

Polymorphism in Abalone Fertilization Proteins Is Consistent with the Neutral Evolution of the Egg's Receptor for Lysin (VERL) and Positive Darwinian Selection of Sperm Lysin

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The evolution of species-specific fertilization in free-spawning marine invertebrates is important for reproductive isolation and may contribute to speciation. The biochemistry and evolution of proteins mediating species-specific fertilization have been extensively studied in the abalone (genus *Haliotis*). The nonenzymatic sperm protein lysin creates a hole in the egg vitelline envelope by species-specifically binding to its egg receptor, VERL. The divergence of lysin is promoted by positive Darwinian selection. In contrast, the evolution of VERL does not depart from neutrality. Here, we cloned a novel nonrepetitive region of VERL and performed an intraspecific polymorphism survey for red (*Haliotis rufescens*) and pink (*Haliotis corrugata*) abalones to explore the evolutionary forces affecting VERL. Six statistical tests showed that the evolution of VERL did not depart from neutrality. Interestingly, there was a subdivision in the VERL sequences in the pink abalone and a lack of heterozygous individuals between groups, suggesting that the evolution of assortative mating may be in progress. These results are consistent with a model which posits that egg VERL is neutrally evolving, perhaps due to its repetitive structure, while sperm lysin is subjected to positive Darwinian selection to maintain efficient interaction of the two proteins during sperm competition.

Introduction

Vertebrates and invertebrates exhibit species-specific fertilization (Metz et al. 1994; Yanagimachi 1988), meaning that homospecific mixtures of sperm and eggs yield zygotes more efficiently than heterospecific mixtures. The molecular basis of this species-specificity has been a long-standing topic of research (Loeb 1916; Lillie 1919; Vacquier 1998). The evolution of species-specific fertilization has recently been investigated to gain insights into the speciation process (Nei and Zhang 1998; Howard 1999). A recurrent theme in the evolution of reproductive proteins is extraordinary sequence divergence driven by positive Darwinian selection in closely related species (Lee, Ota, and Vacquier 1995; Swanson and Vacquier 1995; Metz and Palumbi 1996; Tsauro and Wu 1997; Ferris et al. 1997; Wyckoff, Wang, and Wu 2000). In the vast majority of examples, the evolution of only the male reproductive protein has been investigated.

For example, the sea urchin sperm protein bindin shows high levels of sequence polymorphism and an excess of amino acid replacements compared to silent substitutions (Metz and Palumbi 1996). Recently, it was shown that bindin sequence polymorphism has consequences relating to fertilization success (Palumbi 1999). The fastest evolving protein in *Drosophila* is the male reproductive protein Acp26Aa (Herndon and Wolfner 1995; Schmid and Tautz 1997; Tsauro and Wu 1997; Aguadé 1998). Analysis of sequence divergence be-

tween closely related species has demonstrated that the evolution of Acp26Aa is driven by positive Darwinian selection, but the selective pressure remains a mystery (Tsauro and Wu 1997). Additionally, the female receptor for Acp26Aa remains unknown. The only system in which the evolution of both cognate male and female reproductive proteins has been investigated is that of the abalone (genus *Haliotis*; Swanson and Vacquier 1998).

The abalone system is also the best characterized system for understanding the molecular and evolutionary basis for species-specific fertilization (Swanson et al. 1998). Abalones are large marine archeogastropods with external fertilization. Seven species coexist off the west coast of North America; many have overlapping breeding seasons and habitats. Despite the potential for hybridization, the species remain distinct, with hybrids only rarely being found in the wild (Owen, McLean, and Meyer 1971). The basis for maintaining distinct species could be in part attributed to species-specific fertilization, which can be quantitatively demonstrated in the laboratory (Leighton and Lewis 1982).

Following the release of gametes, the events of abalone fertilization which can exhibit species specificity include chemotaxis of sperm to the egg, induction of the sperm acrosome reaction, dissolution of the egg vitelline envelope (VE), and binding and fusion of the two gametes (Vacquier and Lee 1993; Swanson and Vacquier 1997; Vacquier 1998). The dissolution of the VE has been extensively studied in the abalone and has been demonstrated to exhibit species specificity (Vacquier, Carner, and Stout 1990). Dissolution of the VE is mediated by the sperm protein lysin, which nonenzymatically creates a hole in the VE by stereospecifically competing for hydrogen bonds and hydrophobic interactions among the fibers comprising the VE, leading to the unraveling of the fibers and the creation of a hole in the VE (Lewis, Talbot, and Vacquier 1982). The vitelline envelope receptor for lysin (VERL) is a fibrous mole-

Key words: lysin, VERL, Darwinian selection, speciation, fertilization, abalone, gamete recognition, sperm-egg interaction, sperm competition.

