positive selection in *E. viridis* and *E. vanbrunti*. Along the *E. viridis* branch, there was a hint of elevated levels of replacement substitution. BS/2 Model A was significantly better than the null model (table 4), and 1% of sites were predicted as having $\hat{\omega}_2 = 3.95$, but this signal was apparently too weak to identify a single positively selected site (table 3). Similarly, B/S2 Model B showed a significant improvement in fit over the null model (table 4), and it predicted 0.5% of sites with $\hat{\omega}_2 = 2.70$, but it did not identify any positively selected sites along the foreground lineage. Evidence for positively selected codons along the *E. vanbrunti* lineage was completely absent. Neither B/S3 Model A nor Model B fits the data significantly better than the null models of no positive selection (tables 3 and 4). Taken together, these analyses are conclusive evidence that strong positive selection has acted on a small number of sites in the bindin molecule of *E. lucunter*, but not of the other two species.

Plots of posterior probabilities for assignment of codon sites to classes of different ω values showed that the distribution of positively selected sites along the mature bindin sequence was not random (fig. 2). There was moderate clustering of positively selected sites in the hotspot domain (codons 62–153); 36 of the 67 (53%) positively selected sites occur in this region, even though it encompasses only about 30% of all codons. The conserved core (codons 167–234) showed mostly silent variation, with the exception of five polymorphic sites (170, 178, 188, 190, and 192) under mild positive selection. There is a strong correspondence between sites under positive selection and those that contain substitutions fixed between the species. In particular, the 12 sites with the strongest signal of positive selection are the same 12 sites that are fixed in *E. lucunter* (Fig. 2: marked with diamonds). These residues are well dispersed along the mature bindin.

In contrast to the maximum likelihood models, the McDonald-Kreitman (1991) test failed to demonstrate the action of positive selection for divergence between species. This is also the case in the IWP species of *Echinometra* (Metz and Palumbi 1996) and in *Heliocidaris* (Zigler et al. 2003). The comparison between *E. lucunter* and *E. viridis* yielded 12 fixed and 23 polymorphic silent substitutions, and 13 fixed and 26 polymorphic replacements ($P = 1.0$, 2-tailed Fisher's exact test). Between *E. lucunter* and *E. vanbrunti* there were 10 fixed and 27 polymorphic silent substitutions, and 18 fixed and 35 polymorphic replacements ($P = 0.500$), and between *E. viridis* and *E. vanbrunti* there were 12 fixed and 30 polymorphic silent substitutions, and 5 fixed and 27 polymorphic replacements ($P = 0.266$). Polymerase error may have inflated the polymorphic replacement class, leading to reduced power of this test. Moreover, considering evidence from the ML models that the action of selection on bindin is concentrated on a small number of sites, whereas the rest of the molecule is functionally constrained, the absence of significance in the McDonald-Kreitman test is not surprising.

Table 4
Likelihood Ratio Tests for Branch-Site Models

Models Compared	df	$2\Delta lnL$
E. lucunter lineage		
B-S1 (Model A) vs. S1 (neutral)	2	13.452**
B-S1 (Model B) vs. S3A (discrete, k = 2)	2	25.277***
E. viridis lineage		
B-S2 (Model A) vs. S1 (neutral)	2	11.802**
B-S2 (Model B) vs. S3A (discrete, k = 2)	2	11.752**
E. vanbrunti lineage		
B-S3 (Model A) vs. S1 (neutral)	2	0.508 ns
B-S3 (Model B) vs. S3A (discrete, k = 2)	2	> 0 ns

*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$l; ns = not significant.

