Evolution of Bindin in the Pantropical Sea Urchin *Tripneustes*: Comparisons to Bindin of Other Genera

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Bindin, a sea urchin sperm protein, mediates sperm-egg attachment and membrane fusion and is thus important in species recognition and speciation. Patterns of bindin variation differed among three genera that had been studied previously. In two genera of the superorder Camarodonta, *Echinometra* and *Strongylocentrotus*, both of which contain sympatric species, bindin is highly variable within and between species; a region of the molecule evolves at high rates under strong positive selection. In *Arbacia*, which belongs to the superorder Stirodonta and whose extant species are all allopatric, bindin variation is low, and there is no evidence of positive selection. We cloned and sequenced bindin from *Tripneustes*, a sea urchin that belongs to the Camarodonta but whose three species are found in different oceans. Worldwide sampling of bindin alleles shows that the bindin of *Tripneustes* (1) contains the highly conserved core characteristic of all other bindins characterized to date, (2) has an intron in the same position, and (3) has approximately the same length. Its structure is more like that of bindin from other camarodont sea urchins than to bindin from the stirodont *Arbacia*. The resemblances to other camarodonts include a glycine-rich repeat structure upstream of the core and lack of a hydrophobic domain 3′ of the core, a characteristic of *Arbacia* bindin. Yet the mode of evolution of *Tripneustes* bindin is more like that of *Arbacia*. Differences between bindins of the Caribbean *Tripneustes ventricosus* and the eastern Pacific *T. depressus*, separated for 3 my by the Isthmus of Panama, are limited to four amino acid changes and a single indel. There are no fixed amino acid differences or indels between *T. depressus* from the eastern Pacific and *T. gratilla* from the Indo-Pacific. Bindin of *Tripneustes*, like that of *Arbacia*, also shows no evidence of diversifying selection that would manifest itself in a higher proportion of amino acid replacements than of silent nucleotide substitutions. When the rate of intrageneric bindin divergence is standardized by dividing it by cytochrome oxidase I (COI) divergence, *Tripneustes* and *Arbacia* show a lower ratio of bindin to COI substitutions between the species of each genus than exists between the species of either *Echinometra* or *Strongylocentrotus*. Thus, mode of bindin evolution is not correlated with phylogenetic affinities or molecular structure, but rather with whether the species in a genus are allopatric or sympatric. For a molecule involved in gametic recognition, this would suggest a *pattern* of evolution via reinforcement. However, in bindin the *process* that gave rise to this pattern is not likely to have been selection to avoid hybridization, because there is no excess of amino acid replacements between species versus within species in the bindins of *Echinometra* and *Strongylocentrotus*, as would have been expected if specific recognition were the driving force in their evolution. We suggest instead that the pattern of reinforcement is a secondary effect of the ability of species with rapidly evolving bindins to coexist in sympatry.

Introduction

Explaining how reproductive isolation evolves between marine species is of crucial importance in understanding speciation in the sea. The availability of detailed information on phylogeography (e.g., Palumbi et al. 1997; Lessios et al. 1999; McCartney, Keller, and Lessios 2000; Lessios, Kessing, and Pearse 2001), on temporal isolation (Lessios 1984) and gametic isolation (Strathmann 1981; Lessios and Cunningham 1990; Uehara, Asakura, and Arakaki 1990; Palumbi and Metz 1991), and on alpha taxonomy (Mortensen 1928–1951; Mayr 1954) makes sea urchins good subjects for the study of marine speciation.

Most sea urchins are broadcast spawners, with external fertilization. Reproductive isolation between species could result from distinct spawning times or from species-specific gamete interactions. Congeneric sympatric sea urchins often have overlapping annual (Lessios 1981, 1985; McClary and Barker 1998) or monthly (Lessios 1991) spawning periods. In such cases, reproductive isolation between closely related sea urchins is, at least in part, the product of gametic incompatibility (R. R. Strathmann 1981; M. F. Strathmann 1987 (p. 522); Lessios and Cunningham 1990; Uehara, Asakura, and Arakaki 1990; Palumbi and Metz 1991). Whereas gametic incompatibility could arise between a pair of sea urchin species at any step in the interactions between gametes (e.g., sperm activation, acrosomal reaction, sperm-egg attachment, sperm-egg fusion), it appears that in closely related species it generally emerges during sperm-egg attachment and membrane fusion (see Metz et al. [1994] for discussion). The sea urchin sperm protein bindin mediates both of these processes in sea urchins. Changes in the bindin locus can thus cause gametic incompatibility and convert populations into different species. Bindin is the major insoluble component of the acrosomal vesicle (Vacquier and Moy 1977). It functions as glue between the acrosomal process and the glycoprotein bindin receptors of the vitelline layer of the egg. Bindin and bindin receptors often interact in a species-specific manner (Glabe and Vacquier 1977; Glabe and Lennarz 1979).

The evolution of bindin has been studied in three genera of sea urchins. Metz and Palumbi (1996) studied bindin sequences of the central and western Pacific species of the cosmopolitan genus *Echinometra*. They found many sequence rearrangements and a higher number of nonsynonymous than synonymous substitutions, an indication of positive selection, in a region just 5′ of the conserved bindin core. Biermann (1998) examined bindin in *Strongylocentrotus* and found evidence for positive selection in the same "hotspot" observed in *Echinometra*. Metz, Gomez-Gutierez, and Vacquier (1998) studied the same

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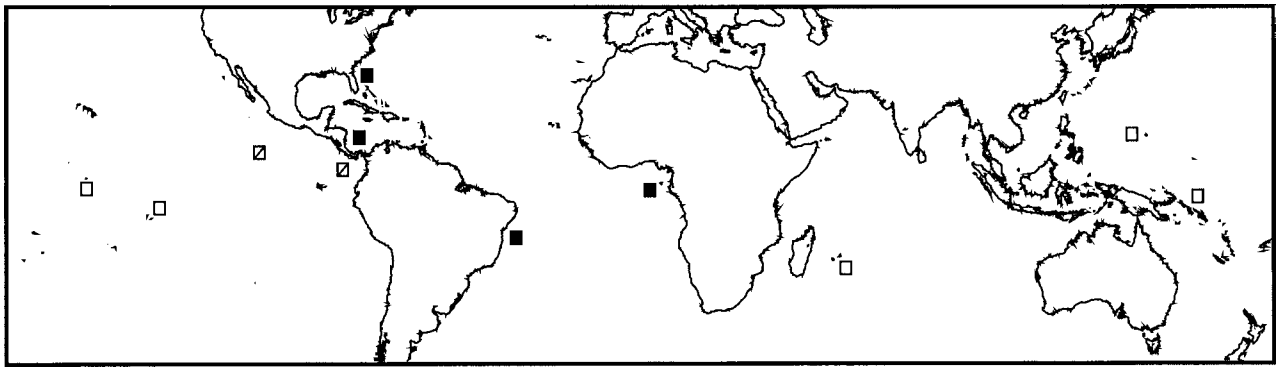