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cule of 1,000 kDa containing approximately 28 repeats of 153 amino acids (Swanson and Vacquier 1997, 1998). In-solution binding kinetics demonstrate that lysin and VERL interact with high affinity (EC_{50} 10 nM). Furthermore, lysin-VERL binding shows positive cooperativity and the same species-specificity as does lysin mediated VE dissolution, indicating that the specificity resides in the two isolated molecules (Swanson and Vacquier 1997).

Lysin is extremely divergent between closely related species (Lee, Ota, and Vacquier 1995) and is among the fastest evolving metazoan proteins yet discovered (Metz, Robles-Sikisaka, and Vacquier 1998). Analysis of the number of nonsynonymous substitutions per nonsynonymous site (d_N) compared with that of synonymous substitutions per synonymous site (d_S) shows that d_N exceeds d_S by as much as fourfold in pairwise comparisons of species (Lee and Vacquier 1992; Lee, Ota, and Vacquier 1995). Lysin is monomorphic in California red abalone, indicating that red abalone lysin evolution proceeds by a series of selective sweeps (Metz, Robles-Sikisaka, and Vacquier 1998). Amino acid replacements are scattered over the entire sequence, but the N- and C-termini are especially divergent between species. Site-directed mutagenesis shows that the species specificity can be attributed, in part, to the N- and C-termini (Lyon and Vacquier 1999).

To gain insights into the selective forces driving the rapid divergence of lysin, the evolution of VERL was previously investigated by sequencing VERL repeats between species of abalone (Swanson and Vacquier 1998). VERL repeats were shown to be more similar within a species than between species. This process of homogenization of repeats has been termed concerted evolution and occurs by unequal crossing over and gene conversion (Elder and Turner 1995). In contrast to lysin from these same species (Lee and Vacquier 1992), the VERL repeats did not show signs of positive Darwinian selection based on d_N/d_S ratios. It was hypothesized that the redundant nature of the VERL molecule reduced the functional constraint of each VERL repeat, leading to relaxed selection on repeats (Swanson and Vacquier 1998).

To investigate the evolutionary forces affecting the divergence of VERL, a polymorphism survey of the VERL locus was conducted. To insure comparison of orthologous regions of the repetitive VERL molecule, we identified the C-terminal VERL repeat (the last repeat) and a novel nonrepetitive portion of the protein. After obtaining polymorphism sequence data for both pink abalone (*Haliotis corrugata*) and red abalone (*Haliotis rufescens*) species, additional tests of neutrality were performed. Some of these tests may be more powerful at detecting certain types of positive Darwinian selection than interspecific d_N/d_S ratios calculated over the entire molecule (Kreitman and Akashi 1995; Aquadro 1997).

Materials and Methods

cDNA and 3' RACE

Abalone ovaries were homogenized in a Waring blender in solution D (4 M guanidinium thiocyanate, 25

mM Na citrate [pH 7], 0.5% sarcosine, and 0.5 M β -mercaptoethanol) as previously described (MacDonald 1987). Insoluble debris was removed by centrifugation at $5,000 \times g$ for 10 min. RNA was selectively precipitated from the supernatant by the addition of potassium acetate to 0.1 M, acetic acid to 1.4 M, and 100% ethanol to final concentration of 75% while vortexing and precipitated at -20°C overnight. RNA was pelleted by centrifuging $10,000 \times g$ for 30 min, and the pellet was dissolved in 52 ml solution D with 4 ml of 5.7 M cesium chloride. Nine milliliters of the resuspended RNA was layered onto 3 ml 5.7 M CsCl with 0.3 M EDTA and centrifuged in a Beckman SW41 rotor at 30,000 rpm for 24 h. The RNA pellet was rinsed with 70% ethanol and resuspended in DEPC-treated water. Poly A⁺ RNA was purified from total RNA using the oligotex kit following the manufacturer's directions (Qiagen). First-strand cDNA was made with an adapter-dT25 primer using BRL superscript polymerase following the manufacturer's directions. PCR was performed with a VERL-specific primer and the adapter primer. Amplified products were gel-purified using Qiagen QiaQuick columns and blunt-end-cloned into pBS. Plasmid DNA was purified using Qiagen plasmid mini kits and sequenced using ABI Prism Big Dye chemistry.