Discussion

Our work on the neotropical species of *Echinometra* shows that, as expected from the mtDNA phylogeny (McCartney, Keller, and Lessios 2000), they display great bindin divergence from IWP species of the same genus. Yet the magnitude of polymorphism and divergence between species within each of these clades is comparable. In addition, regions of bindin in which substitutions and indels tend to be concentrated are similar in all *Echinometra*. The evolution of bindin of the neotropical species also resembles that of the IWP species in showing clear evidence that certain sites are under strong diversifying selection. Thus, our study contributes additional evidence that the mode of interspecific bindin evolution is characteristic for each genus, being rapid and selection-driven in some sea urchin genera (Metz and Palumbi 1996; Biermann 1998; Zigler et al. 2003) but slow in others (Metz, Gomez-Gutierrez, and Vacquier 1998, Zigler and Lessios 2003*a*). Findings specific to the neotropical species of *Echinometra* are that rapid divergence under positive selection is concentrated in the bindin of one of the three species, and that this is associated with the loss of receptivity of eggs to fertilization by heterospecific sperm. It is also noteworthy that the species in which bindin evolution has been the most rapid also has the lowest amount of intraspecific polymorphism, both in amino acid replacement variation and in indels. What can these findings reveal about the function of bindin in conferring prezygotic isolation between species?

Among the neotropical species of *Echinometra*, amino acid sequence divergence was greatest between *E. lucunter* and *E. vanbrunti*, followed by divergence between *E. lucunter* and *E. viridis*, then by divergence between *E. vanbrunti* and *E. viridis*. The rank order of bindin divergence between species is exactly paralleled by the degree to which the eggs of each species reject heterospecific sperm. McCartney and Lessios (2002) found that to fertilize 50% of *E. lucunter* eggs, it takes about 1,700 times more *E. vanbrunti* sperm and 60 times more *E. viridis* sperm than *E. lucunter* sperm. Eggs of *E. viridis* require only 18 times more sperm of *E. vanbrunti* than homospecific sperm, and eggs of *E. vanbrunti* can be fertilized by *E. viridis* sperm at the same rate that they are fertilized by their own. The reciprocal crosses, however, bear no relation to bindin divergence. The concentration of *E. lucunter* sperm needed to fertilize 50% of the eggs of the other two species is only twice as large as the concentration of homospecific sperm. Thus, the modifications of the *E. lucunter* bindin molecule have not rendered it greatly incompatible with the egg bindin receptors of the other two species. That the divergence of the male molecule is correlated with egg incompatibility suggests that bindin evolution must track changes in the bindin receptor. The source of positive selection on bindin sites of *E. lucunter* must be related to the need for recognition and acceptance by the egg bindin receptors. Some (or all) of the 12 amino acid changes fixed in *E. lucunter* must contribute to the signal necessary for recognition by homospecific eggs. Any *E. lucunter* bindin allele that does not carry this signal is at a disadvantage as extreme as that of a heterospecific allele.

The sea urchin egg receptor for bindin has recently been cloned in two species of *Strongylocentrotus* (Kamei and Glabe 2003). Because this molecule is large and complex, understanding its evolution will be a formidable challenge for future research. Until variation in the egg