FIG. 1.—Localities from which bindin alleles were sampled. *Tripneustes ventricosus* (filled boxes) was sampled at Florida; Panama; Salvador, Brazil; and São Tomé. *T. depressus* (crosshatched boxes) was sampled at Isla del Coco and Clipperton Atoll. *T. gratilla* (open boxes) was sampled at Reunion, the Marquesas, Kiritimati, Guam, and Papua New Guinea.

molecule in *Arbacia*. In contrast to the previous studies, they found almost no sequence rearrangements and no evidence for positive selection. Changes in bindin are correlated with prezygotic isolation: species of *Echinometra* and *Strongylocentrotus* are often gametically incompatible (R. R. Strathmann 1981; M. F. Strathmann 1987 [p. 522]; Uehara, Asakura, and Arakaki 1990; Palumbi and Metz 1991), whereas species of *Arbacia* are gametically compatible, at least in the single cross performed by Metz, Gomez-Gutierez, and Vacquier (1998). Patterns of bindin evolution within genera also correlate with the presence of sympatric species. *Echinometra* and *Strongylocentrotus* contain species with overlapping geographical distributions, whereas all species of *Arbacia* are distributed allopatrically.

Metz, Gomez-Gutierez, and Vacquier (1998) examined three hypotheses that might explain the lack of variability of bindin in *Arbacia*: (1) Extensive gene flow over large distances within species of *Arbacia* may limit the potential for rapid bindin evolution. (2) *Arbacia* bindins may be functionally constrained. (3) Non-overlap in geographic distributions of the species of *Arbacia* may have obviated the necessity for the evolution of gamete recognition. A fourth hypothesis is that differences in the mode of evolution of bindin between *Arbacia*, on the one hand, and *Echinometra* and *Strongylocentrotus*, on the other, are due to phylogenetically inherited differences of their genomes. *Arbacia* belongs to the superorder Stirodonta, whereas *Echinometra* and *Strongylocentrotus* belong to the superorder Camarodonta. These superorders last shared a common ancestor approximately 160 MYA (Smith, Lafay, and Christen 1992).

We examined bindin evolution in the genus *Tripneustes*, a member of the superorder Camarodonta (Smith 1988). *Tripneustes* is pantropical and contains three allopatrically distributed species, *T. ventricosus* on both sides of the Atlantic, *T. depressus* in the eastern Pacific, and *T. gratilla* in the central and western Pacific as well as the Indian Ocean. The three species are morphologically very similar, to the degree that it has been suggested that they may constitute a single species (Clark 1912). Indeed, there is no mitochondrial DNA differentiation between *T. depressus* and *T. gratilla*, which suggests that eastern and western Pacific populations of *Tripneustes* belong to the same species (Lessios, Kane, and Robertson, in preparation) *Tripneustes* mature bindin sequences were obtained from all species of the genus, and from all major regions of tropical oceans. We wanted to know whether bindin variation in this genus conformed to the pattern seen in other camarodonts, or whether it resembled that of *Arbacia*. Because *Arbacia* is more distantly related to *Tripneustes* but resembles this genus in containing no extant sympatric species, these comparisons offer insight into the relative roles of phylogeny (and resultant similarities in molecular structure) versus selection against hybridization in the evolution of this important gamete-recognition molecule.

Materials and Methods

Samples

Specimens of *Tripneustes* were collected from locations around the world (fig. 1). Our sampling was intended to cover all mitochondrial DNA (mtDNA) major clades (Lessios, Kane, and Robertson, in preparation), as well as all regions of the tropical oceans. Thus, we sampled two individuals from the eastern Atlantic (an mtDNA clade distinct from the western Atlantic), two from the Caribbean (including Florida), two from Brazil (a subclade of the western Atlantic clade in mtDNA), four from the eastern Pacific, two each from the central and western Pacific, and two from the Indian Ocean.

Identification of Bindin from cDNA

To characterize the first bindin sequence of *Tripneustes*, we isolated poly-A mRNA from the testis of a ripe *Tripneustes ventricosus* from Buena Ventura, Panama, using a Micro Poly (A) Pure Kit (Ambion). Reverse transcription reactions were conducted using Superscript II reverse transcriptase (RT) (Life Technologies) according to the manufacturer's protocol, but with 1 μl of 100 μM MOBY (5′-AAGGATCCGTCGACATCGATAATAC-GACTCACTATAGGGATTTTTTTTTTTTTTTT-3′) as the primer. Using primers MB1130+ (5′-TGCTS-GGTGCSACSAAGATTGA-3′) and core200- (5′-TCYT-CYTCYTCYTGCATIGC-3′), we amplified a fragment of

the core region of bindin from the RT reaction product. These primers correspond to amino acids VLGATKID and AMQEEEE of the core region of bindin (Vacquier, Swanson, and Hellberg 1995). Based on the DNA sequence of the amplified fragment, exact *Tripneustes* bindin core primers were designed for use in 3′ and 5′ rapid amplification of cDNA ends (RACE) (Frohman, Dush, and Martin 1988; Zhang and Frohman 1997). Nested polymerase chain reactions (PCR) for 3′ RACE were carried out on the MOBY-primed testis cDNA with a pair of exact match *Tripneustes* bindin core primers and the 3′ RACE primers out2 (5′-GATCCGTCGACATC-GATAATACG-3′), and in2 (5′-CGATAATACGACT-CACTATAGG-3′). The 5′ RACE was performed using the 5′ RACE system (version 2.0, Life Technologies) according to the manufacturer's protocol.

Genomic Characterizations of Bindin

After the first *Tripneustes* bindin sequence was obtained from cDNA by RACE, we amplified other bindin alleles from genomic DNA. We extracted genomic DNA as described by Lessios et al. (1996) from gonad tissue preserved in ethanol, NaCl-saturated 20% dimethyl-sulfoxide solution, or in liquid nitrogen. Using Tfl DNA polymerase (Epicentre Technologies), we amplified full-length mature bindin alleles from genomic DNA with primers TvF1 (5′-CTTTATCTCGGGGCATCGTC-3′) and TvR1 (5′-CTGAACTTCCAATGGCTTCC-3′). Amplification conditions were as follows: 1 min at 96°C, then 39 cycles of 45 s at 94°C, 30 s at 50°C, 150 s at 72°C and finally 5 min at 72°C. We ran the amplification products in low-melting-point agarose gels. Bands were excised and treated with Gelase (Epicentre Technologies), then cloned using a pMOSBlue blunt-ended cloning kit (Amersham). We screened positive bacterial colonies according to the same PCR protocol stated above, with either the primer pair TvF1 and TvR1 or vector primers T7 and U19. The PCR product from a single positive colony per individual was gel purified and cycle sequenced as described in Lessios et al. (1996), with primers TvF1, TvR1, MB1130+, MB1136- (5′-ARGTCAATCTTSGTSGCCC-3′), TvF3 (5′-TGATGGACCTCAGCAGTGGTGT-3′), and TvR3 (5′-CACAAAATGATGGCTCACAGTT-3′). This combination of primers sequenced both strands of the full mature bindin and its intron. Sequencing was performed on an ABI 377 automated sequencer and edited using Sequencher 3.1 (Gene Codes Corp.). Sequences have been deposited in GenBank (accession numbers AF520207-AF520222).

