Rapidly Evolving Genes and Genetic Systems

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CHAPTER 14

Rates of sea urchin bindin evolution

H. A. Lessios and Kirk S. Zigler

14.1 Introduction

Reproduction at the level of gametic interactions involves activation and attraction of the sperm by egg compounds, induction of the acrosome reaction by the egg jelly, adhesion of the sperm to the egg, and fusion of the two membranes in order to permit the transmission of genetic material. All of these interactions are mediated by molecules. Some of these molecules, such as sea urchin speract, carry out their functions indiscriminately, even if sperm and egg belong to distantly related taxa (Vieira and Miller 2006). Others function in a species-specific or even genotype-specific manner. Selectivity between sperm gamete recognition molecules and their egg receptors is particularly important in organisms with external fertilization, because in the absence of copulation, there are few other opportunities for exercising mate choice. Consequently, such molecules are exposed to the action of selection more directly than molecules with the same function in organisms with internal fertilization. The DNA that codes for gamete recognition molecules often, but not always, evolves rapidly, displaying ratios of amino acid replacement to synonymous substitutions larger than unity, a signature of positive (diversifying) selection (Swanson and Vacquier 2002a, b; Vacquier and Swanson 2011). As a rule, such positive selection is targeted at certain regions of each molecule, presumably involved in gamete selectivity, whereas the rest of the sequence may evolve conservatively under purifying selection, because it performs basic functions essential for fertilization.

The first gamete recognition protein to be characterized was sea urchin bindin (Vacquier and Moy 1977). Bindin DNA was subsequently amplified and sequenced in *Strongylocentrotus purpuratus* by Gao et al. (1986), and then studied with regards to its intra- and interspecific polymorphism with special attention given to detecting positive selection in its exons. These topics have been extensively reviewed (Vacquier et al. 1995; Swanson and Vacquier 2002a, b; Lessios 2007, 2011; Zigler 2008; Palumbi 2009; Vacquier and Swanson 2011). In this chapter, we explore what bindin sequences from various sea urchin species reveal about the rate of evolution of this molecule. Does bindin really evolve in the fast lane?

14.2 Function and structure of bindin

Sea urchin bindin is a protein that coats the acrosome process of sperm after the acrosomal reaction occurs. It interacts with the egg bindin receptor, EBR1, a glycoprotein (Kamei and Glabe 2003), to attach the sperm to the egg's vitelline layer and to fuse membranes of the gametes. The full-length precursor of bindin is cleaved after translation to form the mature molecule. Among the sea urchin species that have been studied to date, the length of mature bindin ranges from 193–418 amino acids (Zigler and Lessios 2003a). The single sea star in which bindin has been characterized was found to contain 793 amino acids (Patino et al. 2009). In both sea urchins and sea stars, there is a single intron separating two exons. Bindins of 11 species of sea urchins from six orders contain a conserved region in the second exon that codes for approximately 55 amino acids. Eighteen amino acids in this conserved region, thought to be involved in membrane fusion (Rocha et al. 2008), have not changed since the extant orders of Echinoidea split from each other, 250 million years ago (mya). Only one amino acid in this region has changed between sea stars and sea urchins in the 500 million years (my)

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that the two echinoderm classes have been evolving independently (Patino et al. 2009; Vacquier and Swanson 2011). The reputation of bindin as a fast-evolving protein is owed to two regions flanking the conserved core, which in some genera have accumulated many point mutations and insertions–deletions. These are the regions that most likely confer fertilization species-specificity (Lopez et al. 1993). The protein moiety of EBR1, which contains 3713–4595 amino acids, has only been sequenced in two species of *Strongylocentrotus* (Kamei and Glabe 2003).

