Rapid Evolution of Fertilization Selectivity and Lysin cDNA Sequences in Teguline Gastropods

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Proteins mediating intercellular recognition face opposing selective forces as they evolve: purifying selection to maintain function, and diversifying selection to alter specificity. Lysin is a 16-kDa protein which enables sperm of free-spawning marine snails to make a hole in the vitelline layer (VE) surrounding conspecific eggs. Previous work on abalone (*Haliotis* spp.) has shown that positive selection promotes rapid interspecific divergence of lysin. Here, we present data on the specificity of VE dissolution by four species of teguline gastropods, along with lysin cDNA sequences. The teguline and abalone lineages diverged over 250 MYA. As in abalone, VE dissolution by lysin in tegulines is species-selective, and positive selection promotes rapid interspecific divergence over the entire mature protein. Nonsynonymous substitution rates, calculated using a mtCOI molecular clock calibrated by two *Tegula* species separated by the Isthmus of Panama, are high (>25 substitutions per site per 10^9 years). However, the extensive replacements in teguline lysins are overwhelmingly conservative with respect to type, charge, and polarity of residues. Predictions of secondary structure suggest that the size and position of α-helices are also conserved, even through pairwise amino acid identities between *Haliotis rufescens* and the different tegulines are less than 15%.

Introduction

Molecules generally evolve at rates inversely proportional to the functional constraints on them (Kimura 1983). Pseudogenes, for example, change faster than their functional progenitors (Li, Gojobori, and Nei 1981), and introns evolve faster than exons (Bodmer and Ashburner 1982). However, many genes encoding proteins whose functions apparently bear directly on fitness also diverge at high rates, including proteins intimately involved with host/pathogen interactions (Hughes, Ota, and Nei 1990; Hughes 1991) and sexual reproduction (Lee, Ota, and Vacquier 1995; Ferris et al. 1997; Civetta and Singh 1998). How do such proteins simultaneously conserve their general function (e.g., peptide presentations or sperm–egg binding) while at the same time rapidly changing their specificity?

The simplest resolution to such conflict is for domains responsible for conserved function to evolve far more slowly than other portions of the molecule which confer specificity. Such regionalization appears to be the case in the class I major histocompatibility complex (MHC) glycoproteins, where nucleotide substitutions promoted by positive selection are concentrated within the antigen-binding cleft; residues outside the cleft are subject to purifying selection (Hughes, Ota, and Nei 1990).

Such obvious regionalization of function does not seem to be the case for the gamete recognition protein lysin. Lysin is released from a vesicle (the acrosome) within the sperm head of free-spawning marine snails after contact with the vitelline envelope (VE), a tough glycoproteinaceous covering surrounding the egg. Lysin dissolves a hole in the VE, thereby allowing the sperm to pass through and fuse with the egg's plasma membrane (Haino-Fukushima 1974; Lewis, Talbot, and Vacquier 1982). In the large marine snails in which lysin has been best studied (abalone, *Haliotis* spp.), VE dissolution by lysin is species-selective; lysin dissolves homospecific VEs more efficiently than it dissolves heterospecific VEs (Vacquier and Lee 1993; Swanson and Vacquier 1997). Interspecific comparisons of lysin cDNAs from multiple species of abalone showed that positive selection has promoted their divergence (Lee, Ota, and Vacquier 1995). Unlike the case for MHC or the sperm–egg recognition protein bindin from sea urchins (Metz and Palumbi 1996), the signal for positive selection is not restricted to particular regions within lysin, but, rather, occurs throughout its length. Another abalone acrosomal protein, formed by gene duplication from lysin, also shows such declocalized positive selection (Swanson and Vacquier 1995).

Functional constraints not evident at the primary structural level may reveal themselves in conserved two- and three-dimensional structures. The structure of lysin from the red abalone (*Haliotis rufescens*) is known to 1.9-Å resolution (Shaw et al. 1993). The molecule contains five α-helices and no β-sheets. Two tracks of basic residues span the length of one side of lysin, and may be involved with disrupting hydrogen bonds that hold the filaments of the VE together. On the side opposite the basic tracks, 11 exposed hydrophobic residues form a hydrophobic patch. This hydrophobic patch is involved with dimerization of lysin (Shaw et al. 1995) and may also play a role in disrupting hydrophobic interactions that may hold the VE filaments together. The basic tracks and the hydrophobic patch are conserved among abalone lysins.

Thus, for gastropod lysins, we have three requisites for studying how conflicting selection for conserved function and changing specificity can be resolved: a

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Abbreviations: VE, vitelline envelope; BSA, bovine serum albumin.

Key words: fertilization, gamete recognition, lysin, protein secondary structure, sperm, *Tegula*.

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clear functional assay (VE dissolution), a rapid divergence exhibiting positive selection, and a well-resolved protein crystal structure. Here, we explore the extent to which lysin can accommodate divergence in specificity while conserving general function by studying lysin from four species of the subfamily Tegulinae: *Norrisia norrisii*, *Tegula aureotincta*, *Tegula brunnea*, and *Tegula funebralis*. The lineages giving rise to the tegulines and to abalone (Superfamilies Trochoidea and Haliotoidea, respectively) are sister groups (Harasewych et al. 1997; Ponder and Lindberg 1997). Fossil data suggest a minimum time since divergence for Trochoidea and Haliotoidea of 250 Myr (Tracey, Todd, and Erwin 1993), far earlier than the origin of extant *Haliotis* species (about 60–70 MYA; Lindberg 1991).

Here, we address three questions concerning teguline lysin: (1) Do lysins from these four species dissolve VEs selectivity? (2) Do lysin cDNAs diverge rapidly between species? If so, does the pattern of substitutions suggest positive selection? (3) Is lysin secondary structure conserved among species with widely divergent primary structures?