Polymorphism PCR

DNA was prepared as described in Metz, Robles-Sikisaka, and Vacquier (1998). Briefly, one peripheral epipodial tentacle was clipped from each living abalone, washed in 20 mM Tris (pH 7.6) and 20 mM EDTA, and homogenized in 300 μl of the same solution containing 10% (vol/vol) chelating resin (Sigma) in a 1.5-ml tube. The tube was boiled for 5 min, vortexed, and centrifuged. PCR amplifications for the last VERL repeat and a portion of C-terminal nonrepeat region were carried out with primers vbend2 (CCAGAGCAAACCTGATCGACTG) and mendR (CCATGATACTCCTATGTGCAG) in a 50- μl reaction containing 50 pmol of each primer, 1 μl *Taq* polymerase, 1 \times *Taq* polymerase buffer (Promega), 1.5 mM MgCl_2 , 0.2 mM of each dNTP, and 1 μl of template DNA. Thermal cycle settings were 94°C for 30 s, 50°C for 30 s, and 72°C for 90 s for 36 cycles. PCR products were purified and sequenced directly. The sequences presented here are available in GenBank under accession numbers AF250892–AF250909.

Sequence Analysis

Sequences were aligned using CLUSTAL W (<http://www2.ebi.ac.uk/clustalw>). Maximum-likelihood analyses were performed using the PAML package (Yang 1999: <http://abacus.gene.ucl.ac.uk/ziheng/paml.html>). For site variation, three likelihood ratio tests were used. First, we compared a neutral model (M1) with two d_N/d_S ratios (0 for conserved sites and 1 for neutral sites) to a selection model (M2) with an additional class of sites with a d_N/d_S ratio estimated from the data (Nielsen and Yang 1998). The second test compared the neutral model (M1) to a selection model (M3) with three d_N/d_S classes estimated

from a discrete distribution (Yang et al. 2000). The third test compared a neutral model (M7) assuming a beta distribution of d_N/d_S among sites (Yang et al. 2000). This is a flexible distribution, but d_N/d_S is limited in the interval (0, 1), where 0 indicates complete constraint and 1 is the expectation under no selective constraint. The alternative selection model (M8) adds an extra class of sites with d_N/d_S estimated from the data, thus allowing for positively selected sites (Yang 1999). Twice the log likelihood difference between the two models was compared with the chi-square distribution, with the degrees of freedom based on the difference between the number of parameters estimated from the models (Nielsen and Yang 1998; Yang 1999; Yang et al. 2000). For the lineage variation, one VERL repeat from each species was randomly chosen for the analysis. Twice the difference between the log likelihood differences of the models was compared with the chi-square distribution with 10 df (Yang 1998). The analysis was repeated several times with different VERL repeats from each species, and similar results were obtained. A neighbor-joining tree was constructed using MEGA (Kumar, Tamura, and Nei 1993) with 1,000 bootstrap replicates and pairwise deletion of gaps. Tajima's (1989) D statistic, Fu and Li's (1993) D^* statistic, Hudson, Kreitman, and Aguadé (1987; HKA) tests, and the McDonald-Kreitman (MK) test (McDonald and Kreitman 1991) were all computed using DnaSP 3.0 (Rozas and Rozas 1999). Significance values for Tajima's D statistic were obtained by coalescence simulations. The HKA test compared the last VERL repeat region with the C-terminal nonrepeat region.

