The Molecular Evolution of Sperm Bindin in Six Species of Sea Urchins (Echinoida: Strongylocentrotidae)

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The acrosomal protein bindin attaches sperm to eggs during sea urchin fertilization. Complementary to ongoing functional biochemical studies, I take a comparative approach to explore the molecular evolution of bindin in a group of closely related free-spawning echinoid species. Two alleles of the mature bindin gene were sequenced for each of six species in the sea urchin family Strongylocentrotidae. The nucleotide sequences diverged by at least 1% per Myr at both silent and replacement sites. Two short sections flanking the conserved block show an excess of nonsynonymous substitutions. Each is homologous to a region that had been identified as a target of selection in other sea urchin comparisons. A large proportion of the bindin-coding sequence consists of a highly variable repeat region. Bindin sequences, even including the large intron, could not resolve the branching order among five of the species.

Introduction

For several sympatric groups of free-spawning marine animals, gamete recognition proteins have been found to be under positive selection for interspecific divergence (Vacquier and Lee 1993; Swanson and Vacquier 1995; Metz and Palumbi 1996). The sea urchin family Strongylocentrotidae contains at least nine externally fertilizing species, all of which occur in the North Pacific with partly overlapping ranges (Jensen 1974; Bazhin 1998). If their sperm bindin is involved in reproductive isolation, evolutionary analyses of the gene may indicate natural selection or functional domains. This is particularly interesting, as the genus *Strongylocentrotus* has been the primary model system for biochemical analyses of bindin (e.g., Glabe and Clark 1991; Minor et al. 1991).

Bindin is involved in the species-specific recognition between sperm and eggs in echinoid echinoderms, serving to bind sperm cells to the egg surface. The protein is exocytosed from the vesicle at the tip of the sperm head when sperm undergo the acrosome reaction (Vacquier and Moy 1977; Gao et al. 1986). Although other phases during sperm–egg recognition can be species-specific (SeGall and Lennarz 1979; unpublished data), the interaction of bindin with the egg surface is a key stage, one that has received much recent attention (e.g., Foltz and Lennarz 1993; Hofmann and Glabe 1994; Vacquier, Swanson, and Hellberg 1995; Cameron et al. 1996; Metz and Palumbi 1996).

Ideally, both sides of a recognition system should be examined jointly, but it is probably not a straightforward matter to pinpoint key recognition sites in the gene for the putative sperm receptor on the egg surface (Glabe and Vacquier 1978; Giusti, Hoang, and Foltz 1997; Mauk et al. 1997). This is because the interaction of bindin with its receptor is a multistep process involving both the receptor's carbohydrate components and the polypeptide backbone (Foltz 1994; Stears and Lennarz 1997). Bindin, on the contrary, is 100% protein (Minor, Gao, and Davidson 1989), and therefore potentially contains specificity information in its primary structure. With recombinant deletion mutants, Lopez, Miraglia, and Glabe (1993) showed that either of the two variable domains of bindin, on either side of the conserved middle region, is sufficient to impart specific gamete agglutination between *Strongylocentrotus franciscanus* and *Strongylocentrotus purpuratus.* Minor, Britten, and Davidson (1993) identified a number of peptides, small bindin segments of these two congeners, that interfere with sperm–egg binding. On average, the joint effect of three peptides was necessary to inhibit fertilization. One 30-residue peptide, matching the *S. franciscanus* sequence, showed inhibitory effects significantly different between the two species.

Strongylocentrotus franciscanus and *S. purpuratus* have overlapping ranges and spawning seasons (Strathmann 1987). However, they are separated evolutionarily by at least 20 Myr (Smith 1988), during which accumulation of silent or multiple substitutions may have obscured molecular traces of initial divergent selection. It may be more informative to compare mating barriers in more recently derived species (Palumbi 1994; Coyne and Orr 1997). This study analyzes sperm bindin for the species that bridge the phylogenetic gap between *S. franciscanus* and *S. purpuratus.* Two alleles of the entire mature bindin were sequenced for each of six species, and their molecular evolution is compared between exons and introns and to a protein-coding region of mitochondrial DNA.

Materials and Methods

Samples and Sequencing

The six sea urchin species were sampled from around the Pacific Ocean between 1994 and 1996. The species abbreviations are as follows: drob, *Strongylocentrotus droebachiensis*; pall, *Strongylocentrotus pallidus*; purp, *S. purpuratus*; poly, *Strongylocentrotus polyacanthus*; Allo, *Allocentrotus fragilis*; Hemi, *Hemicentrotus pulcherrimus.* PurpCA and Allo3 were from California, Allo7 was from the Eastern Bering Sea, and

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the *Hemicentrotus* individual (both bindin alleles sequenced) was from Japan. ''F'' stands for Friday Harbor, Wash.; ''K'' for Kamchatka, Siberia, and ''R'' for Resolute, Northwest Territories. The species identity of each animal was confirmed with mitochondrial DNA. Part of the ATPase subunit 6 gene was sequenced for those individuals that were not included in a larger mitochondrial phylogenetic study (Kessing 1991; unpublished data). Sequences from the mitochondrial project provided an independent locus for all of the species in this study, referred to as ''mtDNA'' below. The sequences include two regions of mitochondrial DNA: one covering 145 codons of the CO1 gene, corresponding to positions 6835–7270 in the complete *S. purpuratus* mitochondrial sequence (Jacobs et al. 1988), and the other stretching from the end of CO2 through ATPase 8 into ATPase 6 (positions 8356–9005 in Jacobs et al. 1988), excluding the Lys-tRNA.