Materials and Methods

Dissolution Assays

Tegula brunnea were collected at Pacific Grove, Calif.; the three other species were gathered from San Diego, Calif. Gametes were obtained by cracking shells, removing ripe gonads, and shaking gametes free into chilled seawater. Gametes from 2 to 14 individuals were used to obtain sufficient material; thus, assays cannot discern any possible intraspecific variation in species selectivity of dissolution.

Eggs were washed three times by settling, the last of these (and all subsequent) washes being in 1 mg/ml BSA seawater. VEs were isolated from other egg components by disruption with two strokes of a Teflon-glass Dounce homogenizer, followed by gentle centrifugation ($200 \times g$ for 5 min) at 4°C. The resulting supernant (including yolk) was drawn off, leaving settled VEs at the bottom of the tube. This process was repeated until only clean VEs remained (Lewis, Talbot, and Vacquier 1982). VE preps were stored in seawater (10 mM sodium azide) at 4°C.

Sperm were pelleted at $1{,}000 \times g$ (15 min) and resuspended in two volumes of seawater. Acrosomal exudate was produced by freezing this suspension, thawing on ice, vortexing, and centrifugation at $10{,}000 \times g$ (30 min, 4°C). The resulting supernantant (containing lysin and other acrosomal proteins) was drawn off and stored in 100 mM Tris (pH 8.0), 10 mM sodium azide in seawater (4°C or −70°C). Protein concentrations were determined using the BCA method (Pierce), with BSA as a standard.

Because lysin is among the most abundant proteins in *Tegula* sperm (personal observation) and no other acrosomal proteins dissolve VEs (Swanson and Vacquier 1995; personal observation), the acrosomal exudates were used to evaluate the ability of lysin to dissolve homospecific and heterospecific VEs. The small volumes of material available precluded spectrophotometric assays. Instead, qualitative dissolution assays were carried out as follows: Serial 1:1 dilutions (in 100 mM Tris [pH 8] seawater) of the acrosomal exudates were made in microtiter plates. Two microliters of VE suspensions (three to six individual VEs) were added to these 20-μl acrosomal protein solutions. After covering with parafilm, dissolutions proceed overnight at 22°C. VE dissolution was scored by observation with an inverted microscope on the following qualitative scale: 1 (no dissolution whatsoever), 2 (VEs begin to thin), 3 (VEs inflate; still round, but diameter noticeably larger), 4 (VEs torn apart, no longer round, but still visible), 5 (VEs no longer visible). Dissolution experiments were performed simultaneously using the same preparations of VEs and acrosomal proteins. Because the proportion of lysin in the acrosomal preparations could vary among species, detailed cross-species dissolution comparisons are not warranted.

Protein Isolation and Sequencing

Proteins in the acrosomal exudates of *T. aureotincta* and *T. funebralis* were separated on 15% acrylamide SDS-PAGE and transferred to a polyvinyldifluoride membrane in 10 mM CAPS buffer (pH 11), 10% methanol. The polyvinyldifluoride membrane was stained with Coomassie blue and destained in 50% methanol. Bands of 16 kDa (the second most abundant protein in these two species and the same size as lysin in *Haliotis* spp.) were excised, and amino-terminal sequences were determined by gas phase microsequencing.

mRNA Isolation, cDNA Synthesis, and Sequencing

Total RNA was isolated by homogenizing the testes of one ripe male in 9 ml of Solution D (Chomczynski and Sacchi 1987) without sarkosyl, 0.5 ml β-mercaptoethanol in a glass Dounce homogenizer and then centrifuged at $5{,}000 \times g$ (15 min, 4°C). The supernantant was decanted and then mixed with 0.5 ml 2 M potassium acetate (pH 5.5) and 0.3 ml 3 M acetic acid. Five milliliters of 100% ethanol was slowly added, and the resulting solution was transferred to a Corex tube and precipitated at −20°C overnight. RNA was pelleted by centrifugation at $9{,}000 \times g$ (20 min, 4°C). The resulting pellet was resuspended in 8.5 ml Solution D (no sarkosyl, no β-mercaptoethanol), then layered over 4 ml of 5.7 M CsCl and spun for 20 h in a Beckman SW41 rotor at 30,000 rpm at 18°C. The resulting pellet was resuspended in 0.5 ml water. mRNA was isolated from this total RNA preparation using oligo-dT spin separation columns (Invitrogen) following the manufacturer's instructions.

A *T. brunnea* testis cDNA library (Stratagene) was constructed following the manufacturer's instructions. cDNA for the other species was produced by reverse transcription using either oligo-dT_{25} or primer TPFI3 (see below).

Lysin cDNA was initially amplified by PCR using the *T. brunnea* testes cDNA library as template. Unpublished lysin cDNA sequence from *Tegula pfeifferi* (generously provided by K. Haino-Fukushima) was used to

design a primer near the poly-A signal sequence (TPFI3: 5′-TGAAGCCCTAAATATACATTTATT-3′), which was paired with the T3 vector primer. Forward primers were then designed using conserved 5′ signal sequences from *T. brunnea* and *T. pfeifferi* (5′-ATGAARGGTGCNGTN-CTNTGY-3′) and used along with TPFI3 to amplify lysin cDNA from *N. norrisii* and *T. aureotincta*. Lysin from *T. funebralis* was initially amplified using an internal forward primer based on amino acid sequences (5′-TAYGTNGAYTTYGGTMGNGTNAAYAAYGG-3′) paired with TPFI3. An internal reverse primer (5′-TGG-CCCATTGGCTGTAGTTGTTCC-3′) was used in combination with oligo-dC_{22} to amplify the 5′ end of *T. funebralis* lysin from dG-tailed cDNA template.