A total of 12 mutations unique to a single allele (singletons) were observed among 16 mature bindin sequences with a combined length of 10,200 bp. Singleton mutations may represent true differences, or they may arise from cloning and polymerase error during amplification. Thus, the upper limit of sequencing error in the study was 0.12%.

Phylogenetic Analysis

Sequences were aligned by eye with the computer program Se-Al (version 1.0, written by A. Rambaut). A bindin sequence obtained from *Lytechinus variegatus* was used as an outgroup to root bindin phylogenetic trees. The *L. variegatus* bindin cDNA sequence reported by Minor et al. (1991) was not used because it differs in sequence at four amino acids of the core region, which are identical in 30 other *Lytechinus* bindin sequences (unpublished data) and all of the *Tripneustes* bindin alleles presented here. To calculate the best-fit model for constructing a tree, we entered the bindin coding sequences (full mature bindin plus 23 codons of preprobindin sequences) of *Tripneustes* in Modeltest version 3.06 (Posada and Crandall 1998). Comparison of the log-likelihood ratios of nested models performed by Modeltest indicated that the simplest model with a significantly better fit to the data than other models was that of Tamura and Nei (1993). Allowing for site-specific rate categories according to codon position did not greatly improve the likelihood of the model. We used PAUP* 4.0b6 (Swofford 1998) to conduct Neighbor-Joining (Saitou and Nei 1987) phylogenetic analyses on the bindin coding sequences based on Tamura and Nei distances. Maximum parsimony and maximum likelihood (ML) trees were also constructed in PAUP. Five codons (62–66 in fig. 2) that could not be unambiguously aligned between *Tripneustes* and *Lytechinus* sequences were excluded from the phylogenetic analysis. Intron sequences were not used in the final analysis because they could not be aligned between *Lytechinus* and *Tripneustes*. Analyses that included the intron but left the *Tripneustes* tree unrooted produced the same intrageneric topology as analyses limited to the coding sequences.

Statistical Tests for Selection

We divided the mature bindin sequences into three regions, based on previous observations of patterns of bindin variation in other genera: (1) a hotspot region 5′ from the conserved core (amino acids 62–93 in fig. 2), corresponding to that observed in both *Echinometra* (Metz and Palumbi 1996) and *Strongylocentrotus* (Biermann 1998); (2) the conserved core (amino acids 98–163); and (3) the rest of the molecule. We used MEGA version 2.1 (Kumar et al. 2001) to calculate the proportion of synonymous (d_S) and nonsynonymous (d_N) differences by the Pamilo and Bianchi (1993) and Li (1993) (PBL) method in each of the three regions. Two separate analyses were carried out, one comparing all sequences to one another, the other comparing alleles from the Pacific species (*T. depressus* and *T. gratilla*) to those from the Atlantic (*T. ventricosus*). 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 (Zhang, Kumar, and Nei 1997), under Nei and Gojobori's (1986) model of evolution.

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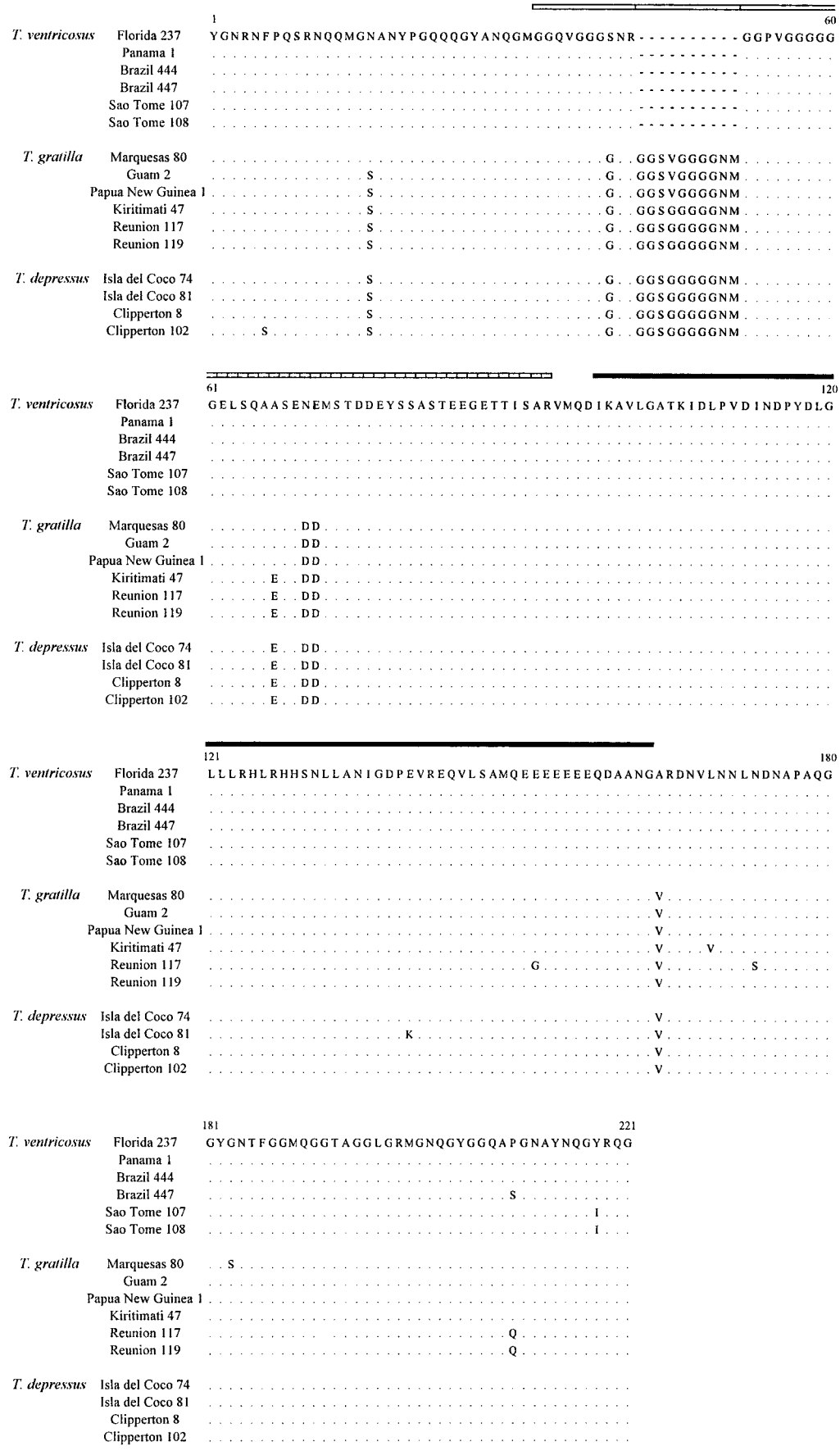
FIG. 2.—Amino acid sequence alignments of alleles in mature bindin of *Tripneustes*. Periods indicate identity to the first sequence, dashes indicate gaps. The three 10 amino acid repeats are overscored by open boxes, the hotspot region by a striped box, and the core by a black box.