PCR primers were designed for near the 3′ end of prebindin upstream of the cleavage site between prepro- and mature bindin, and, after RT-PCR, for the untranslated region 3′ of the stop codon. Sequencing primers were spaced no more than 300 bp apart on both strands. Primer locations and sequences are available from the author.

DNA was isolated from gonad tissue, PCR-amplified, and initially sequenced directly (with Sequenase from Amersham, following the method of Khorana, Gagel, and Cote 1994). However, because of the frequency of heterozygous length mutations, most PCR products had to be cloned before sequencing (pGEM-T vector from Promega, transformation into XL Blue *Escherichia coli*). In spite of using DNA polymerases with proofreading ability (ExTaq and LATaq from TaKaRa, Bio-X-Act from BioExpress, Expand High Fidelity from Boehringer Mannheim), a substantial number of point mutations and recombinations between alleles were evident in the cloned PCR products (e.g., Bradley and Hillis 1997). Hence, at least two clones were sequenced in their entirety for each allele; whenever there was a discrepancy, an additional clone was sequenced until two identical sequences were obtained. Mitochondrial DNA was always sequenced directly. The mature bindin sequences have been deposited in GenBank under accession numbers AF077309–AF077321.

Sequence Analyses

GCG (Wisconsin Package, Version 9.1, Madison, Wis.) was used to explore the sequence structure and provided the initial alignment (PileUp, endweighted), which was then corrected manually. Because of questionable homology, both the repeat region and the polyglycine stretch were excluded from phylogenetic sequence analyses or treated separately.

Nucleotide diversity between the two alleles from each species (π; Nei 1987) and net interspecific divergence (D_a, Nei 1987) were calculated with DnaSP (Rozas and Rozas 1997) in sliding windows along the sequence. Windows were 100 nt in length and moved in steps of 25 nt. Both nonsynonymous and silent substitutions were included. Amino acid frequencies and codon usage, as well as base composition percentages of the nucleotide sequences, were calculated for one allele per species in MEGA (Kumar, Tamura, and Nei 1993).

The number of nonsynonymous (amino acid replacement) substitutions per nonsynonymous site and the number of synonymous (silent) substitutions per silent site (D_n and D_s, respectively; Lee, Ota, and Vacquier 1995; Hughes 1997) were estimated by Nei and Gojobori's (1986) method. D_n and D_s and their standard errors were computed in MEGA (Kumar, Tamura, and Nei 1993) for sliding windows along the sequence. Windows were 30 codons in length and moved by 10 codons for each step, making the plot directly comparable to figure 3 in Metz and Palumbi (1996). When either D_n or D_s exceeded 0.4 for a particular window, the p distance (proportion of differences) was used instead of the Jukes-Cantor corrected distance for both D_n and D_s in that window (Kumar, Tamura, and Nei 1993).

Repeat Network

Because orthology/paralogy relationships in the repeat region are uncertain, the individual segments were taken apart. A name (single letter) was assigned to each of the variations of the motif to visualize the order of their occurrence in the alleles. There are too few informative sites in the 21-nt repeat to construct an overall bifurcating tree of the 29 different motifs. Therefore, a mutational network was constructed from a pairwise distance matrix of individual repeats (Templeton, Crandall, and Sing 1992; Crandall and Templeton 1993). This method was developed for population-level data with small numbers of changes; it evaluates parsimonious connections between alleles on a pairwise basis. Networks are useful for population data (including a ''population'' of repeats within a gene), because they include ancestral sequences as internal nodes and allow the depiction of ambiguities (Crandall and Templeton 1996; Fitch 1996).

Phylogenetic Sequence Analyses

Phylogenetic trees were reconstructed with the help of PAUP* (test versions 4.0.0d59–63). A minimum-evolution (distance) tree delineates the overall relationships of 13 bindin sequences, excluding the repeat region (fig. 6). The total number of nucleotides from the (nonrepeat) coding region and the intron, aligned including the *S. franciscanus* coding sequence (Minor et al. 1991), was 1,558. I employed LogDet distances and rooted the tree at the midpoint. Regions with alignment gaps were excluded from pairwise comparisons only. The tree topology, and particularly the full bootstrap support joining the two sequences from each species, was robust under various distance measures.

Bindin sequence phylogenies were further explored with only one sequence per species, with *Hemicentrotus* as outgroup. Due to the small number of parsimony-informative sites in bindin (table 2), little confidence can be placed in parsimony-based phylogenies. Maximum-likelihood and distance trees were also constructed separately for the nonrepeat bindin-coding sequences and for the intron. A 1,005-nt region of the mitochondrial