PCR reactions contained each primer at 0.5 μM (except that concentrations of degenerate primers were increased in direct proportion to their degeneracy), *Taq* polymerase at 10 U/ml, TaqExtender (Stratagene) at 10 U/ml, 1 × TaqExtender buffer, 0.2 mM each dNTP, and 1–2 μl of template DNA in a total volume of 50 μl. Thermal profiles consisted of 30 cycles of 50 s at 94°C, 3 min at 40°C, and 1.5 min at 72°C.

PCR products were either sequenced directly using amplification primers or blunt-end cloned into pBlue-Script SK^-, which was then used to transfect DH5α-competent *Escherichia coli* cells. Both strands were sequenced using ABI Prism FS chemistry on an ABI 370 automated sequencer. The five new sequences presented here have the following DDBJ/EMBL/GenBank accession numbers: AF132336–AF132340.

Analysis of Protein and cDNA Sequences

Searches for proteins with sequences similar to each of the mature teguline lysins were performed using BLASTp. Molecular weights and isoelectric points for mature lysins were calculated using MacVector.

cDNA sequences from the four teguline lysins varied by only one residue, so they were aligned by eye. *Haliotis rufescens* sequence was aligned with these four based on results of BLASTp searches and conserved signal sequence. Proportions of nonsynonymous (D_n) and synonymous (D_s) substitutions per site were calculated by the method of Nei and Gojobori (1986) using MEGA (Kumar, Tamura, and Nei 1993). Although this approach is standard and provides a good estimate of these parameters when sequences divergence is moderate, synonymous substitutions will be overestimated when substitution rates are high (Muse 1996). Maximum-likelihood estimates of nonsynonymous (K_a) and synonymous (K_s) substitution rates were also obtained using the program of Muse (1996). The program SCR (Hughes, Ota, and Nei 1990) was used to calculate the proportion of nonsynonymous changes that were conservative or radical with respect to charge, polarity, and amino acid type (as defined by Miyata, Miyazama, and Yasunga 1979). We used *t*-tests to determine whether conservative replacements were statistically more frequent than radical ones. The scaled χ^2 method was used to assess codon usage bias (Shields et al. 1988). Nucleotide biases were calculated following Irwin, Kocher, and Wilson (1991).

Divergence times between pairs of teguline species were estimated using a molecular clock based on a 639-bp fragment of mitochondrial cytochrome oxidase I (mtCOI). The clock was calibrated using a pair of *Tegula* species isolated by the rise of the Isthmus of Panama, *T. verrucosa* and *T. viridula* (Vermeij 1978, p. 270). Although species presently separated by the Isthmus may have diverged long before the Isthmus' rise (Knowlton et al. 1993), this particular pair belongs to a subgenus which arose just 4 MYA (Campbell 1993) and likely split just 3 MYA. Times of divergence were estimated in two ways. Because of the strong transition : transversion bias in teguline mtCOI (Hellberg 1998), Kimura (1980) two-parameter distances were used. Because among-site rate heterogeneity can produce saturation effects (especially when the calibration species diverged more recently than the species whose divergence times are to be estimated), we also used silent transversions (Irwin, Kocher, and Wilson 1991). mtCOI sequences for all species studied here (except *T. viridula*) have been published previously (Hellberg 1998).

To help assess whether the lysin genes are orthologous, a phylogenetic tree based on the coding region of the lysin cDNA was constructed using maximum parsimony (PAUP 3.1.1; Swofford 1993) and compared with parsimony trees based on mtDNA (Hellberg 1998). Gaps were excluded, and transitions and transversions were equally weighted. Bootstrap analyses (1,000 replicates, random addition of taxa, hueristic searches) were used to assess the reliability of inferred clades. For both the lysin and the mtDNA trees, all available taxa were included in order to break up long branches.

Secondary structure of lysin was inferred using PHDsec (http:///www.embl-heidelberg.de/predictprotein/phd_pred.html; Rost and Sander 1993, 1994). PHDsec employs neural networks trained on observed position-specific replacements in proteins of known structure to make predictions for secondary structure at individual sites in a target protein of unknown secondary structure. These initial predictions are refined by observed replacements in aligned input proteins. The program was run using each of the four Californian teguline lysins in turn as target proteins, with the remaining three as reference proteins. Predictions were repeated using inferred amino acid sequence from *T. pfeifferi* lysin (Haino-Fukushima, personal communication) as an additional reference protein.

Distances between pairs of cysteine residues within teguline lysins were estimated based on the positions of homologous residues in *H. rufescens* lysin as determined by Mage 3.1 (Richardson 1994).

Results

Acrosomal proteins from the four teguline species dissolve homospecific VEs at far lower concentrations than those required to begin to dissolve heterospecific VEs (fig. 1). The VEs of *T. funebralis* are the most resistant to dissolution by heterospecific acrosomal proteins, with no VE breakdown evident at any heterospecific concentration tested. VEs of *N. norrisii* are most