1 60
T. ventricosus Florida 237 YGNRNFPQSRNQQMGNANYPGQQQGYANQGMGGQVGGGSNR----------GGPVGGGGG
Panama 1
Brazil 444
Brazil 447
Sao Tome 107
Sao Tome 108
T. gratilla Marquesas 80 G..GGSVGGGGNM
Guam 2 S G..GGSVGGGGNM
Papua New Guinea 1 S G..GGSVGGGGNM
Kiritimati 47 S G..GGSGGGGGNM
Reunion 117 S G..GGSGGGGGNM
Reunion 119 S G..GGSGGGGGNM
T. depressus Isla del Coco 74 S G..GGSGGGGGNM
Isla del Coco 81 S G..GGSGGGGGNM
Clipperton 8 S G..GGSGGGGGNM
Clipperton 102 S S G..GGSGGGGGNM
61 120
T. ventricosus Florida 237 GELSQAASENEMSTDDEYSSASTEEGETTISARVMQDIKAVLGATKIDLPVDINDPYDLG
Panama 1
Brazil 444
Brazil 447
Sao Tome 107
Sao Tome 108
T. gratilla Marquesas 80 DD
Guam 2 DD
Papua New Guinea 1 DD
Kiritimati 47 E..DD
Reunion 117 E..DD
Reunion 119 E..DD
T. depressus Isla del Coco 74 E..DD
Isla del Coco 81 E..DD
Clipperton 8 E..DD
Clipperton 102 E..DD
121 180
T. ventricosus Florida 237 LLLRHLRHHSNLLANIGDPEVREQVLSAMQEEEEEEEEQDAANGARDNVLNNLNDNAPAQG
Panama 1
Brazil 444
Brazil 447
Sao Tome 107
Sao Tome 108
T. gratilla Marquesas 80 V
Guam 2 V
Papua New Guinea 1 V
Kiritimati 47 V V
Reunion 117 G V S
Reunion 119 V
T. depressus Isla del Coco 74 V
Isla del Coco 81 K V
Clipperton 8 V
Clipperton 102 V
181 221
T. ventricosus Florida 237 GYGNTFGGMQGGTAGGLGRMGNQGYGGQAPGNAYNQGYRQG
Panama 1
Brazil 444
Brazil 447 S
Sao Tome 107 I
Sao Tome 108 I
T. gratilla Marquesas 80 S
Guam 2
Papua New Guinea 1
Kiritimati 47
Reunion 117 Q
Reunion 119 Q
T. depressus Isla del Coco 74
Isla del Coco 81
Clipperton 8
Clipperton 102

To test for the possibility that selection might be acting at sites scattered throughout the bindin molecule and not in specific regions, we implemented a series of models in PAML version 3.0 (Yang 2000; Yang et al. 2000) based on the neighbor-joining tree of the unique bindin alleles. We calculated the likelihood of this tree under two neutral models (M1 and M7) that do not allow for positively selected sites and under three alternate models (M2, M3, and M8) that do (see Swanson, Aquadro, and Vacquier 2001). Then, we compared the log-likelihoods between the neutral and selection models. We also used PAML to test for evidence of changing d_N/d_S ratios along different lineages of the neighbor-joining tree by first calculating the likelihood for a model that kept the d_N/d_S ratio constant across the tree and then calculating the likelihood for a model that allowed each branch to have a separate d_N/d_S ratio. Finally, we tested for selection on bindin using the McDonald-Kreitman (1991) test to compare the d_N/d_S ratios within and between species.

Comparative Rates of Bindin Evolution

To ask whether bindin evolves faster in some genera than in others, we compared our data for *Tripneustes* bindin to all full-length mature bindin sequences of Metz and Palumbi (1996) for *Echinometra*, of Biermann (1998) for *Strongylocentrotus*, and of Metz, Gomez-Gutierez, and Vacquier (1998) for *Arbacia*. To calculate rates, we standardized intrageneric bindin differentiation by dividing d_N and d_S for the entire bindin molecule by the interspecific Kimura (1980) two-parameter (K2P) genetic distance of the mitochondrial cytochrome oxidase I (COI) gene. Average COI divergence between species of *Tripneustes* was calculated from all sequences included in Lessios, Kane, and Robertson (in preparation), of *Echinometra* from all sequences in Palumbi et al. (1997), and of *Strongylocentrotus* from all sequences in Kessing (1991). Because Metz, Gomez-Gutierez, and Vacquier (1998) sequenced a different region of COI, we obtained our own sequences from 102 individuals of four species of *Arbacia*, covering the same 640 bp that were sequenced in *Tripneustes*. This region completely overlaps the 450 bp sequenced by Palumbi et al. (1997) in *Echinometra* and most of the 440 bp sequenced by Kessing (1991) in *Strongylocentrotus*.

Mature bindin sequences within the same genus were aligned by eye. Sequences from different genera were generally too divergent outside of the core region to be aligned with confidence. Thus, only intrageneric comparisons were feasible. In contrast to *Tripneustes* and *Arbacia*, in which alignments could be made for the whole mature bindin, the glycine-rich repeat regions in *Echinometra* and *Strongylocentrotus* bindins could not be aligned unambiguously. These regions (111 codons 3′ of the core in *Strongylocentrotus*, 26 codons 5′ of the core and 32 codons 3′ of the core in *Echinometra*) were excluded from this analysis. Bindin intron sequences of *Arbacia*, *Tripneustes*, and *Strongylocentrotus* were aligned by eye for each genus. Intron sequences for *Echinometra* were not included, because they were unavailable in GenBank. Inversions in the bindin intron were aligned between sequences that shared them and were considered as gaps in sequences with the opposite inversion.