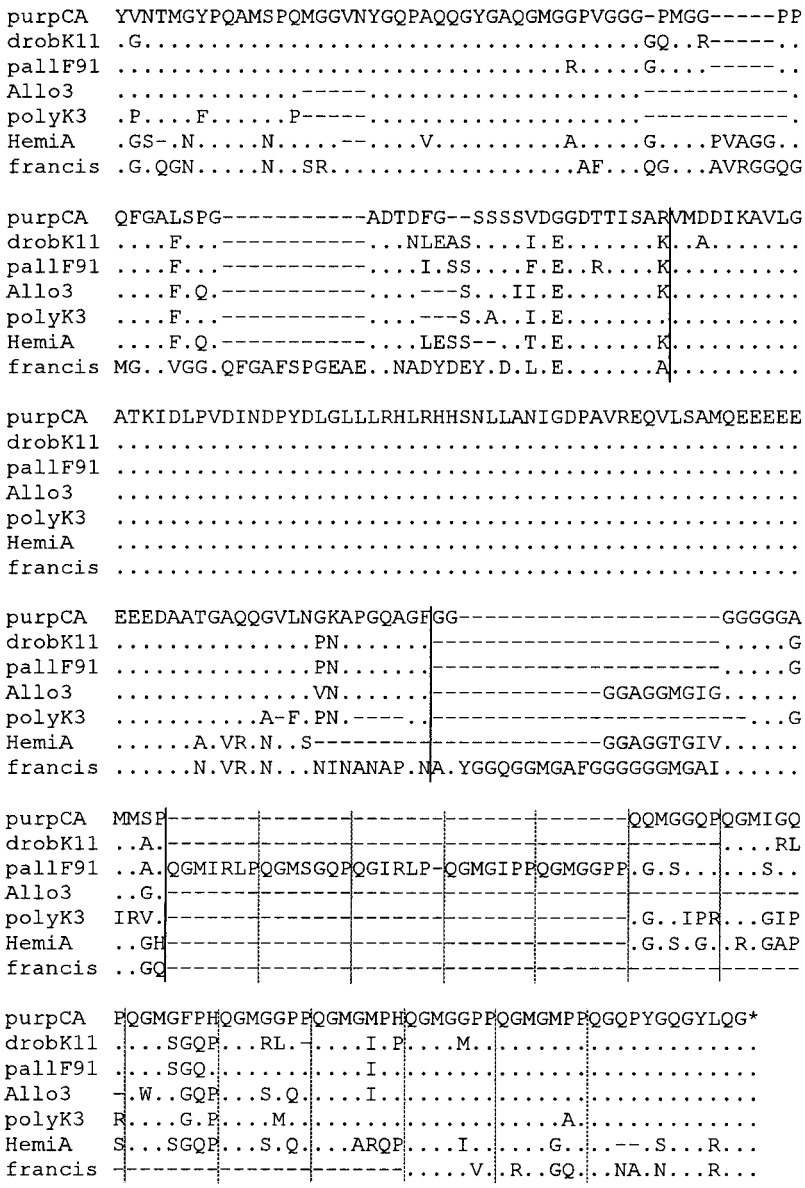

FIG. 1.—Bindin amino acid sequences (one per species). The first black line denotes the intron position and the start of the conserved block; the next two lines bracket the glycine-rich region. The repeats—arbitrarily right-aligned—are separated by light lines. francis = published *S. franciscanus* sequence (Minor et al. 1991).

genome (Kessing 1991; unpublished data) was used to independently examine the relationships between the species and genera.

Results

General Structure

As expected from intergeneric comparisons (Glabe and Clark 1991; Minor et al. 1991; Metz and Palumbi 1996), the central coding region of mature bindin is highly conserved within and across species (fig. 1). Just upstream, a large intron inserts in strongylocentrotid sea urchins in a conserved position (fig. 2; Metz and Palumbi 1996; Metz, Gomez-Gutierrez, and Vacquier 1998). It is more than twice as long as the intron found in the genus *Echinometra* (Metz and Palumbi 1996), and generally conserved enough to permit alignment across species. The only exception is the 5′ half of the intron in *A. fragilis*, which is extremely divergent from this region in the sibling species. Because positional homology is not attainable when aligning that region, *Allocentrotus* was omitted from the main graphs in figure 2 (but see the dotted line), and for phylogenetic analyses employing the intron, this 364-bp region was replaced by ''unknown'' characters. It contains multiple long runs of single bases in *Allocentrotus* sample 3, which caused stuttering of both the PCR and sequencing enzymes. Probably for the same reason, it was not possible to determine this part of the intron sequence for the other *Allocentrotus* individual (sample 7).

Nucleotide and Amino Acid Composition

Base composition in bindin differs significantly between the three codon positions, with small numbers of

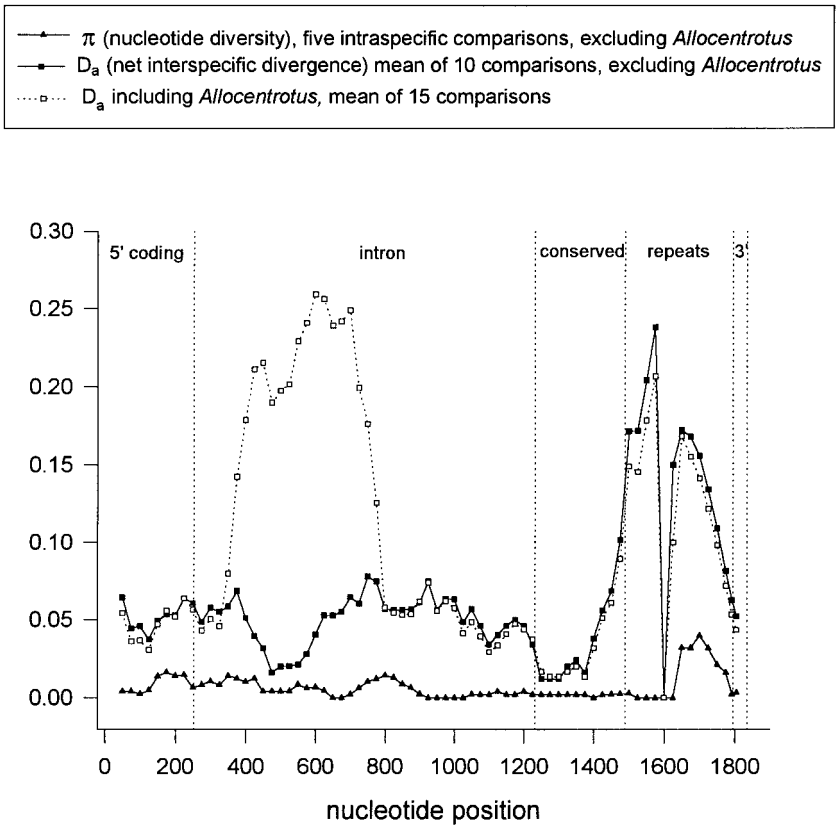

FIG. 2.—Sliding-window comparison of nucleotide diversity π (Nei 1987; between two alleles within each of five species) and of net interspecific divergence D_a (number of net nucleotide substitutions per site; Nei 1987). The repeated amino acid motifs (''repeats'') were right-aligned.