14.3 Rate of bindin evolution

Bindin has been sequenced in 11 genera of sea urchins, but intrageneric variation, which permits insights in the evolution of the molecule, has been studied in only seven: *Echinometra* (Metz and Palumbi 1996; McCartney and Lessios 2004), *Strongylocentrotus* (Biermann 1998), *Arbacia* (Metz et al. 1998a), *Tripneustes* (Zigler and Lessios 2003b), *Heliocidaris* (Zigler et al. 2003), *Lytechinus* (Zigler and Lessios 2004), and *Paracentrotus* (Calderon et al. 2009, 2010). Selection on bindin in all of these genera has been studied as the ratio of amino acid replacement to silent substitutions ($\omega = d_N/d_S$). By this criterion, there is evidence of positive selection ($\omega > 1$) in *Echinometra*, *Strongylocentrotus*, *Heliocidaris*, and *Paracentrotus*, but not in *Arbacia*, *Tripneustes*, and *Lytechinus*. In addition to being an indication of selection at the nucleotide level, the ω ratio would also be a good measure of relative rates of adaptive evolution if silent sites evolved at the same rate in all genera. This, however, is not the case in bindin. Bindins with higher rates of nonsynonymous substitution also have higher rates of synonymous substitution (Zigler and Lessios 2003b). This correlation has also been observed in other molecules such as alcohol dehydrogenase, ATP synthetase, cyclophilin 1, or enolase (e.g. Dunn et al. 2001), and there are a number of hypotheses as to its cause. While it is typically thought to arise from some form of codon bias, codon usage in sea urchin bindin is very equitable (Zigler and Lessios 2003a). Thus, due to different codon biases, comparing ω ratios between bindins of different genera may lead to erroneous conclusions regarding evolutionary rates. To compare the absolute rate of evolution between genera we need to determine the number of nonsynonymous substitutions per nonsynonymous site that accumulate per unit time. Such a calculation requires evidence of dates of divergence. In this chapter, we will use the interspecific divergence of cytochrome oxidase I (COI) as a proxy for the time since speciation. Calibrated by the rise of the Isthmus of Panama, approximately 3 mya, COI of sea urchins diverges at an average rate of 3.6 % per my (Lessios 2008).

Gauged by divergence in COI, average rates of adaptive divergence of bindin within a genus vary between 2.80×10^{-3} nonsynonymous substitutions per nonsynonymous site per my ($d_N my^{-1}$) in *Arbacia* and 22.4×10^{-3} $d_N my^{-1}$ in *Strongylocentrotus* (Table 14.1). As one might expect, genera in which bindin evolves under positive selection, show amino acid divergence rates almost four times higher than genera in which bindin appears to be under purifying selection: the average substitution rate in *Strongylocentrotus*, *Echinometra*, and *Heliocidaris* is 20.4×10^{-3} $d_N my^{-1}$ whereas in *Arbacia*, *Tripneustes*, *Lytechinus*, *Pseudoboletia*, and *Diadema*, it is 5.96×10^{-3} $d_N my^{-1}$. The question we would like to answer is how these rates of adaptive evolution compare with those of other proteins, both of those that have been deemed to evolve rapidly in other taxa, and those that carry out other functions in sea urchins.

Fig. 14. 1 presents a comparison of the rates of adaptive evolution of bindin to seven other classes of reproductive proteins from five groups of organisms. These are all proteins that are generally considered as fast-evolving. Because COI in different taxa evolves at different rates, it is necessary to apply taxon-specific calibrations to calculate divergence rates. To estimate absolute rates of protein evolution, we have assumed that COI evolves at an average rate of 3.6% per my in sea urchins (Lessios 2008), 2.7% per MY in gastropods (Lessios 2008), 2.3% per my in insects (Papadopoulou et al. 2010), and 1.6% per my in hominids (Kumar et al. 2005). Estimated in this manner, the evolutionary rates of bindins in different genera of sea urchins, even those found to be under selection, are slower than that of reproductive proteins of gastropods or insects. They are more comparable to those of

Table 14.1 Pairwise divergence in bindin and in cytochrome oxidase I (COI) of selected species of sea urchin genera in which bindin variation has been studied. **K2P**: Kimura two-parameter distance; $\mathbf{d_N}$: amino acid substitutions per non-synonymous site; $\mathbf{d_S}$: synonymous substitutions per synonymous site; **MY**: million years. Estimated rates of divergence of bindin are based on the assumption that COI in sea urchins diverges at a rate of 0.036 per site per my.

