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SYMPOSIUM

Speciation Genes in Free-Spawning Marine Invertebrates

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Synopsis Research on speciation of marine organisms has lagged behind that of terrestrial ones, but the study of the evolution of molecules involved in the adhesion of gametes in free-spawning invertebrates is an exception. Here I review the function, species-specificity, and molecular variation of loci coding for bindin in sea urchins, lysin in abalone and their egg receptors, in an effort to assess the degree to which they contribute to the emergence of reproductive isolation during the speciation process. Bindin is a protein that mediates binding of the sperm to the vitelline envelope (VE) of the egg and the fusion of the gametes' membranes, whereas lysin is a protein involved only in binding to the VE. Both of these molecules are important in species recognition by the gametes, but they rarely constitute absolute blocks to interspecific hybridization. Intraspecific polymorphism is high in bindin, but low in lysin. Polymorphism in bindin is maintained by frequency-dependent selection due to sexual conflict arising from the danger of polyspermy under high densities of sperm. Monomorphism in lysin is the result of purifying selection arising from the need for species recognition. Interspecific divergence in lysin is due to strong positive selection, and the same is true for bindin of four out of seven genera of sea urchins studied to date. The differences between the sea urchin genera in the strength of selection can only partially be explained by the hypothesis of reinforcement. The egg receptor for lysin (VERL) is a glycoprotein with 22 repeats, 20 of which have evolved neutrally and homogenized by concerted evolution, whereas the first two repeats are under positive selection. Selection on lysin has been generated by the need to track changes in VERL, permitted by the redundant structure of this molecule. Both lysin and bindin are important in reproductive isolation, probably had a role in speciation, but it is hard to determine whether they meet the strictest criteria of "speciation loci," defined as genes whose differentiation has caused speciation.

Introduction

Because of the relative inaccessibility of specimens and the difficulty of direct observations, the study of marine organisms has always lagged behind that of terrestrial ones. This has been particularly true in research on speciation, because, in addition to these problems, most marine organisms have long generation times and are difficult to keep in captivity. With only a few exceptions (e.g., Ellison and Burton 2006) their formal genetics cannot be discerned through controlled matings. The "New Synthesis" was constructed with practically no information from the marine realm, except for that provided by fossils (Simpson 1944). Only 3.5% of 691 entries referring to various species in the index of Dobzhansky's (1970) "Genetics of the Evolutionary Process" concern marine organisms. Similarly, the index of Mayr's (1963) "Animal Species and Evolution" contains 731 references to species, only 33 of which are marine. Thus, marine organisms have played a supporting role in research on speciation, mostly serving to show that principles worked out in terrestrial species also hold true for marine ones (Mayr 1954; Palumbi and Lessios 2005).

One exception to the minor role of marine organisms in speciation research is the study of reproductive isolation mediated through the interactions of gametes in invertebrates with external fertilization. There are multiple reasons why this enterprise has advanced more than others. One is the relative ease with which such gametes can be handled in the laboratory to detect blocks to interspecific fertilization.

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Studies of gamete species-specificity of animals date back to the beginning of the past century (e.g., Lillie 1921). A related reason is the long tradition of using marine invertebrates, particularly sea urchins, for studies of development (Harvey 1956). The knowledge gained by early embryologists from experimental crosses to study development led to studies aiming to identify molecules involved in fertilization (e.g., Vacquier and Payne 1973; Glabe and Vacquier 1977; Vacquier and Moy 1977; Brandriff et al. 1978; Vacquier 1978). With the advent of techniques to amplify and sequence nucleic acids, the identification of fertilization proteins was only one step away from permitting studies of genetic control of such molecules (Gao et al. 1986; Glabe and Clark 1991).

From the point of view of research on speciation, the advantage of studying gametic isolation in free-spawning marine invertebrates, in addition to its simple genetic control, is that it has a high probability of identifying the genetic basis of prezygotic isolating barriers. There is no courtship other than possible prespawning chemical communication between the sexes. Genetically determined habitat separation and allochronic reproduction are the only intrinsic isolating barriers that precede interactions between the gametes in the sequence of events that will produce the next generation. It is, thus, natural that evolutionary biologists have focused their efforts on gamete molecules in an attempt to understand whether the evolution of these molecules can offer clues about the establishment of reproductive isolation, and thus of speciation. The question I address in this review is whether, based on what has been found to date, any of these molecules can be said to be controlled by ''speciation genes.''