Ts and large numbers of Gs, especially in the first position, and many purines in the second codon position (not shown). There is a significant difference in base composition between the bindin-coding region and the intron ($P < 0.00001$, *G*-test with Williams correction; Sokal and Rohlf 1995); i.e., the bias against T's and for Gs is not specific to this part of the chromosome in general. Base composition in coding mitochondrial DNA differs significantly from that in the coding bindin sequence, but not from that in the bindin intron ($P < 0.00001$ and $P = 0.211$, respectively).

The conserved region, which is involved in the binding process (Miraglia and Glabe 1993), has a relatively balanced amino acid composition. It contains a large number of nonpolar residues, many aspartic and glutamic acids, and positively charged residues. A tract of glutamic acid residues near the N-terminus of the conserved block (fig. 1) represents the only area of the protein that is clearly alpha-helical, negatively charged, and hydrophilic. The regions flanking the conserved central part contain a large proportion of turn residues like glycines and prolines; i.e., bindin appears to not form much secondary structure, and so far, it has not been possible to crystallize it for X-ray crystallography. Almost all codons are being used, in fairly equal proportions (not shown).

Repeats

The number of repeats of a seven-amino-acid motif near the C-terminus of the protein is highly variable in this family: *S. purpuratus*, *S. polyacanthus*, and *Hemicentrotus* typically have 7 of these repeats, *Allocentrotus* has 5, *S. droebachiensis* has between 6 and 9, and *S. pallidus* has around 12 (figs. 1 and 3). This region apparently diversified within this group. The basal strongylocentrotid, *S. franciscanus*, has only two repeats, and *Lytechinus variegatus*, in a different family, has this motif only once (Minor et al. 1991).

Figure 3 lists the nucleotide and amino acid sequences of the individual repeats and the order in which they occur in representatives of the different species. Letters with an asterisk represent repeats that differ from the nonasterisked equivalents by a silent substitution; different letters stand for repeats that differ at the protein level. The repeats contain 18–21 nt, three of which are completely invariant; hence, there is too little information to reconstruct their evolution unequivocally.

Figure 4 shows the relationships between nucleotide sequences of individual repeats as a cladistic network. Small zeroes indicate inferred haplotypes that were not present among the repeat samples. Thick black bars show direct connections between haplotypes which are parsimonious (A. R. Templeton's Mathematica program ParsProb; Templeton, Crandall, and Sing 1992). Connections with triple lines are marginally parsimonious (94% probability), with 2 bases out of 21 substituted (one inferred intermediate). Changes between repeats that differ by three substitutions are not parsimonious

A	CAAGGGATA---AGACTTCCG	QGI-RLP
B	CAAGGGATG---AGACTTCCG	QGM-RLP
C	CAAGGGATGGCCAGGCAACCT	QGMARQP
D	CAAGGGATGGGCTTTCCACAT	QGMGFPH
E	CAAGGGATGGGCGGACCACCT	QGMGGPP
E*	CAAGGGATGGGCGGACCACCC	QGMGGPP
F	CAAGGGATGGGCGGACAACCT	QGMGGQP
G	CAAGGGATGGGCGGGGTTCCT	QGMGGVP
H	CAAGGGATGGGCATACCACAT	QGMGIPH
I	CAAGGGATGGGCATACCACCT	QGMGIPP
J	CAAGGGATGGGCATACCACGT	QGMGIPR
J*	CAAGGGATGGGTATACCACGT	QGMGIPR
K	CAAGGGATGGGCATGGCACCT	QGMGMAP
L	CAAGGGATGGGCATGCCACAT	QGMGMPH
M	CAAGGGATGGGCATGCCACCT	QGMGMPP
N	CAAGGGATGGGCATGCCACAA	QGMGMPQ
O	CAAGGGATGATCGGACAACCT	QGMIGQP
P	CAAGGGATGATAAGACATCCT	QGMIRHP
Q	CAAGGGATGATCAGACTTCCT	QGMIRLP
R	CAAGGGATGAGCGGTGGTCCT	QGMSGGP
S	CAAGGGATGAGCGGACAACAT	QGMSGQH
T	CAAGGGATGAGCGGACAACCT	QGMSGQP
T*	CAAGGGATGAGTGGACAACCT	QGMSGQP
U	CAAGGAATGGGTGGACAAGGT	QGMGGQG
V	CAACAGATGGGCGGACAACCT	QQMGGQP
W	CAAAGGATGGGCGCACCTTCC	QRMGAPS
X	CAAAGAATGGGTGGACAACCT	QRMGGQP
Y	CAATGGATGGGCGGACAACCT	QWMGGQP
Z	ACATGGATGGGCGGACAAGCT	TWMGGQA

S. purpuratus	V-O-D-E*-L-E-M
S. droebachiensis-1	Q-T-B-I-M-M
S. droebachiensis-2	T-P-T-J-J-E-N-E-M
S. pallidus-1	Q-T-A-I-E-T-T-S-F-H-E-M
S. pallidus-2	Z-T-S-E-T-T-S-E-H-E-M
Allocentrotus	Y-T-H-E-M
S. polyacanthus	J*-J-E-M-L-E-K
Hemicentrotus	R-W-T*-T-C-I-E
S. franciscanus	G-X
Lytechinus	U

FIG. 3.—Individual repeats from the 3′ region of sperm bindin. Each variation of the motif is given a one-letter name, and its nucleotide and amino acid sequences are shown. Asterisks denote silent changes. The order of occurrence of these motifs in representatives of the different species is shown below. For *S. droebachiensis* and *S. pallidus*, individual ''1'' is from the Pacific Ocean, and individual ''2'' is from the Atlantic Ocean.

according to Templeton's criteria; i.e., if 3 out of 21 bases have mutated, we expect that invisible multiple hits have occurred. These connections are tentatively marked by thin lines through two inferred intermediates. Motifs that differ by more than three changes remain unconnected.