Genus	Species	Species	Bindin d_N	COI d_S	Bindin d_N/ K2P	Bindin d_S/ COI K2P	Bindin d_N/ COI K2P	MY	Reference
Arbacia	*lixula*	*punctulata*	0.007	0.069	0.090	0.072	0.764	0.0026	Metz et al. 1998a
Arbacia	*lixula*	*stellata=incisa*	0.007	0.096	0.134	0.053	0.716	0.0019	
Arbacia	*lixula*	*dufresnei*	0.016	0.071	0.124	0.129	0.570	0.0046	
Arbacia	*punctulata*	*stellata=incisa*	0.003	0.088	0.139	0.022	0.635	0.0008	
Arbacia	*punctulata*	*dufresnei*	0.011	0.059	0.124	0.085	0.477	0.0031	
Arbacia	*stellata=incisa*	*dufresnei*	0.013	0.071	0.119	0.105	0.597	0.0038	
Heliocidaris	*erythrogramma*	*tuberculata*	0.069	0.149	0.147	0.469	1.014	0.0169	Zigler et al. 2003
Tripneustes	*ventricosus*	*gratilla+depressus*	0.016	0.026	0.087	0.187	0.293	0.0067	Zigler and Lessios 2003
Echinometra	*oblonga*	*mathaei*	0.021	0.054	0.023	0.905	2.328	0.0326	Metz and Palumbi 1996
Echinometra	*oblonga*	*type A*	0.024	0.076	0.032	0.757	2.371	0.0273	
Echinometra	*mathaei*	*type A*	0.028	0.051	0.024	1.169	2.107	0.0421	
Echinometra	*lucunter*	*viridis*	0.022	0.047	0.050	0.440	0.940	0.0158	McCartney and Lessios 2004
Echinometra	*lucunter*	*vanbrunti*	0.026	0.046	0.102	0.255	0.451	0.0092	
Echinometra	*viridis*	*vanbrunti*	0.014	0.083	0.126	0.111	0.659	0.0040	
Lytechinus	*pictus*	*variegatus*	0.013	0.105	0.135	0.096	0.778	0.0035	Zigler and Lessios 2004
Lytechinus	*variegatus*	*williamsi*	0.006	0.022	0.017	0.353	1.294	0.0127	
Lytechinus	*semituberculatus*	*pictus*	0.025	0.073	0.114	0.219	0.640	0.0079	
Lytechinus	*euerces*	*Sphaerechinus granularis*	0.019	0.100	0.089	0.213	1.124	0.0077	
Pseudoboletia	*indiana*	*maculata*	0.006	0.024	0.073	0.082	0.329	0.0030	Zigler et al. (in press)
Strongylocentrotus	*purpuratus*	*pallidus*	0.021	0.062	0.072	0.287	0.863	0.0103	Biermann 1998
Strongylocentrotus purpuratus	*pallidus*	*droebachiensis*	0.031	0.086	0.075	0.418	1.148	0.0150	
Strongylocentrotus	*purpuratus*	*H. pulcherrimus*	0.073	0.158	0.104	0.704	1.514	0.0253	
Strongylocentrotus	*pallidus*	*droebachiensis*	0.025	0.036	0.035	0.715	1.011	0.0257	
Strongylocentrotus	*pallidus*	*H. pulcherrimus*	0.066	0.119	0.070	0.941	1.696	0.0339	
Strongylocentrotus	*droebachiensis*	*H. pulcherrimus*	0.063	0.139	0.094	0.672	1.481	0.0242	

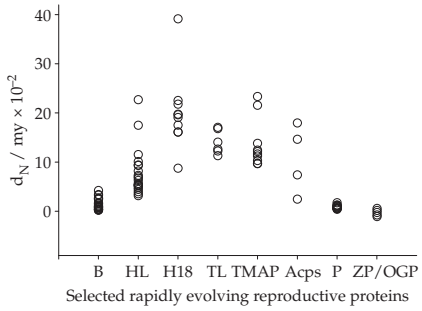

Figure 14.1 Bindin evolution relative to known fast-evolving reproductive proteins from other taxa. Non-synonymous substitutions per non-synonymous site (d_N) per million years, between congeneric species (except in hominids, in which they are within the same family) in sea urchin bindin (B) (data from references in Table 14.1), abalone lysin (HL) and 18 kD protein (H18) (data from Metz et al. 1998b), *Tegula* lysin (TL), and the mature region of TMAP protein (TMAP) (data from Hellberg and Vacquier 1999; Hellberg et al. 2000), *Drosophila* Acp26Aa and Acp36DE (Acps) (data from Tsaur and Wu 1997), hominid protamine 1 and 2 (P), ZP2, ZP3 and oviductal glycoprotein (ZP/OGP) (data from Wyckoff et al. 2000).

protamines, zona pellucida proteins, and oviductal glycoprotein in hominids. Adjustments to the assumed rate of COI evolution, or even an assumption of a universal COI clock, would not change this conclusion. Thus, by the standard of other fast-evolving reproductive proteins from other invertebrates, bindin evolves only at moderate rates.