Strongylocentrotus polyacanthus was not included in the analysis, because COI data were not available for this species. *S. franciscanus* was also excluded, because its bindin sequences could not be unambiguously aligned with the sequences from the rest of the genus. *T. depressus* and *T. gratilla* sequences were treated as coming from the same species, because neither their bindin nor their COI sequences (Lessios, Kane, and Robertson, in preparation) are reciprocally monophyletic. Finally, we included *Hemicentrotus pulcherrimus* in the *Strongylocentrotus* analysis, because both COI (Kessing 1991) and bindin (Biermann 1998) data indicate that it is nested within this genus.

Codon Bias

We estimated codon bias for each full-length mature bindin allele from *Arbacia*, *Tripneustes*, *Echinometra*, and *Strongylocentrotus* with the program CODONS (Lloyd and Sharp 1992) by calculating the effective number of codons (ENC) (Wright 1990). These ENC values can range from 20 (same codon always used for an amino acid) to 61 (random use of each codon for every amino acid). A separate calculation was carried out for each allele, and then the values were averaged for each genus.

Results

Structure of the Molecule

We recovered 16 full-length mature bindin alleles from *Tripneustes*. Twelve of these alleles were unique sequences. Total aligned bindin sequences of *Tripneustes* include 69 bp of preprobindin, 633 or 663 bp of mature bindin, 835–855 bp of intron, and 31 bp of 3′ untranslated region. Like the mature bindins from other genera, those from *Tripneustes* contain no cysteine or tryptophan residues (fig. 2). Their length (211 or 221 amino acids) is approximately the same as that of other known bindins (Vacquier, Swanson, and Hellberg 1995).

Tripneustes bindins have the highly conserved core observed in bindins from all other studied genera. Of 66 amino acids defined as the core in this study (98–163 in fig. 2), 65 are identical to those of *Lytechinus variegatus*, and 64 are identical to the bindin sequences of *S. purpuratus* (Gao et al. 1986), *S. franciscanus* (Minor et al. 1991), and *A. punctulata* (Glabe and Clark 1991). All 18 amino acids (119–136 in fig. 2) implicated in membrane fusion (Ulrich et al. 1998, 1999) are identical to those in all other known bindins.

At a point 5′ of the core, *Tripneustes* bindins contain a glycine-rich repeat GG(Q/S/P) (V/G)GGG(G/S) (N/G) (S/M/G) reminiscent of glycine-rich motifs present in *Echinometra* and *Strongylocentrotus* but absent in *Arbacia*. This repeat is present in two copies in *T. ventricosus*, and in three copies in *T. depressus* and *T. gratilla* (fig. 2). This is the only insertion/deletion (indel) observed in *Tripneustes* mature bindin. Hydrophobicity plots of *Tripneustes* bindin are similar to those of other Camarodont sea urchins (Zigler and Lessios in preparation); they

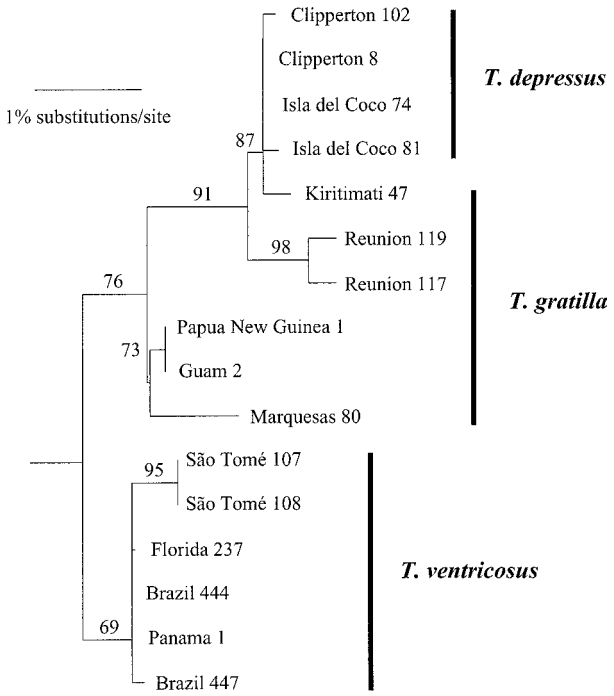


FIG. 3.—Neighbor-Joining tree of sequences of preprobindin and mature bindin of *Tripneustes*, using Tamura and Nei (1993) genetic distances. Bootstrap support (1,000 replicates) of >50% is noted next to nodes. The tree is rooted on a bindin sequence of *Lytechinus variegatus*.

do not contain the 3′ hydrophobic domain observed in *Arbacia* bindin (Glabe and Clark 1991).

The single intron is in the same location as in all other bindins studied to date (after amino acid 93 in fig. 2). It contains a region of approximately 75 bp just inside its 5′ end that is inverted in half of the *Tripneustes* alleles with respect to other *Tripneustes* alleles. All *T. ventricosus* alleles, plus three alleles of *T. gratilla* ("Marquesas 80," "Guam 2," and "Papua New Guinea 1"), have one form of the inversion, whereas all *T. depressus* alleles and the rest of the *T. gratilla* alleles have the other form.

Genealogy of Alleles

Figure 3 depicts the genealogy of the sequenced bindin alleles, reconstructed by neighbor joining, using only coding sequences (mature bindin and preprobindin sequences). Neighbor joining, using Tamura and Nei (1993) distances, divides the genealogy into Atlantic and Pacific clades. Parsimony and ML methods place the "Guam 2," "Marquesas 80," and "Papua New Guinea 1" alleles with the Atlantic alleles. That different methods of phylogenetic reconstruction result in roots placed between different nodes of the *Tripneustes* bindin tree is probably due to the large number of changes between *Tripneustes* and *Lytechinus*, relative to the changes within *Tripneustes*. Mitochondrial DNA divides the Atlantic and Pacific haplotypes into reciprocally monophyletic clades (Lessios, Kane, and Robertson, in preparation)

There are four fixed amino acid substitutions and one indel that distinguish the Atlantic *Tripneustes* bindin alleles from all Pacific bindin alleles. Two of these substitutions (at amino acids 70 and 71 in fig. 2) occur in the "hotspot" region of *Echinometra* (Metz and Palumbi 1996) and *Strongylocentrotus* (Biermann 1998). Within the Atlantic clade, the two alleles from São Tomé off the coast of Africa form a well-supported monophyletic entity. Unlike the pattern seen in mtDNA, in which E. and W. Atlantic populations form reciprocally monophyletic sister clades (Lessios, Kane, and Robertson, in preparation), the São Tomé bindin alleles are nested within the ones from the W. Atlantic.