Results

Analysis of Divergence Between Red and Pink Abalones

Previous analyses of d_N/d_S ratios calculated using the entire VERL repeat sequence did not indicate positive selection (Swanson and Vacquier 1998). Failure of d_N/d_S ratios for the entire VERL repeat to show signs of positive selection could arise from selection at only a few amino acid sites (Nielsen and Yang 1998; Yang et al. 2000) or from episodic positive selection followed by periods of purifying selection (Messier and Stewart 1997; Yang 1998). To test for departures from neutrality at the VERL locus, the divergence data between species were analyzed using maximum-likelihood ratio tests (Nielsen and Yang 1998; Yang 1998; Yang et al. 2000). Maximum-likelihood methods were used to analyze the sequences with variation of the d_N/d_S ratio among sites or among lineages. In both cases, a neutral model was compared to a selection model and twice the log likelihood difference between the models was compared to the chi-square distribution. For site variation, three models of codon evolution were used (as described in Materials and Methods). These covered a range of codon substitution models, ranging from two d_N/d_S ratios (0 for conserved and 1 for neutral) to a beta distribution with d_N/d_S ratios estimated from the data (Yang et al. 2000). For the lineage variation analysis, the neutral model maintained a constant d_N/d_S ratio among lineages,

Table 1
Maximum-Likelihood Analysis of VERL Divergence

Model	Neutral	Positive	$2\Delta^a$	df	P
Site variation					
M1 vs. M2.	-1,099.54	-1,098.72	1.6	2	0.45
M1 vs. M3.	-1,099.54	-1,098.69	1.7	4	0.79
M7 vs. M8.	-1,099.54	-1,098.59	2.0	2	0.34
Lineage variation . . .					
	-1,104.39	-1,101.14	6.5	10	0.77

^a Twice the log likelihood difference between the two models.

while the selection model estimated separate d_N/d_S ratios for each lineage (Yang 1998). Variation of the d_N/d_S ratios between either sites or lineages was not detected (table 1).

Identification of a Novel Nonrepetitive Region of VERL

Using 3' RACE, a novel nonrepeat region was found 3' of the last VERL repeat. This region is 348 amino acids in length and rich in cysteines (fig. 1). This extreme C-terminus of VERL contains a stretch of 25 hydrophobic amino acids that could form a transmembrane domain lacking a cytoplasmic tail. Twenty-two amino acids upstream of the hydrophobic region is a furin protease cleavage site (RRKRR). This domain structure is analogous to the vertebrate zona pellucida (ZP) egg coat protein ZP2 (Wassarman and Mortillo 1991; Tian, Gong, and Lennarz 1999) and may represent a common mechanism for assembling elevated animal egg coats. As hypothesized for the ZP, the abalone VERL may be assembled on the egg cell surface tethered to the membrane by a single transmembrane domain of one component protein. Once assembly is complete, the VERL may be cleaved by furin to form the elevated envelope surrounding the unfertilized egg (Litscher, Qi, and Wassarman 1999).

Divergence between the pink and red abalone species was concentrated in the last VERL repeat (fig. 2) and the region immediately following it. The latter region shows some similarity to VERL repeats and may represent degenerate repeat sequence, which may be due to a reduced homogenizing effect of concerted evolution at the ends of repeat arrays (McAllister and Werren 1999). Much of the C-terminal nonrepeat region is conserved between species. The increased level of divergence in the repeat region may be functionally significant, since lysin binds the VERL repeats (Swanson and Vacquier 1997).

Polymorphism in VERL

A region consisting of 126 residues of the last VERL repeat and 108 residues of the 3' nonrepetitive region was sequenced from 11 pink abalones and 10 red abalones. The pink abalones were all collected at San Diego, while the red abalones were collected from San Diego and Mendocino (a separation of ~1,200 km). Polymorphisms were found in both species. Red abalone VERL showed lower levels of polymorphism than pink