How do rates of bindin evolution compare to rates of evolution among other sea urchin proteins? To answer this question, we compared all protein coding DNA sequences of *Lytechinus variegatus* in GenBank to their closest matches in the *Strongylocentrotus purpuratus* complete genome. With the exception of *S. purpuratus*, more genes have been sequenced from *Lytechinus variegatus* than any other species of sea urchin. *Lytechinus* and *Strongylocentrotus* diverged approximately 60 mya. Sequences were available for 90 *L. variegatus* genes. The protein sequence of each gene was compared between the two species via protein-protein BLAST to GenBank's 'non-redundant (nr) protein sequences' database. The closest match to a *S. purpuratus* protein was noted, and the two protein sequences were aligned using Clustal in MEGA (v. 4.0). We then used MEGA to calculate the p-distance between the aligned protein sequences. We identified matches for 85 of the 90 *Lytechinus* genes. The five genes that did not have a match may be: (1) missing from the annotated *Strongylocentrotus* genome; (2) lost in the *Strongylocentrotus* lineage; or (3) mis-annotated in their original *Lytechinus* entry. The set of genes that we compared contained proteins with various functions, including many involved in reproduction, and also in development, cytoskeleton formation, cell attachment, and stress responses. After ranking the divergences of the 85 proteins, that of bindin was the sixth largest, with a p-distance of 0.326 for the full-length molecule and 0.314 for the mature portion. Of the five proteins with divergence values higher than bindin, vitellogenin and SFE-1 also carry out functions related to reproduction, whereas the other three were involved in development. Considering the inevitable bias of proteins available for comparison, the conclusion from this comparison is that bindin evolves at moderately fast rates in relation to other sea urchin proteins.

14.4 Possible reasons for different evolutionary rates in bindin

Why does bindin in four sea urchin genera evolve more rapidly under strong positive selection, than in three other genera in which it is subject to purifying selection? In the absence of data regarding variation in its egg receptor, the answer can only be speculative. Possible reasons for this lack of pattern have been thoroughly reviewed (Lessios 2007, 2011; Zigler 2008; Palumbi 2009). Here we present a summary of the hypotheses that have been proposed so far.

One possibility is that positive selection of bindin arises from the need for species recognition when two closely related species are in danger of hybridizing with each other. We will call this the 'reinforcement hypothesis.' This name does not imply that speciation by reinforcement has actually taken place, but rather that bindin alleles resembling those of a sympatric species—and thus allowing gamete wastage in inferior hybrids—have been selected against. A broad-brush picture of comparisons between genera is consistent with this hypothesis. When bindin rates of divergence of species that are entirely allopatric with respect to congeners are compared to those of species that may have a higher probability of hybridization, those of the for-

mer are clustered around lower values than those of the latter (Fig. 14.2). Genera with many sympatric species, such as *Strongylocentrotus*, and *Echinometra* tend to have the highest rates of interspecific bindin divergence. Not all the data, however, are consistent with the reinforcement hypothesis. Contrary to what is expected from selection for species recognition, bindin is polymorphic and shows the signature of positive selection not just between species but also between alleles of the same species (Metz and Palumbi 1996; Lessios 2007, 2011). A pattern of character displacement is present in one species of Pacific *Echinometra* (Geyer and Palumbi 2003) in partial geographic overlap with its sister species but not in an Atlantic species of the same genus that also needs to contend with the challenge of a sister species existing over part of its range (Geyer and Lessios 2009). Given the present evidence, the hypothesis that reinforcement in sympatry accelerates bindin divergence is as likely as the hypothesis that divergence in bindin, due to other causes, allows for sympatric coexistence.

Another possibility for the differences in rates of bindin evolution could be that they are correlated to the relative age of species in different sea urchin genera. If, as Civetta and Singh (1998) have suggested, episodes of divergence in reproductive molecules are concentrated at the time of speciation, and if selection on these molecules is subsequently relaxed, younger species would show higher rates of bindin differentiation than older ones. This hypothesis is not supported by the data. Sea urchins tend to conform to 'Jordan's rule' (Jordan 1905). Young sister species tend to be distributed on either side of a geographic barrier, and only older species become sympatric with the passage of time (Lessios 2010). Thus, allopatric species are, in general, younger than sympatric ones, and if bindin divergence were accelerated during speciation then slowed down, they should show more differences in this molecule per unit time than sympatric ones. The opposite is true (Fig. 14.2).