In the Pacific, bindin alleles of *T. depressus*, off the coast of America, and of *T. gratilla* from the central and Indo-West Pacific do not sort according to nominal species or collection locality. One allele from Kiritimati, which, according to collecting location should belong to *T. gratilla*, actually groups with *T. depressus*. The *T. depressus* alleles (plus the Kiritimati allele) is a sister group to the *T. gratilla* sequences from Reunion, in the Indian Ocean. The other three *T. gratilla* sequences are basal in the Pacific clade. The basal position of these three alleles is consistent with the observed intron inversion: each of these alleles has the *T. ventricosus* form of the inversion, whereas the rest of the Pacific individuals have the other form. There are no fixed nonsynonymous changes between *T. gratilla* and *T. depressus*. The close affinity of bindin from the two Pacific species mirrors a similar lack of differentiation of mtDNA sequences (Lessios, Kane, and Robertson, in preparation). Apparently, gene flow between the eastern and central Pacific is either continuing or has been very recently interrupted.

Adaptive Evolution

Relative rates of change of the different regions of the *Tripneustes* bindin molecule are qualitatively similar to patterns of bindin evolution in other Camarodont sea urchins. Rates of nonsynonymous change are highest in the hotspot region, lowest in the core, and intermediate in the rest of the molecule (table 1). Only nonsynonymous changes are observed in the hotspot region. However, in contrast to the pattern in *Echinometra* and *Strongylocentrotus*, the excess of nonsynonymous changes in the hotspot region is not significant in any pairwise comparison. *Tripneustes* bindins also lack the large number of potentially important indels (Palumbi 1999) seen in the other Camarodont genera. In addition, using the models implemented in PAML, we found no evidence for positively selected sites dispersed throughout the molecule. The likelihood of models that allowed for positively selected sites was not significantly higher than that of models that did not (table 2). Nor did we find any evidence for significant variation in d_N/d_S ratios between lineages. Allowing a different d_N/d_S ratio for each branch in the phylogeny did not produce a significantly better model than a model with a single d_N/d_S ratio for the entire tree (table 2). Finally, a comparison of the ratios of replacement to silent differences within and between species was not significantly different from neutral expectation (McDonald and Kreitman 1991). Four replacement substitutions and zero silent substitutions are fixed between Pacific (*T. gratilla* and *T. depressus*) and Atlantic (*T.*

Table 1
Replacement (d_N) and Silent (d_S) Substitutions per Site in Three Regions of *Tripneustes* Mature Bindin

	All Comparisons[a]			Atlantic vs. Pacific[a]		
Region	d_N[b]	d_S[b]	d_N/d_S	d_N[b]	d_S[b]	d_N/d_S
Hotspot[c]	0.021	0.000	>1[d]	0.038	0.000	>1[d]
Core[c]	0.002	0.022	0.09[d]	0.002	0.027	0.06[d]
Rest of molecule	0.013	0.028	0.45[d]	0.019	0.033	0.58[d]

[a] Means of pairwise comparisons.
[b] d_N and d_S calculated by the Pamilo and Bianchi (1993) and Li (1993) method.
[c] Hotspot: amino acids 62–93 in figure 2, core: amino acids 98–163 in figure 2.
[d] Not significantly greater than the neutral expectation for any pairwise comparison by Fisher's exact test using the Nei and Gojobori (1986) model of evolution.

ventricosus) individuals, and there are 12 replacement polymorphisms and 12 silent polymorphisms within the two groups (Fisher's exact test $P = 0.11$).

Rate of Bindin Evolution

To compare the rate of relative change of amino acid replacement of bindin within each genus, we calculated interspecific K2P, d_N and d_S (PBL) for the full mature bindin and the intron, then divided these values by mitochondrial COI genetic distances (K2P) (table 3). If a universal calibration for a "COI clock" is assumed, then divergence in this molecule represents a proxy of the time that species have remained separate, and thus helps standardize the bindin divergence for the age of the species. The comparison of ratios of either bindin K2P or d_N to COI genetic distance indicates that there are large differences between the genera in the overall rates of change in the bindin molecule. Because these are pairwise comparisons, each ratio is not independent of all others, so they cannot be compared statistically. Nevertheless, the trend is clear. All ratios of bindin K2P and especially d_N values to COI distances in *Arbacia* and *Tripneustes* are smaller than all such ratios in *Echinometra* or *Strongylocentrotus*. *Arbacia* shows larger interspecific COI distances relative to those of the other genera, which could be due either to older ages of its species or to higher rates of COI divergence. The COI divergence between *A. punctulata* and *A. stellata* across the Isthmus of Panama is equivalent to that of six other genera thus separated (Lessios, Kessing, and Pearse 2001), which would suggest that rate variation in COI is low, and thus that it correctly indicates most species of *Arbacia* to be older than those of the other genera. If as Metz, Gomez-Gutierez, and Vacquier (1998) suggested, Atlantic and Pacific species of *Arbacia* were separated before the completion of the Panama isthmus, then rate of COI divergence in this genus would be slower than we assume, and the differences in rates of bindin evolution between the genera would be even more pronounced.

Interestingly, the higher rate of bindin evolution in *Strongylocentrotus* and *Echinometra* relative to *Arbacia* or *Tripneustes* does not appear to be limited to adaptive changes. The comparison of bindin d_S to COI divergence ratios follows the same pattern as that of comparisons of bindin d_N to COI divergence. Every interspecific comparison in *Arbacia* or *Tripneustes* produces a lower ratio than in *Strongylocentrotus* or *Echinometra* (table 3). A positive correlation across molecules between rate of substitution in replacement and silent sites has been found in many organisms (Wolfe and Sharp 1993; Akashi 1995, 1997; Comeron and Aguadé 1996; Alvarez-Valin, Jabbari, and Bernardi 1998; Comeron and Kreitman 1998; Dunn, Bielawski, and Yang 2001). The correlation appears to also exist across lineages (Dunn, Bielawski, and Yang 2001). Mutational bias, nonindependence of point mutations, and codon-level selection have been offered as explanations for the phenomenon. Almost all explanations involve some form of codon bias, but ENC values in bindin show that codon usage in this molecule is universally equitable (means per genus: *Tripneustes*, 59.2; *Arbacia*, 44.9; *Strongylocentrotus*, 52.2; *Echinometra*, 55.1).

The trend seen for the silent sites of the expressed region does not extend to the intron. Intron sequences are not available for *Echinometra*, but a comparison between *Arbacia* and *Tripneustes*, on the one hand, and *Strongylocentrotus* on the other, indicates that the ratio of intron to COI divergence is not different between the two groups, which is what would be expected if both the intron and COI evolve linearly with time.