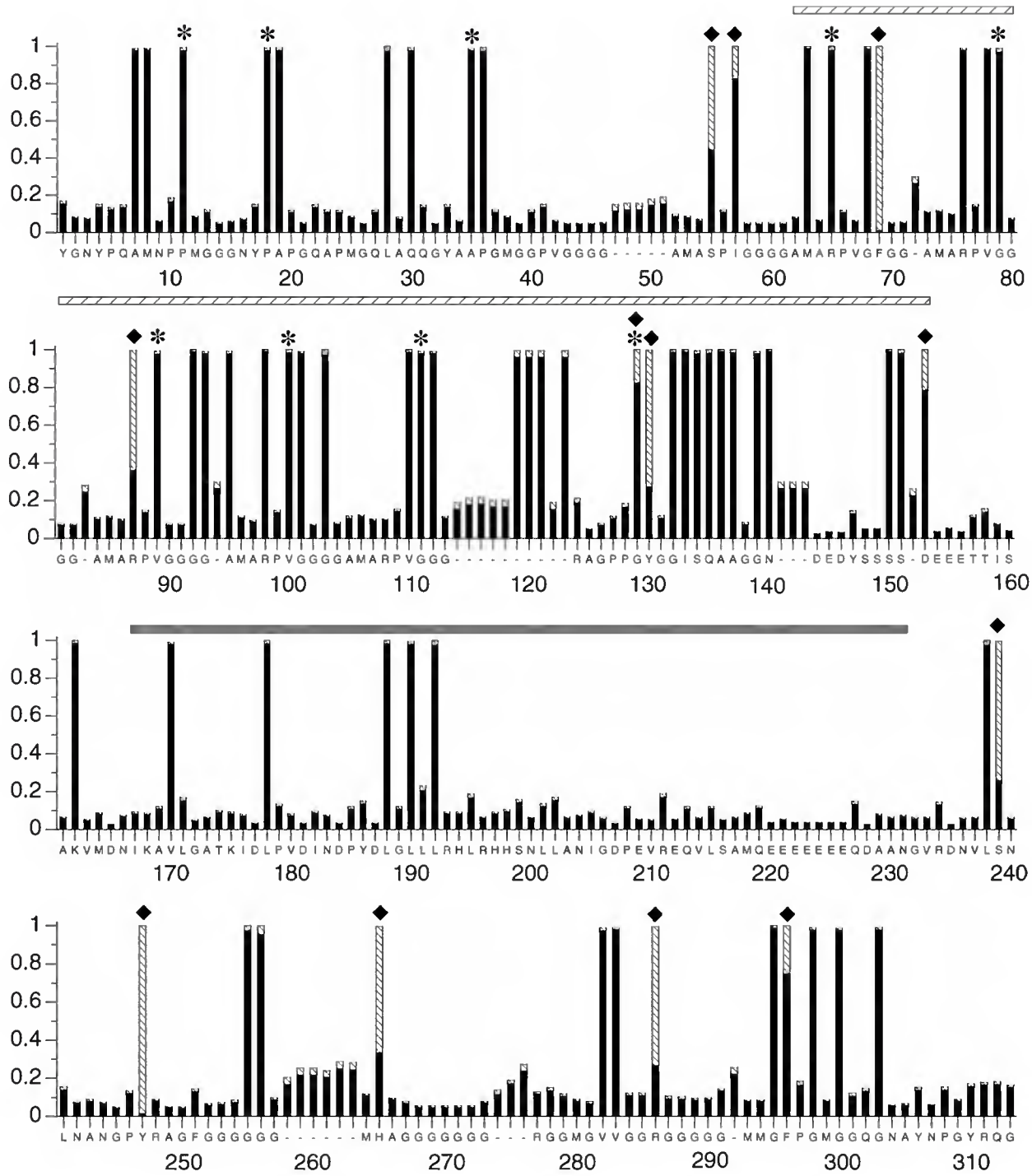

FIG. 2.—Posterior probabilities of codon sites assigned to classes with different values of ω (ratio of amino acid replacement to silent substitutions). Shown along the horizontal axis is the amino acid position and residue in the reference sequence (*E. lucunter* clone G2.4-9). The fraction of bars with solid fill indicates the posterior probability (P_i:vertical axis) of assignment to codon class II ($\hat{\omega}_1 = 1.279$). The hatched bar portion equals P_i for codon class III ($\hat{\omega}_2 = 7.755$). P_i for codon class I ($\hat{\omega}_0 = 0.001$) is 1 minus the sum of the other probabilities. Omega values were calculated using branch-site model B/S1 Model B (table 4). Diamonds identify codon sites with substitutions fixed in *E. lucunter*; asterisks indicate codon sites with substitutions fixed in the other two species. Solid gray bar overlies the core region; hatched bar overlies the hot-spot region.

receptor has been studied, firm conclusions regarding its evolution cannot be drawn. Nevertheless, several hypotheses can be suggested as to the causes of divergence of *E. lucunter* egg bindin receptors (and thus of bindin). The hypotheses pertain to the possibility that the modifications are (1) the result of selection against hybridization, (2) the product of sympatric speciation, or (3) indirect outcomes of intraspecific processes.