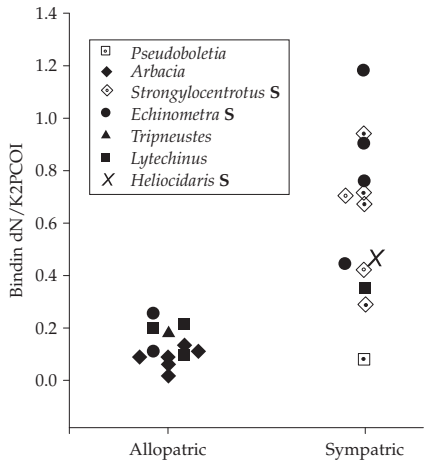

Figure 14.2 Comparison of interspecific rates of bindin divergence between genera. Amino acid replacement substitutions (d_N) per replacement site in bindin divided by Kimura-two-parameter distance in cytochrome oxidase I (COI K2P) in allopatric and sympatric species of eight genera of sea urchins. A species is considered as 'allopatric' if its range does not overlap with that of another member of the same genus. Genera in which bindin has been shown to be under selection are marked in the legend with **S**.

The most credible hypothesis to date for differences in the rates of bindin evolution is that they are caused by differences in the intensity of sexual selection and sexual conflict. Using variation in bindin genotypes of females as a proxy for variation in the bindin receptor (with which bindin is expected to show linkage disequilibrium), Palumbi (1999) has found that sexual selection exists in *Echinometra mathaei*. Eggs are fertilized at higher rates by sperm carrying the same bindin allele. Using the same proxy, Levitan and Farrell (2006) and Levitan and Stapper (2010) showed in *Strongylocentrotus franciscanus* and *S. purpuratus* that sperm density and the danger of polyspermy establish different selective regimes for various bindin alleles. At low sperm densities, most offspring are produced by the union of sperm and egg possessing bindin alleles that are most common in the population. At high sperm densities, rare alleles leave behind the most offspring, because common alleles, causing fast fertilization, result in polyspermic zygotes, which fail to develop. Thus, there is always selection on males to effect fast fertilization, but females in high sperm densities benefit from having alleles that retard fertilization: a typical sexual conflict situation. Depending on ecological conditions, sexual conflict can occur in some populations but not others, thus resulting in different rates of bindin evolution.

14.5 Conclusions and future prospects

In comparison to other invertebrate reproductive proteins, bindin evolves moderately rapidly in some genera and slowly in others. Selective reasons for the differences that cause these dissimilarities in rates are still the subject of speculation, but they may well be related to fertilization environments and intraspecific processes. Interspecific processes, such as reinforcement, can also not be ruled out. There may well be no universal explanation for the presence or absence of positive selection in different sea urchin taxa. Gametic proteins are often brought up as examples of rapid evolution. Fast evolution is certainly true for each of these proteins in the particular genus in which they have been studied. However, in a great many of the documented cases of fast molecular evolution, the evidence comes only from a small fraction of taxa. Data on sea urchin bindin, though far from covering the entire echinoid class, derive from multiple genera. This broader taxonomic coverage alone may explain why more diversity in the mode of evolution of this molecule has been documented than has been found in other invertebrate reproductive proteins.

Future laboratory studies linking the structure of different bindin alleles with the specificity of fertilization would be of great benefit in understanding the evolution of this molecule. We already know which amino acids evolve under selection, but we will need to determine the functional reasons for such selection. Additional understanding of the sources of natural selection on this molecule and the rate of its evolution would come from comparative studies that link fertilization ecology in nature with the success of particular bindin alleles. Simply characterizing species as sympatric or allopatric on the basis of their geographic distribution is not adequate for determining the role of reinforcement or other interspecific processes in bindin evolution. Ultimately, interest in the evolution of bindin and similar molecules stems from our desire to understand the process of speciation and the role of sexual selection in the evolution of reproductive isolation. In that respect, assessing the importance of bindin as a reproductive isolation barrier between species relies on studies that are not aimed directly at this molecule alone. Whether bindin is involved in speciation depends not just on the species-specificity of its interactions with its receptor but on the probability that gametes of two closely related sea urchin species will encounter each other in nature. Even when gametic interactions are, in fact, species-specific, it is still necessary to determine whether bindin or some other molecule, acting earlier in the sequence of fertilization, is responsible. Thus, information on habitat separation, reproductive timing, and pre-spawning chemical communication as well as on the role of other reproductive molecules is important in understanding whether intra- or interspecific interactions mold the evolution of the bindin. Most of all, we will need to link variation of bindin to variation in its egg receptor. The study of EBR1 has been retarded by its enormous size. Recent advances in techniques for massive DNA sequencing have made it practical to gather data on individual variation in large stretches of genetic material, and will no doubt soon be applied to this problem.

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