Discussion

Tripneustes Bindin Structure and Divergence

The bindin of *Tripneustes* contains the highly conserved core characteristic of all other bindins studied to date, has an intron in the same position, and has

Table 2
Maximum Likelihood Testing for Variation in the Ratio of Replacement Substitutions to Silent Substitutions Among Sites and Lineages

Models Compared[a]	L[b] (1st Model)	L[b] (2nd Model)	$2\Delta L$[c]	df	P[d]
Variation Among Sites					
M1 vs. M2	−1102.08	−1101.83	0.5	2	0.78
M1 vs. M3	−1102.08	−1101.68	0.8	4	0.94
M7 vs. M8	−1102.08	−1101.82	0.52	2	0.77
Variation Among Lineages					
Model 0 vs. model b	−1103.14	−1093.65	18.98	21	0.59

[a] For model specifics, see Yang et al. (2000) and Yang (2000).
[b] Log likelihood.
[c] The test statistic is twice the log likelihood difference of the two models.
[d] Probability from the chi-square distribution.

Table 3
Intrageneric Differences in Mature Bindin, Bindin Intron, and Mitochondrial Cytochrome Oxidase I in All Genera for Which Data Exist

Genus	Species	Species	Bindin[a]			Intron[a]	COI[a]	Bindin K2P/	Bindin d_N/	Bindin d_S/	Intron K2P/
			K2P[b]	dN[c]	dS[c]	K2P[b]	K2P[b]	COI K2P	COI K2P	COI K2P	COI K2P
Arbacia	*lixula*	*punctulata*	0.021	0.007	0.069	0.053	0.090	0.233	0.072	0.764	0.583
	lixula	*incisa*	0.029	0.007	0.096	0.179	0.134	0.217	0.053	0.716	1.343
	lixula	*dufresnei*	0.030	0.016	0.071	0.153	0.124	0.242	0.129	0.570	1.233
	punctulata	*incisa*	0.024	0.003	0.088	0.159	0.139	0.173	0.022	0.635	1.143
	punctulata	*dufresnei*	0.025	0.011	0.059	0.134	0.124	0.202	0.085	0.477	1.081
	incisa	*dufresnei*	0.028	0.013	0.071	0.100	0.119	0.235	0.105	0.597	0.838
Tripneustes	*ventricosus*	*gratilla+depressus*	0.020	0.016	0.026	0.043	0.087	0.224	0.187	0.293	0.491
Echinometra	*oblonga*	*mathaei*	0.029	0.021	0.054		0.023	1.237	0.905	2.328	
	oblonga	*type A*	0.036	0.024	0.076		0.032	1.118	0.757	2.371	
	mathaei	*type A*	0.032	0.028	0.051		0.024	1.314	1.169	2.107	
Strongylocentrotus	*purpuratus*	*pallidus*	0.031	0.021	0.062	0.053	0.072	0.425	0.287	0.863	0.740
	purpuratus	*droebachiensis*	0.044	0.031	0.086	0.048	0.075	0.588	0.418	1.148	0.643
	purpuratus	*H. pulcherrimus*	0.092	0.073	0.158	0.090	0.104	0.886	0.704	1.514	0.865
	pallidus	*droebachiensis*	0.030	0.025	0.036	0.032	0.035	0.843	0.715	1.011	0.915
	pallidus	*H. pulcherrimus*	0.081	0.066	0.119	0.073	0.070	1.159	0.941	1.696	1.047
	droebachiensis	*H. pulcherrimus*	0.083	0.063	0.139	0.066	0.094	0.887	0.672	1.481	0.705

NOTE.—See text for the source of the data.

[a] Distances are the means of all pairwise comparisons.

[b] Kimura 2-parameter (K2P) method (Kimura 1980).

[c] Replacement substitutions (d_N) and silent substitution (d_S) rates calculated by the Pamilo and Bianchi (1993) and Li (1993) method.

approximately the same length. Its structure resembles that of the bindins of other Camarodont sea urchins (*Echinometra*, *Strongylocentrotus*, and *Lytechinus*) more than the bindin of the Stirodont *Arbacia*. The resemblances include the glycine-rich repeat structure 5′ of the core and the lack of the 3′ hydrophobic domain of *Arbacia* bindin. These similarities to the other Camarodonts are not surprising, given that *Tripneustes* last shared a common ancestor with *Echinometra*, *Strongylocentrotus*, and *Lytechinus* 25–60 MYA, and with *Arbacia* 120–180 MYA (Smith 1989; Smith, Lafay, and Christen 1992).

Despite the similarities in structure of *Tripneustes* bindin with that of other Camarodont sea urchins, its mode of evolution appears to be more like that of *Arbacia*. In contrast to *Echinometra* or *Strongylocentrotus* bindin, *Tripneustes* bindin has evolved slowly. For example, bindin of *T. ventricosus* from the Caribbean differs from bindin of the eastern Pacific *T. depressus* by only four fixed amino acid changes and a single indel. These two species were presumably separated from each other for more than 3 million years by the Isthmus of Panama (Coates and Obando 1996). There are no fixed amino acid differences or indels between T. *depressus* and *T. gratilla* from the Indo-Pacific, despite the tremendous geographical distance separating the eastern Pacific from the West Indian Ocean. This level of differentiation is almost as low as that seen in the bindin of *Arbacia*, in which only a single indel distinguishes species on either side of Central America (Metz, Gomez-Gutierez, and Vacquier 1998). It contrasts with differentiation in *Echinometra*, in which species separated for less than 1.5 MY have 7 fixed amino acid differences (Metz and Palumbi 1996) and in *Strongylocentrotus* in which *S. purpuratus* and *S. droebachiensis*, separated for less than 3 MY, show 21 amino acid differences (Biermann 1998). In addition, bindin of *Tripneustes*, like that of *Arbacia*, has only one indel, whereas indels are much more numerous in *Echinometra* and *Strongylocentrotus*. Such indels may be functionally important in gamete recognition (Palumbi 1999). Bindin of *Tripneustes*, like that of *Arbacia*, shows no evidence of diversifying selection that would manifest itself in significantly more replacement substitutions than silent nucleotide substitutions. Finally, *Tripneustes* and *Arbacia* show a lower ratio of bindin to COI substitutions than *Echinometra* or *Strongylocentrotus*, despite the inevitable underestimation of bindin divergence in the latter two genera, arising from the exclusion from the analysis of the most variable regions, which could not be aligned. What could account for such similarities between distantly related taxa and differences between closely related ones?