The network permits inferences about whether identical motifs are more likely to be convergent (possibly the ''J'' repeats in *S. droebachiensis*-2 and *S. polyacanthus*) or orthologous to each other (repeats ''Q-T-A/B-I'' in *S. droebachiensis*-1 and *S. pallidus*-1; figs. 3 and 4). Only about one in seven mutations among the repeats in figure 4 is a synonymous change. The unusual amino acid composition of the repeats alone is not responsible for the excess of replacements: random point mutations imposed on the motifs resulted, on average, in 1 change out of 4.6 being silent.

In spite of the uncertain homology when the repeats are aligned in the order found in the gene, the magnitude of both polymorphism and divergence in this region is striking (fig. 2). The repeated segments appear to predispose bindin to rapid evolutionary change but, at the same time, make it difficult to recognize whether natural selection has promoted this mutability (Hughes 1991). Upstream of the tandem duplications is a glycine-rich stretch that is also very variable in length. Especially in these regions of bindin, blocks of similar sequence appear to have been retained between species since recombination events during the divergence of these lineages (fig. 1).

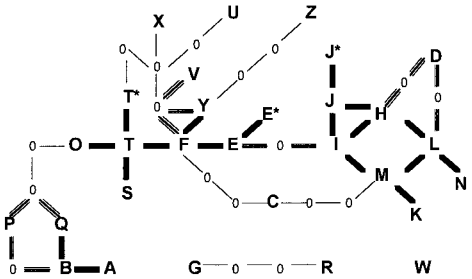

FIG. 4.—Relationships between nucleotide sequences of individual repeats from the 3′ region of sperm bindin (fig. 3). A cladistic network was constructed based on the parsimony criteria by Templeton, Crandall, and Sing (1992). Only the heavy bars and the triple lines symbolize parsimonious connections (see text). Small zeroes indicate inferred intermediate haplotypes. Repeat variants that differ by more than three changes from any others were not connected to the main network.

Synonymous and Nonsynonymous Substitution Rates

D_n and D_s increase linearly with the phylogenetic distance of the alleles compared (plot not shown). The rate (per available site) of amino-acid-altering substitutions was slightly lower than that of silent changes in almost all pairwise comparisons. It is nevertheless very high compared to those for most other proteins: D_n/D_s ratios for bindin are generally an order of magnitude higher than those for mitochondrial genes (see also fig. 5). The many insertions and deletions between bindin sequences can be considered nonsynonymous changes as well (Aguadé, Miyashita, and Langley 1992), and are not included in the D_n/D_s ratios. Moreover, the substitutions among repeats are almost exclusively nonsynonymous, but the repeat region was excluded from pairwise comparisons due to alignment ambiguity.

The sliding-window comparison clearly shows the absence of replacement substitutions in the conserved region (fig. 5, codons 100–150). Both D_n and D_s vary widely along the rest of the gene, with D_n significantly exceeding D_s in three locations when the five most closely related species are compared (marked by asterisks in fig. 5, including two regions composed of two adjacent significant windows each). Because one of these three windows is in the repeat region, it was not analyzed further. For the other two significant windows (white boxes in fig. 5), D_n and D_s were contrasted on a pairwise basis between individual species (table 1).

Phylogenetic Trees

Bindin and mitochondrial DNA sequences indicate that both *Allocentrotus* and *Hemicentrotus* fall phylogenetically within the genus *Strongylocentrotus* (fig. 6).