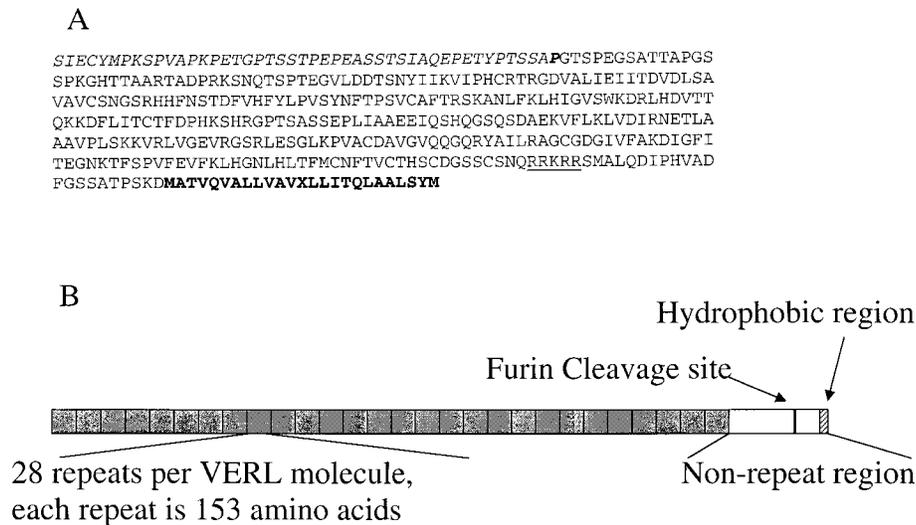


FIG. 1.—Identification of the 3' end of VERL. A, Deduced amino acid sequence of VERL carboxyl terminus. A portion of the last VERL repeat is shown in italics, with the last residue (P) in bold; the furin cleavage site is underlined, and the hydrophobic region is in bold. B, Schematic representation of the VERL molecule. The sequence 5' of the first repeat remains unknown.

abalone VERL. Tajima's *D* statistic for red abalone VERL ($D = -1.4$) had a relatively large negative value that would be consistent with recovery from a recent selective sweep. However, it did not differ significantly from the equilibrium neutral expectation (table 2). Fu and Li's test statistic yielded similar results (table 2). The lack of a signal of selection from other statistical tests (see below) argues against a recent selective sweep. Furthermore, the levels of polymorphism of red abalone VERL were comparable to those seen in the mitochondrial CO1 gene of this species (table 2; Metz, Robles-Sikisaka, and Vacquier 1998).

In contrast to red abalone VERL, pink abalone VERL is more polymorphic. Tajima's *D* statistic ($D = +1.1$) was positive but not significantly different from neutral expectations (table 2). Fu and Li's test statistic gave similar results (table 2). However, despite the individuals coming from the same population, a neighbor-joining tree showed a distinct population subdivision (fig. 3). Of the 11 individuals sequenced, 10 were homozygotes for one of the two types. These two types, present in equal frequencies, differed by both amino acid replacements and insertions/deletions (fig. 4). Remarkably, only one individual was found to be a heterozygote between the two groups, as observed in the sequencing chromatograms for both point mutations and the sequencing going out of register at the insertion site. This lack of heterozygotes between the two VERL repeat types of pink abalone suggests that the evolution of assortative mating may be in progress. The same individ-

uals did not show this subdivision at the lysin gene (see below), indicating that the process generating the subdivision is specific to the VERL locus. Alternatively, the lack of heterozygotes could have arisen from biased PCR amplification. However, if this were so, PCR would be unlikely to have amplified two separate types in equal numbers. Furthermore, heterozygous individuals at silent sites were present in both groups at sites not distinguishing the two groups, as seen in sequencing chromatograms, indicating that two alleles were amplified in the PCR.

Given the subdivision in pink abalone egg VERL, we initiated a search for similar subdivision in pink abalone sperm lysin. Since the entire genomic sequence for lysin remains undetermined, we were limited to analyzing a portion of the lysin gene from these individuals. Unlike red abalone lysin, which is monomorphic (Metz, Robles-Sikisaka, and Vacquier 1998), pink abalone lysin does contain polymorphic sites. Our limited initial sample of pink abalone lysin polymorphism does not cluster into two groups, as does pink abalone VERL. The frequency distribution of the first pink abalone lysin exon shows a significantly negative Tajima's *D* statistic (table 2; $D = -1.6$; $P = 0.03$), consistent with recovery from a recent selective sweep. The fifth pink abalone lysin exon does not show signs of a selective sweep (table 2). Fu and Li's test statistic gives similar results (table 2). However, recombination between these regions is possible given the large introns found in lysin. Such

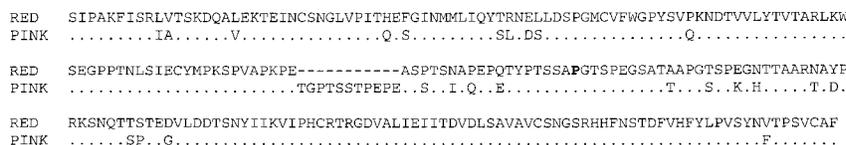


FIG. 2.—Divergence of the last VERL repeat and the carboxyl-terminal region. Dots denote identity to the red sequence; dashes are inserted for alignment. The bold P marks the end of the VERL repeat and the beginning of the nonrepetitive carboxyl-terminus.