The somewhat grandiose term ''speciation gene'' has been used liberally in the literature with different meanings, ranging from any gene involved in reproductive isolation, regardless of the role of its divergence in forming new species (Orr et al. 2004; Orr 2005; Presgraves 2010; Rieseberg and Blackman 2010) to genes that actually cause the evolution of reproductive isolation by diverging before other loci (Rieseberg and Blackman 2010; Nosil and Schluter 2011). Other terms with similar meanings are ''isolation genes'' (Rieseberg et al. 2004) and ''barrier genes'' (Noor and Feder 2006). The idea that certain loci are predominantly responsible for speciation, even if the rest of the genome of two incipient species remains porous to gene flow, is based on the ''genic view'' of speciation (Wu 2001). In keeping with this view, Nosil and Schluter (2011) specified three criteria for characterizing a locus as a speciation gene: (1) that it affects reproductive isolation between species, (2) that its divergence preceded the completion of speciation, and (3) that its divergence had a large effect on the cessation of genetic exchange relative to that of other genes. Most animal genes to which this label has been attached are involved in postzygotic isolation by rendering hybrids unfit (e.g., Orr et al. 2004; Wu and Ting 2004; Orr 2005; Presgraves 2010). Prezygotic barriers could also have a great effect in keeping species from interbreeding, and they may well have diverged in the absence of postzygotic barriers. If so, there is ample justification for also discussing genes involved in prezygotic isolation as potential speciation genes (e.g., Noor and Feder 2006; Rieseberg and Blackman 2010). The question of how well a locus meets the criteria specified by Nosil and Schluter (2011) is not one of terminology, but rather of the role it plays in speciation. Here I review what is known about molecules that determine interactions of gametes within and between species of two kinds of marine invertebrates and ask how important their evolution is likely to be in forming new species.

Fertilization molecules of free-spawning invertebrates as barriers to interspecific gene flow

The process that starts with the shedding of gametes into the water and ends with the production of zygotes involves: (1) activation and attraction of the sperm by compounds released by the egg, (2) interaction of the sperm with the egg's jelly layer, which provokes the eversion of the acrosome process, (3) binding of the sperm to the vitelline envelope (VE) of the egg, (4) penetration of the egg's vitelline layer, and (5) fusion of membranes of the sperm and egg and injection of the sperm's DNA into the egg (Vacquier 1998). Many molecules on gamete surfaces are known to mediate these steps and many more remain to be discovered (reviews by Ward and Kopf 1993; Vacquier 1998; Neill and Vacquier 2004; Kaupp et al. 2006; Vieira and Miller 2006; Hirohashi et al. 2008; Kaupp et al. 2008). An important question regarding their role in speciation is the degree to which each contributes to reproductive isolation between sister species. Some of these molecules have a greater effect than do others, but it is rare for a single type to constitute an absolute barrier on its own. Many of them have been shown not to act in a species-specific manner in crosses between gametes of closely related species (Suzuki and Yoshino 1992; Vieira and Miller 2006). However, if complete prezygotic reproductive isolation is only achieved

step by step through the combined action of many molecules (and in the case of habitat separation and allochronic isolation, by the action of many loci reacting epistatically to produce a trait), any gene that contributes to the final outcome is of interest in speciation. Thus, the concentration of studies on the evolution of a few molecules—even in ignorance of the isolating contribution of other loci—is justified. This review will concentrate on two systems, bindin in sea urchins and lysin in abalone and their respective receptors. Though variation in other molecules has also been documented (e.g., Riginos and McDonald 2003; Biermann et al. 2004; Mah et al. 2005; Riginos et al. 2006; Springer and Crespi 2007; Moy et al. 2008; Springer et al. 2008), bindin and lysin are the two loci that have been studied to sufficient detail to allow conclusions (or at least informed speculations) about their contribution to speciation.