An attractive hypothesis to account for gamete incompatibility and for positive selection on *E. lucunter*

bindin is reinforcement of reproductive isolation (Butlin 1989; Howard 1993; Noor 1999). Though *E. lucunter* is the only species of *Echinometra* off the coast of Brazil and in the central and eastern Atlantic, in the Caribbean it is sympatric with *E. viridis*. It is therefore conceivable that differences in bindin of this species have evolved to avoid hybridization. The asymmetry of cross-fertilization (and thus the asymmetry in bindin modifications) might be explained by the bathymetric distribution of the two species (Lessios and Cunningham 1990). *E. viridis* reaches by far its greatest abundance at depths of 1 to 15 m, whereas *E. lucunter* is restricted almost entirely to very shallow (<1 m) depths. Scattered individuals of *E. viridis* are occasionally found in the *E. lucunter* zone, but the reverse is seldom observed. Because sperm from the few *E. viridis* in shallow water could potentially fertilize the eggs of many *E. lucunter* females, the latter species would be under more intense selection to avoid hybridization. This selection would result in modification of bindin receptors of *E. lucunter* to accept bindin alleles that were absent from *E. viridis*. As an incidental result, selection on *E. lucunter* egg receptors to avoid fertilization by *E. viridis* could also isolate it from the allopatric *E. vanbrunti*. This explanation could also account for the reduced bindin polymorphism in *E. lucunter*. There is, however, a solid reason for rejecting it. *E. lucunter* eggs are much more incompatible with sperm of the allopatric *E. vanbrunti* than they are with the sympatric *E. viridis* (Lessios and Cunningham 1990; McCartney and Lessios 2002), and *E. lucunter* bindin is also more divergent from that of the allopatric species than of the sympatric one. Reinforcement could not produce differences in this direction. The second possibility that might be suggested by the data showing rapid evolution of bindin in *E. lucunter* is that it is the cause, rather than the result, of speciation from *E. viridis*. Van Doorn, Luttikhuizen, and Weissing (2001) have proposed a model of sympatric speciation that appears to be particularly relevant. According to this model, linkage between a locus controlling a reproductive protein, such as bindin, and a locus controlling ecological preferences, such as bathymetric distribution, can lead to sympatric speciation.

However, the asymmetry of gametic interaction between the neotropical species of *Echinometra* provides evidence against models that would implicate divergence of bindin as the cause of sympatric speciation. Given that sperm of *E. lucunter* can easily fertilize eggs of *E. viridis*, gametic incompatibility, alone, could not genetically isolate the two species in sympatry. Yet their gene pools remain completely distinct. Two allozyme surveys (Lessios 1979; Bermingham and Lessios 1993) failed to detect any hybrids in two loci diagnostic between the two species, and two mtDNA studies (Bermingham and Lessios 1993; McCartney, Keller, and Lessios 2000) failed to detect any haplotypes of the "wrong" species in individuals of distinctive specific morphology. Thus a second isolating mechanism, prezygotic or postzygotic, must account for the genetic distinctiveness of the species. This greatly detracts from the credibility of any hypothesis that invokes divergence in gamete recognition loci as the process responsible for initiating speciation between the Atlantic species of *Echinometra*. Asymmetry in gametic incompatibility is the rule among closely related sympatric sea urchins (e.g., Strathmann 1981; Lessios and Cunningham 1990; Minor et al. 1991; Aslan and Uehara 1997; Rachman, Uehara, and Pearse 2001; McCartney and Lessios 2002; Levitan 2002; McClary and Sewell 2003; but see Palumbi and Metz 1991). This suggests that our finding, that modifications of bindin in one species do not necessarily render the sperm incompatible with the eggs of others, may be common. If so, even though divergence in reproductive proteins may be important in establishing reproductive barriers after secondary contact, it would not be a frequent cause of sympatric speciation in sea urchins.