Table 2
Abalone Polymorphism Summary Statistics

Locus	<i>N</i>	Length (bp)	π (%)	Tajima's <i>D</i>	Fu and Li's <i>D</i> *
Pink lysin exon 1	10	621	0.2	-1.6 (<i>P</i> = 0.03)	-1.8 (<i>P</i> > 0.5)
Pink lysin exon 5	5	373	0.7	0.0 (<i>P</i> = 0.54)	0.0 (<i>P</i> > 0.1)
Pink VERL	10	757	1.5	+1.1 (<i>P</i> = 0.88)	0.9 (<i>P</i> > 0.1)
Red lysin ^a	6	1,555	0.0	—	—
Red VERL	8	1,165	0.2	-1.4 (<i>P</i> = 0.07)	-1.6 (<i>P</i> > 0.1)
Red G-protein intron ^a	6	750	1.4	+0.4 (<i>P</i> = 0.54)	0.3 (<i>P</i> > 0.1)
Red mtCO1 ^a	11	528	0.4	-0.2 (<i>P</i> = 0.42)	0.3 (<i>P</i> > 0.1)

^a Taken from Metz, Robles-Sikisaka, and Vacquier (1998)

recombination could weaken any signs of hitchhiking associated with selective sweeps in other parts of lysin.

The VERL polymorphism and divergence data were used to test for departures from neutrality using a variety of statistical tests; no departures from neutrality were detected. First, the nonrepeat C-terminal region of VERL was compared to the last VERL repeat with the HKA test (table 3). If evolution is neutral, the ratio of divergence to polymorphism should be the same for the two regions. In all comparisons using the HKA test there was no departure from neutrality. The pink abalone HKA comparison remained nonsignificant when the two subdivided VERL sequences were analyzed separately

(table 3). Our HKA tests used linked regions of VERL; the comparison to an unlinked neutral locus will be important goal for future work. The ratios of silent and replacement changes between species was then compared with the MK test. If evolution is neutral, the silent/replacement ratios should be similar for both the polymorphic changes and fixed divergent changes. For VERL, the ratios were identical, indicating no departure from neutrality. Finally, we calculated d_N/d_S for all possible pairwise comparisons between the polymorphic VERL repeats. In no case was d_N significantly greater than d_S (data not shown).

Discussion

Previous analysis of interspecific divergence data suggested that the evolution of the abalone VERL did not depart from neutrality (Swanson and Vacquier 1998). This was in stark contrast to lysin, which is one of the fastest evolving metazoan proteins known and perhaps the most robust molecular example of positive Darwinian selection (Metz, Robles-Sikisaka, and Vacquier 1998). Here, we further analyzed the evolutionary forces acting on VERL by performing a polymorphism study of VERL from two species. A previously unreported C-terminal nonrepeat region of VERL was identified to insure comparison of orthologous regions of the molecule. No evidence of a departure from an equilibrium neutral model of evolution was observed at VERL either within or between species.

It had been hypothesized that the redundant and repetitive nature of VERL repeats could lead to relaxed selection on individual VERL repeats within a single VERL molecule (Swanson and Vacquier 1998). Since there may be 28 repeats in each VERL molecule, mutations in one repeat may be only weakly selected against (if at all), since the remaining 27 VERL repeats would remain functional. As long as the mutation does not disrupt the construction of the VE, from the female's perspective it may be tolerated. This would be particularly likely for mutations that affect VERL-lysin interaction, since there is potentially an excess of sperm (as suggested by observed spawning [Stekoll and Shirley 1993] and comparison of other taxa [Yund 2000]), such that even eggs with a reduced VERL-lysin affinity would most likely be fertilized.