Progress of research on bindin and lysin has advanced via different paths. Bindin has been studied more extensively with regard to its intraspecific polymorphisms and has been sampled more broadly throughout the class Echinoidea (Zigler 2008); it has even been characterized in one species of Asteroidea (Patino et al. 2009). Understanding of its evolution, however, has suffered from inadequate documentation of variation in its receptor (Kamei and Glabe 2003). The lysin receptor of abalone has received more attention, and we know much more about variation of its molecular structure between and within species (Swanson and Vacquier 1997, 1998; Galindo et al. 2002; Clark et al. 2009). Lysin, however, has only been studied in the archaeogastropods *Haliotis* (review in Kresge et al. 2001), *Tegula*, and *Norrisia* (Hellberg and Vacquier 1999). A cursory glance at the literature might lead to the impression of wider taxonomic coverage, but the M7 lysin of mussels (Riginos and McDonald 2003; Riginos et al. 2006; Springer and Crespi 2007) is not phylogenetically related to the lysin of archaeogastropods (Vacquier 1998). Similarly, bindin in oysters (Moy et al. 2008; Springer et al. 2008) is not related to bindin in echinoderms.

Structure, function, and species specificity

Bindin

Sea-urchin bindin is a protein that coats the acrosome process after the sperm has come in contact with the egg's jelly layer. It carries out two functions in fertilization. It binds the sperm to the egg's vitelline layer, and it fuses the gamete membranes to permit genetic material to combine (Vacquier et al. 1995). Mature bindin contains 193–418 amino acids. Comparisons of DNA sequences from ten species, belonging to six orders, have revealed the presence of a central region in which 55 amino acids are highly conserved, flanked by two regions showing many point mutations and varying amounts of variation in length (Zigler and Lessios 2003a). The conserved region includes a stretch of 18 amino acids apparently involved in membrane fusion (Ulrich et al. 1998) that have remained unchanged over the entire 250 million year span of extant echinoid evolution (Zigler and Lessios 2003a). The flanking regions confer species specificity (Lopez et al. 1993). Metz et al. (1994) have shown that fertilization specificity between *Echinometra mathaei* and *Echinometra oblonga*, two sister species (Landry et al. 2003), is concentrated at the step of gamete membrane fusion, which strongly suggests that bindin is responsible for reproductive isolation. Gametes of these two species are completely incompatible at moderate concentrations of sperm (Uehara et al. 1990; Metz et al. 1994). This compete barrier, however, is not present in the majority of interspecific gametic interactions. Among 14 pairs of congeneric sea-urchin species in which gametic compatibility was tested, only three were completely incompatible. In nine of them the sperm of one species could not fertilize the eggs of the other, but fertilization in the reverse combination proceeded readily (Zigler et al. 2005; Lessios 2007). In these experiments the gametes of each species were exposed to those of the other in separate containers. The single experiment that exposed eggs to a mixture of sperm of two species showed that there was preferential fertilization by conspecific sperm (Geyer and Palumbi 2005). Nevertheless, the asymmetry in "no choice" experiments indicates that differences in bindin do not always cause bidirectional blocks to fertilization.

The asymmetry in gametic compatibility does not mean that bindin is unrelated to reproductive isolation. Average gametic incompatibility between 14 pairs of species is not correlated with the time since speciation, but it is correlated with adaptive divergence of bindin (Zigler et al. 2005). Most amino acid changes in the genealogy of the bindin of three Neotropical species of *Echinometra* are concentrated on the branch leading to *Echinometra lucunter*, the species in which eggs block heterospecific fertilization, suggesting that bindin evolves in ways that match changes in the bindin receptor. (McCartney and Lessios 2004). This change, however, does not render sperm incapable of also fertilizing