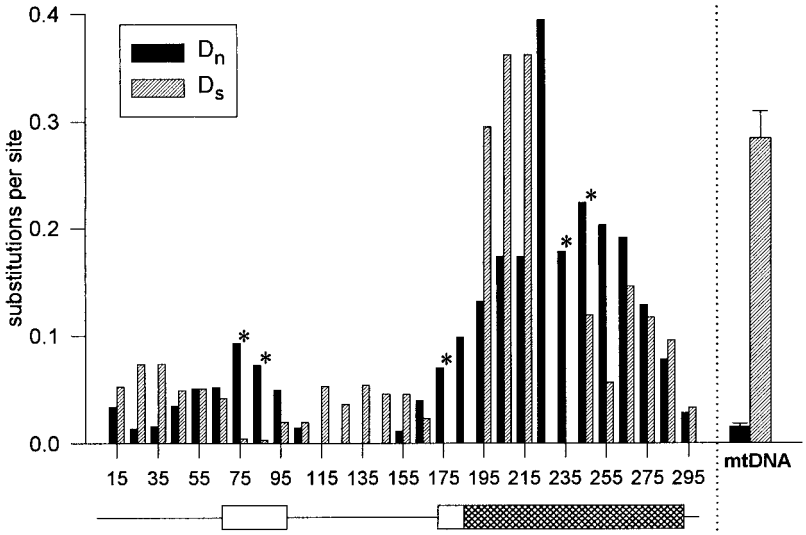

FIG. 5.—Nonsynonymous changes per nonsynonymous site (D_n) and silent changes per silent site (D_s) in sliding windows 30 amino acids in length along the coding sequence of bindin. Pairwise differences between one individual each of *S. purpuratus, S. droebachiensis, S. pallidus, S. polyacanthus,* and *A. fragilis* are shown. Asterisks signify windows in which D_n was significantly greater than D_s (Kumar, Tamura, and Nei 1993). The repeat region is marked by the cross-hatched rectangle below. The open rectangle covers a region that was identified by Metz and Palumbi (1996) as being under positive selection in the sea urchin genus *Echinometra,* and the open square corresponds to a peptide recognized as species-specific in *Strongylocentrotus* by Minor, Britten, and Davidson (1993). The bars on the right show Jukes-Cantor corrected D_n and D_s among these five species for 335 codons of mitochondrial sequence.

Strongylocentrotus franciscanus branched off well before these two monospecific genera diverged from the other strongylocentrotid species. The phylogeny of this family has been impossible to ascertain on the basis of morphological characters (O. Ellers and R. Mooi, personal communication); hence, these sequences represent our best estimate of their relationships.

Table 1
Pairwise Significance *t*-tests (Kumar, Tamura, and Nei 1993) of an Excess of Nonsynonymous Substitutions per Site (D_n) Over Silent Substitutions per Site (D_s)

	purp	drob	pall	Allo	poly
purp		*	*	*	
drob					*
pall					*
Allo					
poly	*			*	

NOTE.—Comparisons are for regions identified by sliding windows (fig. 5). To the upper right of the diagonal are tests for the upstream window (codons 61–100; see also Metz and Palumbi 1996), and tests in the lower left are for the downstream window (codons 161–190; see also Minor, Britten, and Davidson 1993). An asterisk denotes significance at $P < 0.05$. purp = *Strongylocentrotus purpuratus,* drob = *Strongylocentrotus droebachiensis,* pall = *Strongylocentrotus pallidus,* Allo = *Allocentrotus fragilis,* poly = *Strongylocentrotus polyacanthus.*

Collapsing branches with less than 50% bootstrap support resulted in polytomies among the most closely related species for both bindin and the mitochondrial data. *Strongylocentrotus purpuratus, S. droebachiensis, S. pallidus, S. polyacanthus,* and *Allocentrotus fragilis* appear to have diverged so rapidly from each other that the internodes may be too short to permit reconstruction of the branching order. This is consistent with the low number of parsimony-informative sites: for example, out of 132 variable sites in the bindin intron, only 8 are parsimony-informative (table 2). Only a maximum-likelihood bootstrap analysis for the bindin intron supports any groupings among these five species (not shown), but these are not retained in distance trees. The alignable coding regions of bindin do not strongly support any bifurcations (data not shown), and the overall bindin tree

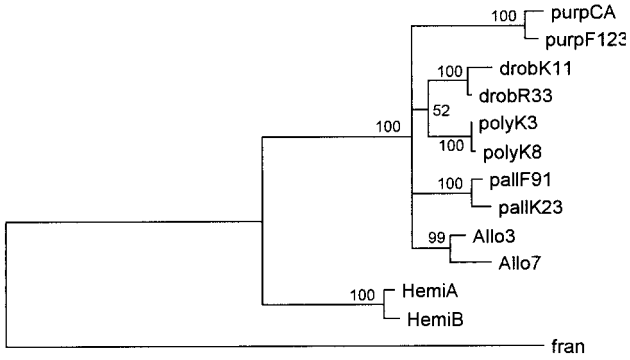

FIG. 6.—Minimum-evolution tree based on LogDet distances between bindin sequences of strongylocentrotid sea urchins (midpoint-rooted). See *Materials and Methods* for species names and sampling locations. The sequences include the nonrepeat coding region and the intron of mature bindin (for *S. franciscanus,* only the coding region is available; Minor et al. 1991). Numbers on nodes indicate bootstrap support (500 replications).

Table 2
Numbers of Characters, as well as Parameters Estimated by Maximum Likelihood, for Different Sequence Subsets in Six Sea Urchin Species (*Strongylocentrotus droebachiensis, Strongylocentrotus pallidus, Strongylocentrotus purpuratus, Strongylocentrotus polyacanthus, Allocentrotus fragilis,* and *Hemicentrotus pulcherrimus*)

	Nonrepeat Coding Bindin	Bindin Intron	mtDNA (coding)
Number of aligned nucleotides	549	979	1,005
Number of variable sites	64	132	179
Parsimony-informative sites	6	8	64
Observed proportion of constant sites	0.883	0.865	0.822
Estimated proportion of invariable sites	0.395	0.0	0.295
Estimated gamma shape parameter	0.845	∞	0.253
Estimated transition/transversion ratio	1.49	0.99	10.32

NOTE.—The nonalignable part of the intron in *Allocentrotus* was excluded.

(fig. 6 shows a minimum-evolution tree) is dominated by the intron sites.

More than a kilobase of mitochondrial coding sequence (table 2) could not resolve the branching order among the five most closely related species either: each optimality criterion or evolutionary model tried resulted in a multifurcation similar to that shown in figure 6.

Discussion

Numerous length mutations in the mature bindin of these six sea urchin species reduce the number of orthologous sites available for evolutionary analysis. Almost one half of the coding region consists of repeated residues and motifs (fig. 1) whose evolutionary dynamics are more complex and much less well understood than those of nucleotide substitutions. Another third of the protein is extremely conserved, which further limits the inference of historical events. Nevertheless, interesting patterns can be gleaned from the sequences.