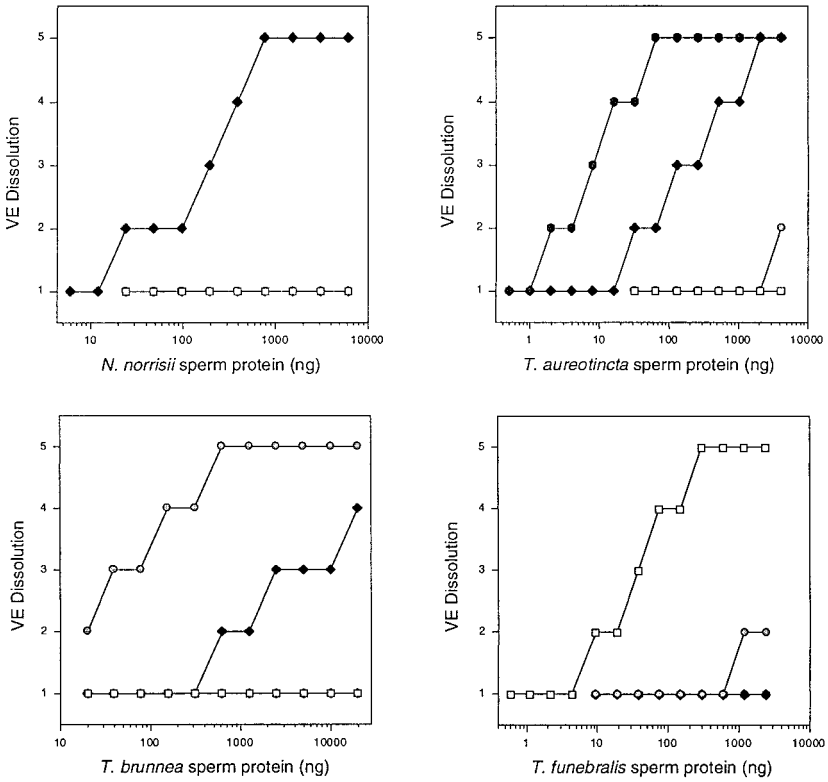

FIG. 1.—Vitelline envelope (VE) dissolution by homospecific and heterospecific acrosomal proteins. Each panel shows dissolution (on a qualitative scale ranging from no dissolution at 1 to complete dissolution at 5) of VEs from four species (*Norrisia norrisii* = black diamonds; *Tegula aureotincta* = dark hexagons; *Tegula brunnea* = shaded circles; *Tegula funebralis* = squares) by acrosomal proteins from the species indicated on the x-axis. Each point on the x-axis has half the lysin of the preceding point.

vulnerable to heterospecific lysins: both *T. aureotincta* and *T. brunnea* dissolve them at concentrations roughly 32 times as high as those required for homospecific VEs. *Norrisia norrisii* lysin is the only lysin which exhibits no ability to dissolve heterospecific VEs at the concentrations tested; thus, the species whose VEs are easiest to dissolve also has the least potent lysin.

The four mature teguline lysins (fig. 2) are 137–138 amino acid residues long, with deduced molecular weights between 16.3 and 16.8 kDa. All are highly pos-

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      -22                  -1 1        10         20         30         42
        ##**#*#** ** *#**  * | |        |    **   |   *      |    *       |
Nno                   ALQWTDV-- SGRYVKIVRHGNVFFGNTEEVWIKGTLIHELDKKANAYCQKH
Tau             CFVVTIVALQWADVYG SMR-VPIVRHGNVDFGRSENGWIKRGAVEEMDKQADKYVKER
Tbr     MKGAVVCFVVTIAALQWTDV-- YGH-RISNVNSNKDFGGVNNGVMKTAIVKALHKKATVWCRQH
Tfu     MKGAVVCFVATIAALQWTNVYG HTPGVRIVQHGYVDFGRVNNGLIKGGMFLEMDNVAKRFCRKH
Hru     MKLLVLCIFAMMATL--AMS-- --RSWHYVEPKFLNKAFEVALKVQIIAGFDRGLVKWLRVHGR
Crystal                                                 aaaaaaaaaaaaaaaaaaaaaaa

              50         60         70         80         90         100     107
        *    * |          |*  # # * #          # #        |     #     |       |
Nno     PTIRMYRDYMLYQGRQKLYNSWNNWSQWFAREIKKLGRSPTRNDYVNLARRLGRDVFMRHLYEAA
Tau     PNLRSYIPMFKYFSKMKVYNMWPNWSNWCATWLRRLNRTPHARDYAACGKIRGREAYMPHLYDVA
Tbr     PHGRPYEPFMRFMNVQRVYTNWNNMSTWAVKELRKMHRRPVTRDYENLGRRIGEHTYMRHVYEVV
Tfu     PSAKPYFQYMRYLNRQRIYGNWNNYSQWARGLVQKLGRKPTSREFANIGRKMGKEMDCEAYFRIV
Hru     TLSTVQKKALYFVNRRVMQTHWANYMLWINKKIDALGRTPVVGDYTRLGAEIGRRIDMAYFYDFL
Crystal     aaaaaaaaaaaaaaaaaaaaaaaaaaaa          aaaaaaaaaaaa    aaaaaa

        110         120         130
          |     **#   |           # *
Nno     WKYKLK--LNPDKRNMLRIPIYKFPVRGVGRFA 138
Tau     VRQNYK-TLNPYEKKILATAPIHLPIRAVGRFA 138
Tbr     SEMRIRPT--PDQIRFTNIKPANLPLRTPGRFG 137
Tfu     VRYRLK--LNPDKRKLLNTPAIDFPIRTLRNWG 138
Hru     KDKNMIPKYLPYMEEINRMRPADVPVKYMGK   136
Crystal aa         aaaaaaaa
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FIG. 2.—Amino acid sequences of lysins deduced from cDNA sequences. −22 to −1 represents signal sequence. Amino-termini (beginning at 1) of lysins from *Tegula aureotincta* (Tau, 10 residues) and *Tegula funebralis* (Tfu, 25 residues) were confirmed by gas phase sequencing. Asterisks indicate identity among the four tegulinids, hashes identity among all five species, and dashes are inserted for alignment. ''Crystal'' shows known α-helical domains of *Haliotis rufescens* (Hru) lysin indicated by ''α''s. Shaded regions indicate predicted α-helices. Numbering refers to the *T. funebralis* sequence. Nno = *Norrisia norrisii*; Tbr = *Tegula brunnea.*