heterospecific eggs. The ability of *E. lucunter* sperm to fertilize eggs of both its sympatric congener *E. viridis* and the allopatric *E. vanbrunti* (Lessios and Cunningham 1990; McCartney and Lessios 2002) indicates that species-specificity is not a "lock and key" mechanism, in which each change in the egg's receptor requires a corresponding change in bindin to preserve compatibility. It is, instead, a "backwards compatible" system, in which changes in one lineage of the receptor require bindin to change in order to maintain reproductive efficiency, but in such a way that it retains some ability to combine with the egg's receptor of other lineages (McCartney and Lessios 2004). The Egg Bindin Receptor (EBR1) is an enormous glycoprotein with a protein moiety consisting of 3713–4595 amino acids. Its entire length has only been sequenced in *Strongylocentrotus purpuratus* and *Strongylocentrotus franciscanus* (Kamei and Glabe 2003), two distantly related species (Biermann et al. 2003) in which gametes are almost completely incompatible (Minor et al. 1991). The EBR1 molecule consists of an N-terminal domain that resembles other extracellular proteases, and of 19 tandem repeats, each made up of two alternating amino-acid domains. Ten repeats closest to the C-terminal differ between the two species of *Strongylocentrotus* and are, thus, likely to be involved in species specificity.

Lysin

In archaeogastropods, the molecules analogous to bindin and EBR1 are lysin and its receptor, most extensively studied in abalone (genus *Haliotis*) (reviewed by Kresge et al. 2001). Abalone sperm cross the egg's jelly layer and undergo the acrosome reaction, which coats a large part of the egg's VE with lysin. Lysin is a 16-kD dimeric protein with 126–138 amino acids. Lysin binds to the Vitelline Envelope Receptor for Lysin (VERL), a 1000 kD glycoprotein, causing its molecules to unravel and create a hole, so that the sperm can reach the cell membrane of the egg (Swanson and Vacquier 1997). The protein moiety of VERL contains 22 tandem repeats, each composed of 153 amino acids (Galindo et al. 2002). Each repeat can bind two molecules of lysin. The two repeats closest to the amino terminal are different from the rest, but repeats 3–22 are very similar to each other. Species-specificity of the lysin-VERL interaction among three species of *Haliotis* has been demonstrated by the larger amount of purified lysin required to dissolve isolated heterospecific VEs (Vacquier et al. 1990; Lewis et al. 1992; Vacquier and Lee 1993). Site-directed mutagenesis has shown that the sections of the molecule that impart species-specific properties are located close to the N and C terminals (Lyon and Vacquier 1999). In contrast to the dual action of sea-urchin bindin, fusion of membranes in abalone is not mediated by lysin, but by a different, 18-kD acrosomal protein (Swanson and Vacquier 1995). The likely receptor of this protein is Vitelline Envelope Zona Pellucida 14 (VEZP14), one of the thirty zona-pellucida-domain proteins discovered to date as being part of the VE of abalone (Aagaard et al. 2010)

Patterns of polymorphism, mode of evolution, and role in speciation

Bindin

Intraspecific and interspecific variation of the bindin locus has been studied in seven genera of sea urchins: *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). In the absence of information regarding variation in the bindin receptor EBR1, studies of bindin have used sequences of bindin in females as a proxy, even though bindin is a sex-limited gene. The rationale of this approach in interspecific comparisons is that the evolution of a species-specific molecule in males must co-evolve with its receptor in females, although the exact mode of this coevolution is not known. In intraspecific polymorphisms, linkage disequilibrium is expected between a gene that controls mate selection in one sex and a gene that codes for the preferred trait in the other, because offspring in every generation would carry both (Fisher 1930; Tomaiuolo and Levitan 2010).

What have the studies of bindin shown with regard to speciation? As one would expect from speciation genes, there is evidence of rapid evolution and positive selection, but the pattern is far from universal. Positive selection, manifested in ratios of amino-acid replacement to silent substitutions ($\omega = d_N/d_S$) significantly higher than unity, has been found in *Echinometra*, *Strongylocentrotus*, *Heliocidaris,* and *Paracentrotus*, but not in *Arbacia, Tripneustes* and *Lytechinus*. *Tripneustes* contains no sympatric species. Most species in *Arbacia* and *Lytechinus* are also allopatric. Thus, the most obvious explanation for the difference between genera regarding the presence or absence of selection on bindin is reinforcement [Reinforcement in its original meaning denotes selection for prezygotic isolation to