Amino acid composition differs notably between the conserved region and the rest of the gene. The fact that the central conserved block contains the larger number of hydrophobic residues agrees with the finding that this part associates with phospholipid bilayers and, hence, presumably with the egg membrane in vivo (Kennedy, DeAngelis, and Glabe 1989). This leaves the two variable ends of bindin to interact with the glycoproteins of the egg in a potentially species-specific manner (Lopez, Miraglia, and Glabe 1993). These glycine-rich ends do not contain many charged residues or obvious features predicting secondary (and higher) structure, consistent with bindin's function as an amorphous glue protein.

Bindin is seasonally expressed in large amounts in the testes (Cameron et al. 1990; Nishioka et al. 1990), and the need for efficient translation is thought to select for biased codon usage (Akashi 1997). Bindin's codon bias appears to be low, however. Without comparison with other nuclear genes, it is difficult to assess whether the selection pressure for preferred codons on bindin is weak, or just ineffective due to effectively small populations (Akashi 1997).

Below, I discuss three additional aspects of bindin evolution: the repeat structure, the high rate of nonsynonymous substitutions, and the lack of a conclusive phylogenetic signal.

Repeats

The evolutionary dynamics of repeated DNA sequences has become a major field of investigation in recent years. Generally, the focus has been on noncoding DNA, especially microsatellites (Stephan 1989; Nielsen 1997*a*). The attention is now shifting, since more and more coding repeats are found to be important in human diseases (Lesch et al. 1996; Mandel 1997). Interestingly, tandem repeats are generally found in adhesive proteins like glue (Martin, Mayeda, and Meyerowitz 1988), byssus (Coyne, Qin, and Waite 1997), and silk (Guerette et al. 1996). Repeats in the latter proteins also form glycine-rich amorphous structures. The only other proteins that commonly contain repetitive arrays are recognition molecules, e.g., in parasitic protozoa (Hughes 1993). Both its adhesive and recognition functions could therefore have favored the repetitive character of bindin. While in the strongylocentrotids, the repeats are most evident in the 3′ part of bindin, in *Echinometra*, repeat structures appear in the other half of bindin, upstream of the conserved block (Metz and Palumbi 1996). It will be interesting to find out whether the repeats are important for the general or specific function of bindin.

Repeat regions are thought to be hot spots for unequal crossing over (Smith 1976), but in addition to length mutations, the bindin repeat region is characterized by numerous, largely nonsilent, point substitutions. Although the parsimony network (fig. 4) and the simulations (see *Results*) are based on conventional models of molecular evolution (random and independent nucleotide substitutions), it is unlikely that recombination per se would result in an excess of nonsynonymous substitutions. Generally, multiple copies of a sequence are constantly being homogenized by gene conversion (Dover et al. 1993; Schlötterer et al. 1994; Odorico and Miller 1997). The extreme diversity of the 3′ bindin repeats, coupled with a scarcity of silent substitutions, is highly suggestive of natural selection as the agent responsible for the maintenance of their varied identities. It appears that mutations induced by chromatid breaks during recombination are not eliminated by the

homogenizing effect of concerted evolution, but are maintained by positive selection.

Rate of Nonsynonymous Substitutions

Two abalone sperm proteins have been shown to be subject to strong directional selection, especially in closely related species (Lee and Vacquier 1992; Swanson and Vacquier 1995). This stimulated the exciting notion that gamete recognition proteins may play a crucial role in speciation in marine invertebrates (Palumbi 1992, 1994). Gamete surfaces could be avoiding microbial attack (Vacquier and Lee 1993) or diversifying by intraspecific processes such as sexual selection (Metz and Palumbi 1996). Alternatively, divergence of the surface molecules could be driven by reinforcement of mating barriers upon secondary contact of two incipient species. In either case, the signature of positive Darwinian selection could be a large number of amino acid replacements between species at these recognition loci.

Among the sea urchin bindins examined here, the number of nonsynonymous substitutions is larger than the number of silent mutations, but on a per-site basis, it is on average slightly lower. However, the mean D_n/D_s ratio is much higher for bindin than for mitochondrial proteins (fig. 5). Because D_n and D_s do not differ significantly in bindin overall, the elevated D_n could be due to a relaxation of purifying selection and not necessarily to the action of directional selection. The extreme conservation of the central block speaks against complete neutrality of the substitutions in bindin. Furthermore, all methods tend to underestimate the D_n/D_s ratio (Tsaur and Wu 1997), and the multiple insertion and deletion differences were not counted (see Aguadé, Miyashita, and Langley 1992).

Two phenomena complicate the interpretation of the observed pattern. We do not know whether certain residues of bindin are of particular importance (as we do, e.g., for MHC molecules; Hughes, Ota, and Nei 1990). If this number is limited, selection may be restricted to certain sites, and the number of replacement substitutions would be underestimated (Clark 1993; Nielsen 1997*b*). Second, Lewontin (1989) suggested that it is impossible to estimate the number of evolutionary events if the degree of constraint varies through time. The divergence of the strongylocentrotid sea urchins examined here occurred at least between 3 and 20 MYA, much earlier than that of the tropical *Echinometra* species (Palumbi and Metz 1991; Metz and Palumbi 1996). Even though I sampled this set of species exhaustively, former diversifying selection could have been obscured by subsequent mutations.

The two alleles sampled within each species differed from each other, even on the amino acid level, except for *S. polyacanthus.* Substantial intraspecific polymorphism is incompatible with strong directional selection and would point to frequency-dependent or balancing selection as the agent responsible for maintenance of diversity (Prout and Clark 1996). Clearly, larger population samples are necessary to distinguish modes of selection.