Table 1
Pairwise Amino Acid Identities and Estimated Per-Site Proportions of Nonsynonymous and Synonymous Nucleotide Substitutions for Coding Regions of Teguline Lysin cDNA

Species Comparison	% Amino Acid Identity	D_n (±SE)	D_s (±SE)	D_n/D_s	K_a/K_s[a]
Nno-Tau	42.3	0.549 (0.057)	0.423 (0.086)	1.30	1.37
Nno-Tbr	33.3	0.703 (0.072)	0.301 (0.068)	2.33***	3.58***
Nno-Tfu	38.4	0.636 (0.064)	0.424 (0.085)	1.50*	1.71*
Tau-Tbr	31.6	0.739 (0.075)	0.637 (0.122)	1.16	1.33
Tau-Tfu	35.8	0.648 (0.066)	0.749 (0.142)	0.86	0.87
Tbr-Tfu	34.1	0.682 (0.070)	0.419 (0.086)	1.63*	1.84**

NOTE.—Nno = *Norrisia norrisii*; Tau = *Tegula aureotincta*; Tbr = *Tegula brunnea*; Tfu = *Tegula funebralis*.
[a] Ratio of replacement to silent substitutions based on maximum-likelihood estimators of Muse (1996).
* Significant at the $P < 0.05$ level.
** Significant at the $P < 0.005$ level.
*** Significant at the $P < 0.0005$ level.

itively charged, with inferred isoelectric points between 10.4 and 11.6. Among the four tegulines, amino acid sequence identity is low, no more than 42.3% for any pairwise comparison (table 1). BLASTp searches using these four teguline lysins recovered both *Haliotis* lysins and lysin from *T. pfeifferi* (Haino-Fukushima et al. 1986). Sequence conservation between the lysin-coding regions of the four tegulines and *H. rufescens* is low. Only 8 of 129 alignable residues are identical for all five species (fig. 2); of these, only 3 occur in all reported *Haliotis* lysin sequences (see fig. 1 in Lee, Ota, and Vacquier 1995). The signal sequence is more conserved (fig. 2).

Rates of nucleotide substitution underlying this extensive protein divergence were calculated using times of divergence estimated using a cytochrome oxidase subunit I molecular clock. The Kimura two-parameter distance for *T. verrucosa* and *T. viridula* was 0.074, or a divergence rate of 2.4% per Myr. Using this value, estimated times of divergence for the four species from which lysin had been obtained ranged from 9.2 to 12.6 Myr (table 2). Substitution rates for the coding region of lysin based on these times of divergence are high: between 51 and 76 nonsynonymous nucleotide substitutions per nonsynonymous site per billion years (table 2). Synonymous rates are slightly lower, but still high (table 2). The substitution rate for the signal sequence (obtained only for *T. brunnea* and *T. funebralis*) is over 10 times as slow: 4.9 substitutions per site per billion years. The mtCOI silent-transversion clock (calibrated using the three transversions between *T. verrucosa* and *T. viridula*) estimated longer times of divergence (18–30 Myr) and hence lower lysin substitution rates than the Kimura two-parameter clock (table 2).

The coding region used for phylogenetic analysis consisted of 420 sites, of which 305 were variable and 105 were parsimony-informative. The branching order of the tree based on lysin cDNAs (fig. 3) is concordant with that based on combined mitochondrial genes (see fig. 2 in Hellberg 1998). As with the mtDNA tree, the placement of *T. aureotincta* is somewhat ambiguous.

To test for positive selection, the percentage of nonsynonymous (amino-acid-altering) nucleotide substitutions per nonsynonymous site (D_n) and the percentage of synonymous (silent) nucleotide substitutions per synonymous site (D_s) were calculated over the full length of the lysin-coding regions for each pairwise comparison. D_n values greater than D_s values suggest that positive selection promotes the divergence of sequences. Table 1 shows that D_n is significantly greater than D_s for three of the six possible pairwise comparisons. This is the case regardless of whether the method of Nei and

Table 2
Pairwise Estimates of Nonsynonymous and Synonymous Nucleotide Substitution Rates for Coding Regions of Lysin cDNA Based on Divergence Times Estimated Using a COI Molecular Clock

Species Comparison	COI K2P (SE)	K2P Estimated Divergence[a]	Tv	Tv Estimated Divergence[a]	Lysin Nonsynonymous Rate		Lysin Synonymous Rate	
					K2P[b]	Tv[b]	K2P[b]	Tv[b]
Nno-Tau	0.121 (0.016)	9.8	10	20	56.2	27.4	43.2	21.1
Nno-Tbr	0.113 (0.015)	9.2	9	18	76.4	39.0	32.7	16.7
Nno-Tfu	0.136 (0.017)	11.0	13	26	57.8	24.5	38.5	16.3
Tau-Tbr	0.117 (0.015)	9.5	9	18	77.8	41.0	67.0	35.4
Tau-Tfu	0.155 (0.018)	12.6	15	30	51.4	21.6	59.4	25.0
Tbr-Tfu	0.131 (0.016)	10.6	14	28	64.3	24.4	39.5	15.0

NOTE.—Nno = *Norrisia norrisii*; Tau = *Tegula aureotincta*; Tbr = *Tegula brunnea*; Tfu = *Tegula funebralis*; K2P = Kimura two-parameter distance; Tv = transversion.
[a] Times of divergence (in Myr) estimated using a cytochrome oxidase I molecular clock calibrated by Kimura two-parameter distance (=0.074) or number of synonymous transversions (3) between *Tegula viridula* and *Tegula verrucosa*, separated by the rise of the Isthmus of Panama 3 MYA.
[b] Rates are in substitutions per site per 10^9 years.