It was also hypothesized that the neutral drift of the egg receptor VERL leads to a continually changing target to which lysin must adapt in order to maintain op-

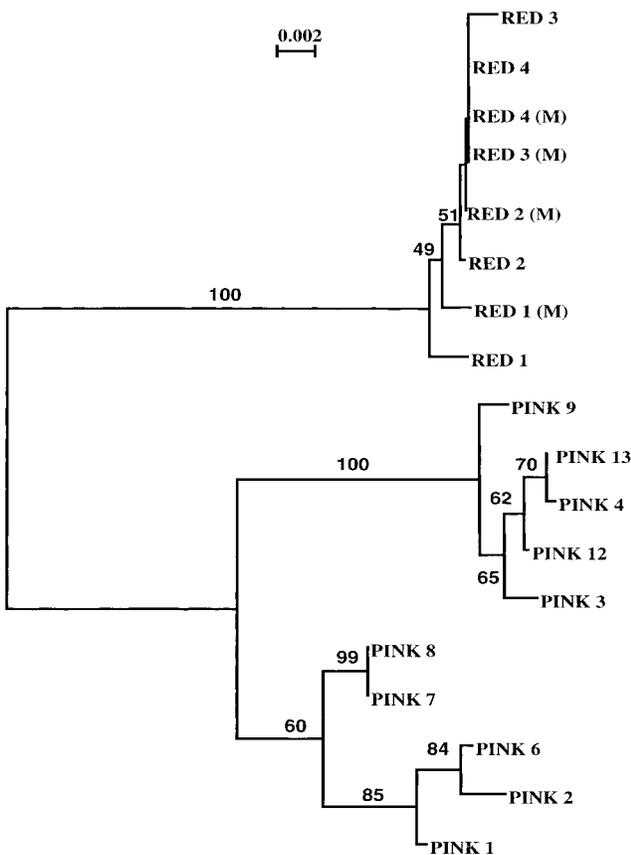


FIG. 3.—Neighbor-joining phylogenetic tree of VERL polymorphism. Numbers on branches represent bootstrap values (1,000 replicates). The pink VERL sequences group into two distinct clades. M = red individuals from Mendocino, California. All other individuals are from San Diego.

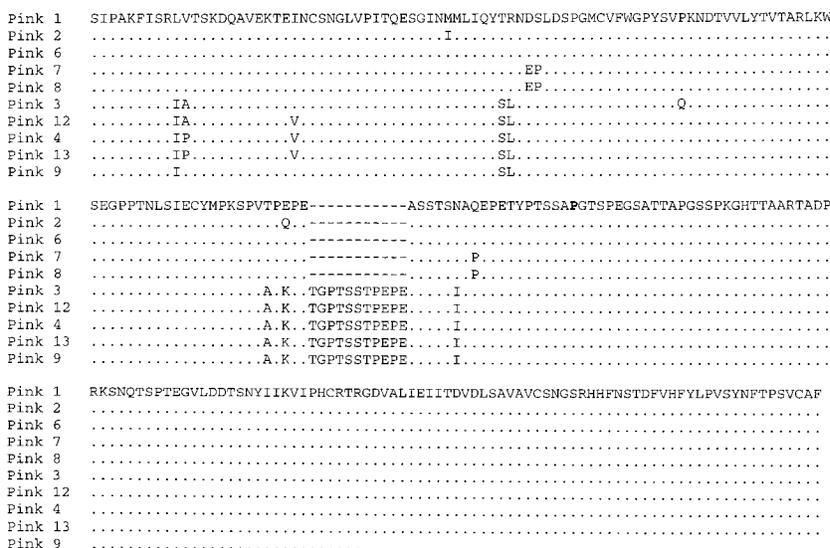


FIG. 4.—Deduced amino acid sequences of the pink VERL polymorphism data. Dots denote identity to the top sequence; dashes are inserted for alignment. The last residue (P) of the VERL repeat is in bold. All of the amino acid replacements are in the VERL repeat.

timal interaction of sperm and egg cognate proteins. Selection would favor lysin variants that bind optimally, since once lysin is released, if the sperm does not fertilize the egg encountered, its genetic material would not be passed to the next generation. Thus, there may be considerable competition among sperm to fertilize the egg. The redundant nature of the VERL molecule is a key part of this hypothesis, as is the homogenization of VERL repeats by concerted evolution for the maintenance of the redundant nature of VERL and thus a single target to which lysin can adapt.