avoid the production of inferior hybrids (Dobzhansky 1970; Butlin 1987), but Butlin's (1987) use of the term "character displacement" to describe selection for species recognition when speciation is complete creates confusion. Brown and Wilson (1956) defined character displacement as the geographic pattern of divergence between species in areas of sympatry. Here I use reinforcement to denote selection for the avoidance of wastage of gametes whether it is due to unfit hybrids or to unsuccessful attempts at interspecific fertilization]. Reinforcement *sensu lato* is also a reasonable hypothesis regarding selection on the bindin of *Heliocidaris*. The range of *H. erythrogramma* partially overlaps with that of its sister species, *H. tuberculata*. In the bindin genealogy of the two species, most of the amino-acid replacements are concentrated on the branch leading to *H. erythrogramma*, the species that has most to lose by hybridization, because, unlike its sister species, it invests in large lecithotrophic eggs (Zigler et al. 2003). Reinforcement is also apparent in a pattern of character displacement [*sensu* (Brown and Wilson 1956)] in *E. oblonga*, the bindin of which is divergent from *Echinometra* "species C" in the western Pacific, where the two species co-occur, but not in the central Pacific, where they do not (Geyer and Palumbi 2003). Reinforcement, however, could not be a universal explanation for positive selection on bindin. The pattern of character displacement in *E. oblonga* is not duplicated in *E. lucunter*. This tropical Atlantic species expanded its range into the Caribbean, so that it now overlaps with the entire range of its sister species, *E. viridis*. There is no present-day gene flow between Atlantic and Caribbean populations of *E. lucunter*. Yet, bindin alleles of *E. lucunter* from the two areas are mixed, and there is no evidence that positive selection is stronger within than without the area of sympatry (Geyer and Lessios 2009). Intraspecific polymorphism in bindin is also not consistent with the hypothesis that reinforcement is the only force that accounts for its adaptive divergence. All bindins sampled to date, both those with high ω ratios and those with low, are polymorphic. In *Echinometra* (Metz and Palumbi 1996) and in *Strongylocentrotus* (Debenham et al. 2000) there is an excess of amino-acid replacement over silent substitutions between alleles of the same species. Selection for species recognition will cause positive selection between species, but it cannot cause it within a species. Thus, reinforcement cannot by itself account for the patterns of variation of bindin, even though it may be involved in accelerating divergence between sympatric species.

If reinforcement cannot be the whole explanation for adaptive divergence between species in bindin, then what can? The emerging consensus in recent literature is that the answer lies in sperm competition, sexual selection, and sexual conflict (Rice 1998; Hayashi et al. 2007), arising from the danger of polyspermy. At low densities of sperm, it behooves both males and females to be efficient in uniting. Under these conditions selection would favor a single allele in bindin and a single allele in its receptor. If the density of sperm is high, however, sperm would compete with each other for higher efficiency, so they can be the first to fertilize, but eggs would benefit from being selective, or from slowing down the rate of sperm attachment and thereby avoiding polyspermy, which is lethal to the zygote. Palumbi (1999) found assortative mating based on bindin genotype in *E. mathaei* in crosses conducted with moderate concentrations of sperm. Levitan and Ferrell (2006) in field experiments with *S. franciscanus* found an interaction between allele frequency and sperm density. When density of sperm was low, the most successful fertilizations were those in which sperm and egg carried the same, most common, bindin allele. When sperm density was high, crosses between different alleles left behind the most offspring. Presumably, under high densities of sperm many of the fertilizations with matched bindin alleles produced lethal polyspermic zygotes, suggesting that under such conditions there is strong sexual conflict. Allele-frequency by sperm-density interactions were also found in field experiments with *Strongylocentrotus purpuratus,* but the results revealed an additional refinement (Levitan and Stapper 2010). Common bindin alleles had the highest reproductive success, but in the group of common haplotypes, males with a proline residue in one site of their bindin were more successful than those with the more common serine. Similarly, females with the most common bindin haplotype were the most successful, but in the group of common female haplotypes those with a relatively rare insertion were more successful than those without it. Matches between males with proline residues and females with insertions were the most successful in contributing to the next generation, yet genotypes with both of these features were not the most common in the population. Levitan and Stapler (2010) explained this pattern as the result of simultaneous action by positive frequency-dependent selection removing new bindin alleles that do not match existing receptors and negative frequency-dependent selection arising from matches so perfect, that they cause polyspermic deaths under high sperm densities.