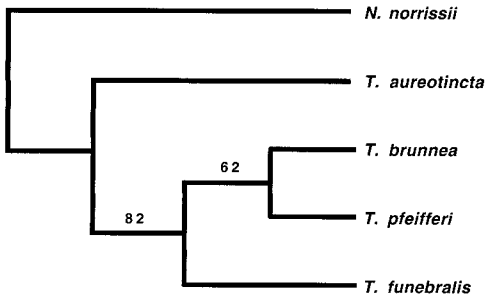

FIG. 3.—The most parsimonious phylogenetic tree for five teguline lysin cDNAs. Bootstrap values (1,000 replicates) >50% are listed above branches.

Gojobori (1986) or that of Muse (1996) was used for calculations.

Biases in nucleotide frequency usages at silent sites or in codon usages could result in underestimates of D_s, thereby leading to misleading conclusions of positive selection. The percentage of G+C shows little bias either over the full coding region (whether it ranges from 43.5 to 48.3) or at third sites (48.6–54.3; table 4). The percentage of C at third sites varies from 23.2 to 28.3. Nucleotide usage biases fall toward the low end of their theoretical range (from 0 = no bias to 1 = maximum bias; Irwin, Kocher, and Wilson 1991). Codon usage biases are likewise low (table 4): values for teguline lysins are lower than those for chymotrypsin and myosin from *Haliotis* spp. (Lee 1994). These data suggest that neither nucleotide nor codon usage bias can account from the significant excess of D_n over D_s seen for three of the six pairwise comparisons.

While D_n/D_s ratios suggest that positive selection promotes divergence of amino acid sequences, the ratios of the proportions of conservative nonsynonymous substitutions per conservative site (p_{NC}) to the proportions of radical substitutions per radical site (p_{NR}) suggest that amino acid replacements are constrained with respect to charge, type, and perhaps polarity (table 3). p_{NC}/p_{NR} ratios of ≈1 suggest that replacements occur without regard to side-chain property. Values of p_{NC} greater than values of p_{NR} suggest that replacements are conservative (Hughes, Ota, and Nei 1990). p_{NC} consistently exceeded p_{NR} when charge and amino acid type were the side-chain properties considered. These differences are statistically significant for all pairwise comparisons (table 3). p_{NC} likewise exceeds p_{NR} with respect to polarity for all pairwise comparisons; however, only one of these comparisons is statistically significant.

Conserved lysin structure is evident not only at the level of replacements, but also in secondary structure. Shaded regions of figure 2 show a high degree of correlation between predicted α-helices in the four teguline lysins and the α-helical structure of *H. rufescens* lysin known from crystallography studies (Shaw et al. 1993). The C-terminal α-helix predicted for *T. aureotincta* and present in *H. rufescens* (fig. 2) was also predicted for *N. norrisii* and *T. funebralis* when *T. pfeifferi* was included in the PHDsec input (results not shown). A predicted break in the second teguline α-helix (residues 63–66 in fig. 2) aligns with a novel kink (at His^{61}) in *H. rufescens* lysin (Shaw et al. 1993).

The presence of cysteine residues constitutes another difference between the teguline lysins and those of *Haliotis.* Lysins from 20 species of *Haliotis* (Lee, Ota, and Vacquier 1995) do not contain a single cysteine in their mature proteins; however, cysteines appear at least once in the coding regions of all four teguline lysins. Lysins from both *T. aureotincta* and *T. funebralis* possess two cysteines which could potentially form disulfide bonds. The two cysteines in *T. aureotincta* (aligning to positions 37 and 97 in the *H. rufescens* lysin) are predicted to be 16.2 Å apart (based on *H. rufescens* structure), probably not close enough to form a disulfide bond. The two cysteines in *T. funebralis* (aligning to positions 69 and 87 in *H. rufescens*) are 11.3 Å apart, perhaps close enough to form a disulfide bond. None of the cysteines in the teguline lysins align with the two invariant cysteines shared by all abalone 18-kDa acrosomal proteins.

Discussion

Species Selectivity of VE Dissolution

As in abalone (Vacquier and Lee 1993), teguline lysins exhibit a high degree of species-selectivity in their ability to dissolve vitelline envelopes from species that diverged no more than 15 MYA (fig. 1). Two previous studies have reported species selectivity of VE dissolution by teguline lysins (Haino 1971; Solano-Estrada et al. 1995), but the former presented no data and the latter

Table 3
Pairwise Comparisons of Conservative (p_{NC}) and Radical (p_{NR}) Replacements ± SEs

Species Comparison	Charge			Polarity			Type		
	p_{NC}	p_{NR}	p_{NC}/p_{NR}	p_{NC}	p_{NR}	p_{NC}/p_{NR}	p_{NC}	p_{NR}	p_{NC}/p_{NR}
Nno-Tau	0.44 (0.037)	0.32 (0.039)	1.37*	0.43 (0.034)	0.32 (0.045)	1.34*	0.55 (0.051)	0.32 (0.031)	1.70***
Nno-Tbr	0.53 (0.038)	0.37 (0.040)	1.44**	0.49 (0.034)	0.40 (0.048)	1.21	0.61 (0.050)	0.40 (0.033)	1.54***
Nno-Tfu	0.51 (0.038)	0.33 (0.039)	1.53**	0.45 (0.034)	0.36 (0.047)	1.25	0.65 (0.050)	0.33 (0.031)	1.96***
Tau-Tbr	0.54 (0.037)	0.39 (0.041)	1.37**	0.50 (0.034)	0.43 (0.047)	1.17	0.58 (0.050)	0.43 (0.033)	1.35*
Tau-Tfu	0.52 (0.037)	0.32 (0.039)	1.63***	0.46 (0.034)	0.38 (0.047)	1.21	0.63 (0.050)	0.35 (0.032)	1.78***
Tbr-Tfu	0.51 (0.038)	0.38 (0.040)	1.36*	0.46 (0.034)	0.43 (0.048)	1.09	0.64 (0.049)	0.37 (0.032)	1.74***

NOTE.—Nno = *Norrisia norrisii*; Tau = *Tegula aureotincta*; Tbr = *Tegula brunnea*; Tfu = *Tegula funebralis.*
* Significant at the $P < 0.05$ level.
** Significant at the $P < 0.005$ level.
*** Significant at the $P < 0.0005$ level.