A final hypothesis to account for the patterns of evolution of bindin in neotropical species of *Echinometra* suggests that divergence between species is the indirect result of forces that act within species, each with its own distinctive fertilization ecology. The intraspecific evolutionary forces implicated in driving the evolution of gamete-recognition proteins include sperm competition, sexual selection, and sexual conflict (reviewed in Swanson and Vacquier 2002). As Metz, Gomez-Gutierrez, and Vacquier (1998) have pointed out, such factors would operate independently of the challenge of sympatric species, so any explanation of the patterns of bindin divergence in neotropical *Echinometra* has to consider how *E. lucunter* differs not just from *E. viridis* but also from *E. vanbrunti*. It is known that the success of sea urchin sperm in fertilizing eggs decreases dramatically with distance between males and females (Pennington 1985; Levitan 1991), whereas sperm competition can be severe in high-density populations (Levitan 1998). When sperm concentration is low, selection on both egg and sperm is for fertilization with little discrimination. When there is an excess of sperm, interlocus sexual conflict is strongest, because avoidance of polyspermy becomes important for the eggs, whereas precedence in fertilization becomes important for the sperm (Rice and Holland 1997; Rice 1998). Sexual selection will also be most intense as sperm becomes locally more abundant in relation to eggs. Both interlocus conflict (Rice and Holland 1997; Holland and Rice 1998) and sexual selection (West-Eberhard 1983; Kirkpatrick and Ravigne 2002) can lead to runaway divergence in traits involved in reproduction. The tendency, demonstrated by Palumbi (1999) in *E. mathaei*, of eggs to select sperm with bindin similar to their own, indicates that sexual selection is present and could accelerate divergence in bindin set in motion by sperm competition and sexual inter-locus conflict. Depending on the degree of sperm competition, sexual conflict and sexual selection can act differently in different species, and they may even affect the susceptibility to fertilization by heterospecific sperm (Levitan 2002).

Why would these intraspecific processes differ between *E. lucunter* and the other two neotropical species? *Echinometra lucunter* reaches extremely high point densities in its preferred habitat, the edge of reef flats, with each individual occupying holes only centimeters away from others (see, for example, Table 1 in Lessios et al. 1984). Thus it is likely that sperm is abundantly available to eggs during spawning events in this species.

High wave energy at the edge of the reef flat is also likely to mix gametes and allow eggs the choice of sperm from different males (Denny, Nelson, and Mead 2002). *E. viridis* can also be locally abundant, but it lives subtidally in the interstices of corals, such that spawning individuals can be more widely separated, and their sperm may be less likely to be mixed by waves. *E. vanbrunti* is found in crevices and under rocks in the intertidal, but it does not reach point densities anywhere near as high as those of *E. lucunter* (personal observation). It is therefore easy to imagine that sperm competition may be more intense in *E. lucunter* than in the other two species. If so, selection would favor more discriminating eggs in *E. lucunter*, and it would initiate change of bindin through sexual selection and sexual inter-locus conflict. Although these processes do not appear to have caused in *Echinometra* the selective sweeps evident in abalone lysin (Metz, Robles-Sikisaka, and Vacquier 1998), the lower polymorphism of bindin in *E. lucunter* could also be their result. Until higher sperm competition in its natural habitat is actually demonstrated in *E. lucunter*, this explanation remains highly speculative, but it does fit the observed differences in variation of bindin in the three *Echinometra* species.

Whatever the causative explanations may be, our results show a clear link between bindin divergence and egg discrimination in comparisons between species. This finding provides empirical support to the supposition that sperm loci are selected to change in order to maintain recognition by a changing egg receptor. Yet our study also shows that this diversifying process is not universal. Our work confirms the trend, established by previous studies of bindin, for different sea urchins to show qualitatively different modes of evolution in this molecule; but it also shows that, even within a genus, selection can act differentially on bindin of different species. The challenge now is to understand the forces that cause bindin to evolve in such diverse ways.

Acknowledgments

We especially thank Vic Vacquier for help and training, and for kindly providing access to his laboratory during the early phases of this project. Gary Moy, Willie Swanson, and Bailey Kessing provided excellent technical suggestions and helpful advice. Ed Metz provided unpublished intron sequences, and Kirk Zigler and Willie Swanson made comments on the manuscript. Support was provided by a National Science Foundation (NSF)/Sloan Postdoctoral Fellowship in Molecular Evolution to M.A.M., and by the Smithsonian Scholarly Studies Program and the Molecular Evolution Program of the Smithsonian Institution.

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Accepted December 2, 2003