Thus, sexual conflict appears to be operating on bindin when concentrations of sperm are high and explains persistent polymorphism which otherwise would be absent in a species-recognition gene, but how is it related to speciation? It is easy to imagine that populations isolated from each other by an external barrier would maintain different population densities for a sufficient length of time, so that different selection pressures would lead to different complements of bindin alleles. Even if densities of sperm were the same between populations, new alleles would arise in each by mutation, attain an initial advantage because of their rarity if sperm concentrations are high, then lose it as they become more common, driving divergence between the isolates until partial, or complete, reproductive isolation is attained (Palumbi 2009). It is particularly easy to envision this process in parapatric speciation, because population densities would be expected to be much smaller at the periphery, so the degree of sexual conflict would be smaller than in the more dense center. Mathematical models suggest that speciation by sexual conflict can even occur sympatrically (Gavrilets and Waxman 2002; Gavrilets and Hayashi 2005). Conditions required by such models, however, are so restrictive, that it is not clear whether they can be found among free-spawning invertebrates. As Gavrilets and Waxman (2002) pointed out, sympatric speciation in their model requires a degree of ecological divergence or spatial separation to allow coexistence of sympatric species. In many cases, sympatric congeneric sea-urchin species occupy different habitats (Lessios 2007), but it is, of course, not known at which point after speciation sympatry and ecological divergence occurred. Ecological divergence is also assumed by an alternative model of sympatric speciation, based on sexual selection in a recognition locus linked to a locus for habitat preference (Van Doorn et al. 2001). Among various models of sympatric speciation, these two appear to be more relevant to free-spawning marine invertebrates, but all hypotheses of sympatric speciation by divergence in bindin need to overcome an additional difficulty. Asymmetric gametic isolation cannot stop gene flow between species. If genes from one species freely enter the genome of the other in sympatry, even in one direction, recombination would be sufficient to prevent the formation of a new species. The effects of gametes' molecules on reproductive isolation may be large, but they do not appear to be large enough to account for speciation in sympatry.

Lysin

Patterns of variation in lysin are more consistent than those of bindin with what would be expected from a species-recognition molecule. Lysin shows a large amount of divergence between species, driven by positive selection both in *Haliotis* (Lee et al. 1995; Yang et al. 2000; Clark et al. 2009) and in *Tegula* (Hellberg and Vacquier 1999; Swanson and Vacquier 2002). The amino-acid sites that show the highest ω-values in abalone are concentrated near the N and C terminals of the molecule (Yang et al. 2000), suggesting that positive selection is associated with species recognition. In contrast to the high variation between species in bindin, abalone lysin shows very low variation within species (Lee et al. 1995; Metz et al. 1998b; Clark et al. 2009). This monomorphism is unlikely to be due to demographic history, because it is not reflected in mitochondrial DNA or in nuclear introns. Metz et al. (1998b) suggested that selective sweeps may have occurred in both lysin and the 18-kD membrane-fusion protein, which is one of the fastest evolving proteins known (Swanson and Vacquier 2002). VERL polymorphism of *Haliotis fulgens* is practically zero, but that of *H. corrugata* is higher (Clark et al. 2009). Clark et al. (2009) were able to use the polymorphism in the latter species to document that the expected linkage disequilibrium arising from coevolution of lysin and its receptor did, in fact, exist, even though the two loci are not physically linked.

Concordant patterns of interspecific variation in the two molecules demonstrate that selection on lysin is acting to maintain its compatibility with conspecific VERL (Clark et al. 2009). Greater similarity between paralogous repeats of VERL within a species than between orthologous repeats from different species suggests that homogenization of these repeats is due to concerted evolution (Swanson and Vacquier 1998). In contrast, repeats 1 and 2 show greater similarity between heterospecific orthologous repeats, and their genealogy reflects that of lysin. Selection could not be detected in repeats 3–22 (Swanson and Vacquier 1998), but a number of amino-acid sites in repeats 1 and 2 show the signature of positive selection (Galindo et al. 2003; Clark et al. 2009). As the VERL molecule can bind 44 lysin ones, some mutations can be tolerated on each repeat without immediate cost to the genotypes that carry it. These mutations could then be incorporated into all repeats through concerted evolution. Swanson and Vacquier (1998) suggested that most of the repeats in VERL evolve neutrally through this process, whereas lysin is under selection to match these changes. The finding