Table 4
Nucleotide and Codon Bias of Lysin

Species	% G+C	3rd-Position % G+C	3rd-Position % C	Nucleotide Bias	3rd-Position Nucleotide Bias	Codon Bias[a]
Nno	43.5	48.6	23.2	0.09	0.04	0.18
Tau	48.3	54.3	28.3	0.08	0.06	0.20
Tbr	47.0	53.3	25.6	0.09	0.07	0.14
Tfu	44.7	49.3	26.1	0.09	0.03	0.13

NOTE.—Nno = *Norrisia norrisii*; Tau = *Tegula aureotincta*; Tbr = *Tegula brunnea*; Tfu = *Tegula funebralis.*
[a] Varies from 0 (=no bias) to 1 (=maximum bias).

compared species from different archeogastropod families which diverged tens of millions of years ago.

Whether this selectivity acts as the primary barrier to interspecific hybridization in the field depends on the synchrony of spawning between individuals of different species. Gonadal conditions of sister species of *Tegula* with overlapping microhabitat distributions covary throughout the year (Watanabe 1982; personal observations), suggesting that the species selectivity of lysin may play an important role in maintaining genetic isolation between species in situ.

Substitution Rates and Positive Selection

Rates of nonsynonymous substitution within the coding region of teguline lysins (table 2) exceed the highest nonsynonymous substitution rates (for γ-interferon) observed between primates and rodents by 7- to 25-fold (Li 1997, p. 180). Although the extract rates of substitution are sensitive to a number of assumptions, the conclusion that lysins evolve at rates may times as fast as those reported for the fastest mammalian genes appears robust (table 2; see also Metz, Robles-Sikisaka, and Vacquier 1998).

Calculations of rates of change are sensitive to estimates of divergence time. If the lysin cDNAs compared here came from paralogous loci whose divergence predated those of the species themselves, inflated estimates of nucleotide substitution would result. This does not seem to be the case. Lysin is a single-copy gene in abalone (Lee 1994). The phylogenetic branching pattern for lysins (fig. 3) is congruent with that based on mtCOI sequences. Noncoding regions of the lysin gene have lower rates of substitution than coding regions (see conserved signal sequence in fig. 2). Furthermore, direct sequencing of lysins from two *Tegula* species confirmed that the cDNAs are expressed, and no species showed any sign of multilocus amplification (which might be expected in the event of a gene duplication, given the limited time for pseudogene extinction in the brief divergence times between these species). These observations in combination suggest that the teguline lysins analyzed here are orthologous genes.

The high estimated rates of substitution could in part stem from the molecular clock used to date times of species divergence. For example, rate heterogeneity between sites could produce underestimates of divergence times for the four species, all of which are more genetically distant at mtCOI than the trans-Isthmusian species pair used to calibrate the clock. This is likely the case for divergence estimates based on Kimura two-parameter distances. However, these estimates are similar to those based on the fossil record. Furthermore, the fossil record contains no members of this abundant and species-rich group before 15 MYA, suggesting a 30-Myr maximum time of divergence. Substitution rates based on this most conservative denominator would still be many times as fast as those of any mammalian gene.

Such high rates of substitution, while extraordinary with respect to most nucleotide sequences, appear to be typical of gastropod sperm fertilization proteins. Similar nonsynonymous substitution rates have been reported for two fertilization proteins from abalone, lysin and a related 18-kDa acrosomal protein (Metz, Robles-Sikisaka, and Vacquier 1998). As was true for these abalone proteins, teguline lysins diverge rapidly not only in an absolute sense, but also relative to other molecules from the same species: nonsynonymous substitution rates for lysin exceed synonymous substitution rates for mtCOI for two of six pairwise comparisons.

Synonymous substitution rates are likewise higher than those for mammalian genes summarized by Li (1997). While this could be due to locally high mutation rates (which would contribute to high nonsynonymous rates as well), the high D_s probably results in part from bias of the Nei and Gojobori estimators. Muse (1996) showed that this approach will overestimate D_s when D_n is high. Maximum-likelihood estimates of D_s (not shown) were slightly lower.

Inflated estimates of D_s make D_n/D_s ratios a conservative test for positive selection. Nonetheless, three of the six pairwise comparisons show a significant excess of nonsynonymous substitutions per nonsynonymous site relative to synonymous substitutions per synonymous site (table 2) regardless of how D_n and D_s were calculated. These high D_n/D_s ratios do not result from biased nucleotide or codon usage (table 4).