Sliding-window comparisons do not identify an excess of nonsynonymous substitutions when all seven species are compared (not shown), but they do point to three regions with significantly large D_n values in the comparison of the five most closely related species (fig. 5). This confirms that proportionally more silent substitutions accumulated on the long branches leading to *H. pulcherrimus* and *S. franciscanus.* Sliding-window analyses are generally used for data exploration only (e.g., Kreitman and Hudson 1991), because they essentially consist of multiple comparisons (a certain proportion of windows are expected to be significantly different by chance), and spatial autocorrelation makes statistical testing complex. They are nonetheless very useful in identifing pattern changes along a sequence.

Because one of the windows in which D_n significantly exceeds D_s falls in the repeat region (cross-hatched bar below the graph in fig. 5), it is not analyzed further because of uncertain homology. It is fascinating that the other two windows (open boxes below the graph in fig. 5) coincide precisely with parts of bindin that have previously been pinpointed as species-specific—each for a different reason. Window 61–100, just upstream of the conserved block, is in the same position as the region identified as being under diversifying selection in the genus *Echinometra* by Metz and Palumbi (1996). Window 161–190, just downstream of the poly-Glu helix at the end of the conserved region, corresponds precisely to the only peptide of 24 tested that species-specifically inhibited fertilization between *S. franciscanus* and *S. purpuratus* (Minor, Britten, and Davidson 1993).

Pairwise comparisons for these two windows reveal five combinations of species that show an excess of D_n in the first window and two (different) species combinations for the second, shorter window (table 1). Neither of the windows shows a significantly high D_n/D_s in comparisons between *S. franciscanus* or *Hemicentrotus* and the other species (not shown). Only the pairs *S. polyacanthus*/*S. purpuratus* and *S. polyacanthus*/*Allocentrotus* have significantly more nonsynonymous substitutions in the downstream window (lower left part of table 1), and this has to be interpreted with caution because of multiple comparisons. However, the mean D_n/D_s ratio between the seven species for this window is extremely high at 1.98 (and this excludes the half of the comparisons that had a D_s value of zero).

The upstream window, although its mean D_n/D_s ratio is ''only'' 1.39 (excluding 9 of 21 comparisons because of zero in the denominator), has five individual species pairs that show an excess of replacement substitutions. Positive selection is indicated between *S. purpuratus* and its three sympatric close relatives, and between *S. polyacanthus* and the only included species it co-occurs with, *S. droebachiensis* and *S. pallidus* (Bazhin 1998). In combination with the signal in *Echinometra* (Metz and Palumbi 1996), this region should clearly be the target for more population genetic and biochemical investigations.

Phylogenetic Information

The maximum-likelihood parameters in table 2 show important differences among data sets: the proportion of invariable sites estimated and the gamma shape parameter (α) differ dramatically between the bindin intron and exons and mtDNA. The inverse of α is the variance of the gamma distribution; i.e., all sites in the bindin intron evolve at essentially the same rate, while the distribution is much more skewed in coding bindin and mtDNA. Combined with its extreme transition over transversion bias, mtDNA appears to require a very different substitution model. When the data sets were combined anyway (into 2.8 kb of ''total-evidence'' nucleotide sequence), an unequivocal resolution of the branching order between these species was still not possible (data not shown). This suggests that the inability to systematize them on the basis of adult morphology may be due not only to ecological convergence and plasticity, but also to a lack of synapomorphies in the morphological data set.

The lack of cladistic information indicates that the time intervals between speciation events were relatively brief (see the short internodes in fig. 6). Interestingly, these two loci were expected to be particularly well suited to untangling the branching order. Mitochondrial DNA has a low effective population size (e.g., Avise 1994) and is therefore expected to coalesce into reciprocal monophyly faster than nuclear loci (Moore 1997). Furthermore, bindin sequences, if involved in speciation, are also expected to separate before an average nuclear gene (Hey 1994). This is because assortative mating within incipient species should occur earlier with respect to a mate recognition gene than with respect to any other gene.

Lee and Vacquier (1995) successfully inferred the phylogenetic relationships for over 20 species of *Haliotis* gastropods from sequences of sperm lysin. In that study, the sperm protein locus contained enough information, even though the strong directional selection on *Haliotis* lysin (Lee, Ota, and Vacquier 1995) would seem to violate the assumptions of some tree-building methods. Indeed, S. Palumbi (personal communication) demonstrated that sperm bindin resolves the relationships among *Echinometra* sea urchins better than does mitochondrial DNA; i.e., the selection on bindin makes its genealogy coalesce into species clades faster than does the genealogy of mtDNA, even though the latter has the smaller effective population size.

In contrast to vertebrates, for which mitochondrial sequence divergence is up to an order of magnitude faster than nuclear divergence, mtDNA in sea urchins seems to evolve at approximately the same rate as does nuclear DNA (Vawter and Brown 1986; Jacobs 1988). The mean distances between bindin sequences of these six strongylocentrotid species range from 2% to 10% in alignable bindin and from 4% to 11% in mtDNA. Most changes in the mitochondrial sequence are silent (mean distance between the six species is less than 2% for nonsynonymous sites and more than 30% for silent sites) and generally clocklike (Bermingham and Lessios 1993), but because mtDNA is one linked unit, assuming that it is a neutral marker may not be justified (Ballard and Kreitman 1994; Hey 1997). Other nuclear genes may, of course, show a slower substitution rate than bindin. The exact evolutionary rate has no bearing on the fact that few mutations occurred between speciation events relative to the many autapomorphies that accumulated since then in both genomes. We may have to accept the existence of effectively hard polytomies (Hoelzer and Melnick 1994), potentially caused by biogeographic events which may not be resolvable millions of years later.

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