that repeats 1 and 2 in VERL were under selection requires a different explanation. Galindo et al. (2003) and Clark et al. (2009) invoke sexual selection or sexual conflict as two possible sources of selection that could have caused speciation in abalone. Given the low polymorphism found in lysin, these processes would not be operating in the same frequency-dependent manner as in bindin. Conversely, the bindin receptor EBR1 is not likely to evolve in a manner similar to VERL, because preliminary data indicate that sequences of EBR1 repeats are variable within, as well as between, sea urchin species, and thus do not fit the hypothesis of concerted evolution (Palumbi 2009).

Conclusions

Are gamete molecules of free-spawning marine invertebrates speciation loci? How large is their role in speciation? Of the three qualifications of a species locus listed by Nosil and Schluter (2011), bindin and lysin (and their receptors) certainly meet the first; they are undoubtedly involved in reproductive isolation, at least in genera in which positive selection has been detected. It is hard to judge whether they meet Nosil's and Schluter's second requirement, that their divergence has preceded divergence of other genes. Bindin in *Echinometra* coalesces within species, whereas a nuclear coding locus does not (Palumbi 2009), the opposite of what Nosil and Schluter envision for a speciation locus. This, however, is most likely due to acceleration of divergence by positive selection on bindin, not because bindin diverged later than the nuclear locus. Bindin of sea-urchin species isolated on either side of the Isthmus of Panama 2–3 million years ago shows the effects of positive selection on bindin. Bindin exons of *Echinometra*, in which positive selection is present, are twice as divergent as those of *Arbacia,* in which selection is absent (Lessios 2008, Table 1). The higher divergence in *Echinometra* is not due to earlier isolation; the bindin intron in *Arbacia* is almost four times as divergent as the intron of *Echinometra*. Nosil and Schluter pointed out that speciation loci will, by definition, precede divergence of other genes when speciation is incomplete. The problem is deciding at which point speciation can be declared as complete. Natural hybrids between sympatric sister species occur in low frequencies both in sea urchins (Lessios 2007) and in abalone (Owen et al. 1971; Brown 1995). Are any similarities between somatic genes of different species due to incomplete speciation, introgression after secondary contact, or incomplete sorting? As more data on nuclear genes accumulate in sea urchins and abalone, an analytical procedure such as the one of Hey's and Nielsen's (2004) Isolation-Migration algorithm may provide the answer, but if speciation genes are under selection, determining the order in which they speciated relative to other genes will remain a challenge. The essence is not in timing but in causation. Did evolution of bindin and lysin actually cause speciation, or did differentiation in these molecules evolve as part of the general divergence in allopatry? Certainly the demonstration of contemporary sexual conflict in bindin has provided a plausible hypothesis of how selection on this gene can result in the evolution of (at least partial) reproductive isolation between populations independently of any other form of divergence. Nosil's and Schluter's third requirement for a speciation locus, that it should have a great effect on reproductive isolation relative to other loci (not only at present but also in the past) will probably never be met satisfactorily for any locus. The best that can be said is that lysin and VERL, as well as bindin, are loci important for reproductive isolation, but that other genes probably have contributed to reproductive isolation as well. The case for a great effect seems to be weaker for bindin, but this may well be the result of wider taxonomic sampling of this molecule, which revealed that generalizations made from a single genus did not apply universally.

In short, lysin and bindin are involved in reproductive isolation, often evolve under strong selection most likely in response to the evolution of their receptors and, depending on the species, may have a great effect, but we do not yet know the magnitude of their effect relative to that of other reproductive barriers. In one genus, affinities of different alleles of bindin have been shown to react in a way that fits the expectations of sexual conflict. We cannot say that divergence in lysin and bindin has *caused* speciation, but we can definitely say that they show evidence of having been important in reproductive isolation over the evolutionary history of species of sea urchins and abalone.

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