The analyses presented here are consistent with previous results demonstrating neutral evolution of the abalone egg receptor, VERL (Swanson and Vacquier 1998), despite strong positive Darwinian selection on the cognate sperm protein lysin (Lee, Ota, and Vacquier 1995; Metz, Robles-Sikisaka, and Vacquier 1998). It appears that lysin is evolving to match changes in a neutrally drifting VERL. Lysin exhibits two to three times the number of amino acid substitutions of VERL (Nei and Zhang 1998). The simplest model of lysin-VERL coevolution may predict a one-to-one correlation for the

divergence of lysin and VERL. However, there is no a priori reason to expect a one-to-one correlation between lysin and VERL divergence. Lysin may need to undergo multiple substitutions for each substitution in VERL. When the repeated nature of VERL is considered, it is possible to construct scenarios in which lysin may require fixation of multiple changes for every single change in VERL in order to maintain optimal interaction with VERL. For example, a mutation in one VERL repeat may not produce a selective force for lysin to change. However, if by chance this becomes more prevalent among the VERL repeats, there would be strong selection for a corresponding change in lysin. At the point where the mutation represents 50% of the repeats, selection may become strong, favoring lysins which interact with the two types of repeats, which may not be optimal for either type alone. When the mutation is present in the majority of repeats, selection may then favor the single lysin variant best adapted to a single VERL repeat type. Thus, one mutation in VERL could lead to multiple rounds of adaptation in lysin.

Lysin functions specifically to dissolve the egg VE and probably does not have other functions (Lewis, Talbot, and Vacquier 1982). Likewise, VERL is a major component of the egg VE and most likely does not have other functions (Swanson and Vacquier 1997). Therefore, the evolutionary forces driving their divergence are most likely related to their roles in fertilization. Although we cannot rule out other molecules being involved in the dissolution of the VE, extensive biochemical data suggest the dissolution of the VE occurs through the specific interaction of lysin and its egg receptor, VERL (Swanson and Vacquier 1997). The binding kinetics of isolated VERL and lysin show high affinity and the same species specificity as lysin-mediated dissolution of intact VEs. Furthermore, the addition of the remaining VE components does not alter the binding kinetics of lysin and VERL, indicating that lysin does not

Table 3
VERL HKA Tests

	Repeat	C-Terminus	<i>P</i>
All Pink			
Polymorphism	14	3	0.07
Divergence	12.5	16.85	
Pink group 1			
Polymorphism	1	1	0.83
Divergence	12.2	17.45	
Pink group 2			
Polymorphism	5	2	0.34
Divergence	12.8	16.25	
All red			
Polymorphism	1	2	0.79
Divergence	12.5	16.85	

have high affinity for the other VE components (unpublished data).

The pink abalone population subdivision observed at the VERL locus suggests the possibility that assortative mating is being established. If so, this process may be in progress, because we do not observe similar subdivision at other pink abalone loci. However, we studied only the last repeat of the repeat array. This repeat is least likely to be subjected to the homogenizing effects of concerted evolution due to its being at the end of the repeat array (McAllister and Werren 1999). It is unknown if other repeats in the repeat array will show similar subdivision. However, subdivision is also observed in the nonrepeat carboxyl-terminal region in silent sites (data not shown). Currently, we are not able to detect corresponding changes in pink abalone lysin. Elucidation of the entire pink lysin genomic structure (or working from cDNA for lysin) will permit these types of analyses. We prefer to pursue the use of genomic DNA, because sampling can be performed non-invasively, which is important given the recent dramatic declines in abalone populations. It is also of interest that the levels of polymorphism tend to correlate between abalone lysin and VERL. For example, low VERL polymorphism in red abalone is associated with a lack of red lysin polymorphism. In contrast, moderate pink lysin polymorphism is associated with relatively high levels of pink VERL polymorphism. Taken together, these observations suggest the coevolution of VERL and lysin. However, polymorphism levels at other loci will have to be studied in order to determine if this is a genomewide effect relating to effective population size or specific to these two loci.

From their analysis of the sea urchin sperm protein bindin, Metz and Palumbi (1996) first suggested the hypothesis that assortative mating in marine invertebrates could evolve through the interaction of male gamete recognition protein with a "tolerant" female receptor. However, evolutionary analysis of the female receptor for bindin has not been possible. Our analyses of the abalone egg VERL is consistent with this hypothesis. The "tolerant" nature of the egg receptor in abalone may result from the egg receptor's being largely a highly repeated structure. Furthermore, relaxed selection on the female locus of a mate recognition system is consistent with a theoretical model for the evolution of assortative mating (Wu 1985). Future studies on the evolution of gamete recognition proteins could provide insights into their role in speciation.

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