One possible explanation might be the relative age of the species in different genera. By COI and intron divergence (but not by the number of silent substitutions in bindin) the extant species of *Arbacia* and *Tripneustes* diverged earlier from each other than did the species of *Echinometra* and *Strongylocentrotus*. If, as Civetta and Singh (1995, 1998) have suggested for sex-related genes of Drosophila, the episodes of adaptive bindin evolution are concentrated to the time that new species are formed, and if positive selection on the molecule is subsequently relaxed, then older species may lose the signature of an excess of replacement substitutions relative to silent substitutions. This explanation cannot, however, be applied to bindin. A concentration of adaptive changes at the moment of speciation would decrease the ratio of replacement substitutions to silent substitutions in older species. Increased time since speciation would also decrease the ratio of bindin to COI divergence. However, longer time since speciation could not decrease the absolute number of amino acid changes fixed between species after they have been accumulated early in divergence. The low number of such replacements in *Arbacia* and *Tripneustes* must therefore indicate that they have never gone through a period of accelerated bindin

divergence. This possibility is also supported by our analysis of the apportionment of replacement and silent substitutions along branches of the *Tripneustes* bindin tree, which failed to show a concentration of adaptive change in younger (or older) lineages.

Metz, Gomez-Gutierez, and Vacquier (1998) considered three hypotheses as possible explanations for the decelerated bindin evolution in *Arbacia*: (1) high gene flow, (2) functional constraint, and (3) degree of overlap of species distributions. First, they suggested that high levels of gene flow and a lack of population subdivision within species of *Arbacia* may limit the rate of bindin evolution. *Tripneustes*, like *Arbacia*, shows low levels of biogeographic subdivision, at least in the Pacific (Lessios, Kane, and Robertson, in preparation). However, it is not clear that these two genera are exceptional among echinoids in that respect. High gene flow over thousands of kilometers is a standard feature of all sea urchin species with planktonic larvae (Palumbi and Wilson 1990; Palumbi et al. 1997; Lessios et al. 1999; McCartney, Keller, and Lessios 2000; Lessios, Kessing, and Pearse 2001). In particular, the observations of minimal population subdivision over a range of several thousand kilometers in Indo-West Pacific *Echinometra* (Palumbi et al. 1997) and in eastern Pacific *S. purpuratus* (Palumbi and Wilson 1990) or *S. franciscanus* (Debenham et al. 2000) indicate that the accelerated interspecific bindin divergence relative to *Arbacia* and *Tripneustes* cannot be attributable to this factor alone.

Second, Metz, Gomez-Gutierez, and Vacquier (1998) proposed that slow bindin evolution in *Arbacia* might be due to functional constraints imposed by its molecular structure. They suggested that lack of repeat elements and indels, as well as the presence of a 3′ hydrophobic domain, represent evolutionary constraints on *Arbacia* bindins that are not shared by bindins of Camarodonts. *Tripneustes* bindin, despite three repeats and a lack of 3′ hydrophobic domain, evolves almost as slowly as *Arbacia* bindin. It appears, therefore, that these features of the molecule do not necessarily affect its evolutionary rate.

The third hypothesis of Metz, Gomez-Gutierez, and Vacquier (1998) was that positive selection, arising from the presence of sympatric congeners, is operating on bindin of *Echinometra* and *Strongylocentrotus*, but is absent in *Arbacia*. *Echinometra* and *Strongylocentrotus* contain sympatric species, which are generally gametically incompatible (R. R. Strathmann 1981; M. F. Strathmann 1987 [p. 522]; Uehara, Asakura, and Arakaki 1990; Palumbi and Metz 1991). All extant species in *Tripneustes* and *Arbacia* are allopatric. There are no data about gametic compatibility between *Tripneustes* species, but *A. punctulata* and *A. incisa* are gametically compatible (Metz, Gomez-Gutierez, and Vacquier 1998). Thus, it is possible that reinforcement of reproductive isolation among the sympatric species of *Echinometra* and *Strongylocentrotus* may explain the observed patterns of bindin evolution. This is a *pattern* of reinforcement (Noor 1997) on the genus level. However, as is also true for these patterns on the population level (Butlin 1987; Noor 1999), we do not know the *process* that produced them. Did the presence of congeners create selective pressures for bindin divergence in *Strongylocentrotus* and *Echinometra*, or did divergence in bindin, caused by selection arising from another cause, permit species in these genera to invade the same area and coexist without fusing or becoming extinct? This question cannot be answered with certainty, but the patterns of intraspecific and interspecific divergence can provide a clue. If selection to avoid hybridization were responsible for the reinforcement pattern, we would have expected an excess of amino acid replacements between species (particularly between sympatric species) of *Echinometra* and *Strongylocentrotus* relative to within species. This however, is not the case in either genus. In the rapidly evolving 40 codon bindin region of *Echinometra*, the ratio of replacement substitutions to silent substitutions between alleles is larger than unity in both intraspecific and interspecific comparisons, and McDonald-Kreitman (1991) tests are not significant (Metz and Palumbi 1996). In the same region of bindin, comparisons between *S. franciscanus* and either *S. purpuratus* or *S. droebachiensis* indicate that the ratio of replacement substitutions to silent substitutions is higher within than between species (Debenham, Brzezinski, and Foltz 2000). Palumbi (1999) has shown experimentally that, in *Echinometra*, males carrying different bindin alleles have different rates of success in fertilizing females, depending on the female bindin genotype, and that intraspecific polymorphism is thus maintained by selection. Such selection could arise from male heterozygote advantage, nontransitive female preferences (Palumbi 1999), or interlocus conflict evolution between the sexes (Rice and Holland 1997). These processes can operate within species whether or not they are sympatric with a congener (Metz, Gomez-Gutierez, and Vacquier 1998). Thus, it is possible that the selective force that accelerates bindin evolution in some genera is not avoidance of hybridization in sympatry. The pattern of reinforcement suggested by the comparison between genera with sympatric and allopatric species may be due to the ability of congeneric species with divergent bindin to coexist.

Because *Echinometra* was the first genus in which evolution of bindin was studied, and because its rapid evolution under positive selection fit patterns found in other genes related to sexual reproduction (Civetta and Singh 1995, 1998; Lee, Ota, and Vacquier 1995; Vacquier, Swanson, and Lee 1997; Ferris et al. 1997; Tsaur and Wu 1997; Aguadé 1999; Hellberg and Vacquier 1999; Wyckoff, Wang, and Wu 2000; Swanson et al. 2001), it has been tacitly assumed that the patterns of positive selection and rapid allele divergence exemplify the mode of evolution of this locus (e.g., Palumbi 1998; Wyckoff, Wang, and Wu 2000; Van Doorn, Luttikhuizen, and Weissing 2001). Biermann's (1998) evidence from *Strongylocentrotus* reinforced this view. However, the information from *Tripneustes* now indicates that what Metz, Gomez-Gutierez, and Vacquier (1998) found in *Arbacia* was not an isolated exception, and not a characteristic of stirodont versus camarodont sea urchins. Why bindin should evolve faster in certain genera of sea urchins and not others remains unclear, but the addition of data from more sea urchin taxa will help determine the factors that promote or retard evolution in this molecule.

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