Gastropod sperm proteins tend to show an inverse relationship between percentage of identity of amino acid residues and D_n/D_s ratios, an observation consistent with saturation effects erasing the signature of natural selection between distantly related sequences. For example, Swanson and Vacquier (1995) found $D_n/D_s > 3$ for all three pairwise comparisons of 18-kDa abalone sperm proteins with identifiers >75%, but $D_n/D_s \leq 1$ for more distant contrasts. However, the correlation is not absolute. Lee and Vacquier (1993; see their table 1) found $D_n/D_s > 1$ for four of six lysin cDNAs with identities >80% and for three of eight comparisons with

identities between 70% and 80%, but also for one comparison with an identity of 65%. As should be expected, contrasts with high estimates of D_s often show D_n/D_s ratios equal to or below unity, although, as with percentage of identity, exceptions occur. Lee, Ota, and Vacquier (1995) observed $D_n/D_s > 1$ for all lysin cDNAs with $D_s < 0.2$ (their fig. 3), but a few comparisons with $D_s > 0.5$ still produced $D_n/D_s > 1$. In combination, these observations suggest that the power to infer positive selection when estimates of D_s are high (>0.2) is limited. Nevertheless, D_n/D_s ratios significantly greater than one were found for some teguline lysins with <40% amino acid identity, lower than those for any abalone proteins evincing positive selection. This might be due to lower constraints on primary sequence in teguline lysins relative to those in *Haliotis.* Greater freedom to vary could allow teguline lysins to accrue more nonsynonymous substitutions, and maintain them for a longer time, before silent substitutions saturate. Consistent with this explanation, divergence of *Haliotis* lysins reaches only about 55% (Lee, Ota, and Vacquier 1995), less than seen here for tegulines, even though divergence times for extant haliotids predate those of the tegulines.

Structural Constraints on Lysin

Molecules which mediate intercellular recognition face conflicting selective pressures as they evolve. On the one hand, evolving targets (be they pathogens or complementary gamete recognition proteins) may exert strong selection for changing specificity. Teguline lysins demonstrate high species selectivity in their ability to dissolve VEs (fig. 1), and the extensive divergence of these proteins offers no shortage of candidate replacements which could alter this specificity. On the other hand, these replacements must somehow be constrained so that lysin could maintain its dissolution function over the 250 Myr since the *Tegula* and *Haliotis* lineages diverged.

That lysin maintains its dissolution function is no trivial problem, as evidenced by a similarly divergent fertilization protein which has lost the ability to dissolve VEs. The two major proteins found in the sperm of *H. rufescens* (lysin and an 18-kDa protein) share 16.7% amino acid identity, slightly more than the 13%–16% amino acid identity shared between *Haliotis* and *Tegula* lysins (pairwise calculation excluding indels). However, although all lysins dissolve homospecific VEs readily, the 18-kDa protein shows no such ability (Swanson and Vacquier 1995).

Some recognition molecules whose specificities change over time possess invariant regions which may be responsible for conserved functions. For example, while portions of the sperm-egg attachment protein bindin from sea urchins evince positive selection between closely related species (Metz and Palumbi 1996), over one quarter of the mature protein's length remains invariant between species which diverged over 200 MYA (Minor et al. 1991; Vacquier, Swanson, and Hellberg 1995). A portion of this conserved region has been implicated in bindin's conserved membrane fusagenic function (Ulrich et al. 1998). Such regional conservation is not the case for lysin. Only eight residues are conserved between *H. rufescens* and all four tegulines (fig. 2), and these occur at interhelical loops and at the beginnings of helices over the length of the molecule and are not concentrated in one region.

Although no region within lysin is obviously conserved among species, the rapid amino acid replacements characterizing teguline lysin evolution are largely conservative ones. p_{NC} was consistently greater than p_{NR} for all types of amino acid replacements examined, suggesting that the majority of replacements in lysin are conservative with respect to type, polarity, and charge (table 2). Conservative replacements characterized the rapidly evolving 18-kDa abalone acrosomal protein as well (Swanson and Vacquier 1995). Visual inspection also suggests that the location of positively charged residues in lysin is conserved: of the 23 residues which comprise the basic tracks in *H. rufescens* lysin, all except two have positively charged residues within three residues of corresponding positions in teguline lysins (fig. 2).

The excess of conservative replacements must result from selective constrains on the secondary structure of lysin. Predicted α-helices of teguline lysins correspond closely to both those known from the crystalline structure of *H. rufescens* lysin and those predicted for *H. rufescens* (fig. 2). Conserved secondary structure may be necessary to preserve the dissolution function of lysin, but it is certainly not sufficient: the 18-kDa sperm protein from *H. rufescens* has an α-helical structure that corresponds to that of the lysin of that same species even more closely than do the teguline lysins, yet it cannot dissolve homospecific VEs (Swanson and Vacquier 1995).

Conclusions

Comparisons among the four teguline lysins analyzed here evince the same rapid divergence previously seen among abalone lysins (Lee, Ota, and Vacquier 1995; Metz, Robles-Sikisaka, and Vacquier 1998). Thus, rapid divergence and positive selection appear to have been features of lysin's evolution at least since the teguline and abalone lineages diverged over 250 MYA, and not just recent occurrences, as in some other rapidly evolving proteins associated with sexual reproduction (e.g., Ting et al. 1998).

Although many replacements differentiate the lysins of tegulines and abalone, their secondary structures are highly conserved. This shared secondary structure is similar to that of an 18-kDa abalone acrosomal protein, which shares greater amino acid identity with abalone lysin than does any teguline lysin. However, unlike lysins, the 18-kDa protein cannot dissolve VEs. These acrosomal proteins thus offer another example of the imperfect correlation between sequence divergence and functional change (Golding and Dean 1998).

Both *Haliotis* and *Tegula* contain a number of sister species that occur along the same coastline, suggesting that reproductive isolation evolved in the absence of sustained barriers to dispersal (Hellberg 1998). Thus,

knowledge of the mechanism driving lysin's rapid divergence may provide clues as to how speciation occurs in the sea. The egg-borne receptor for lysin is tandemly repeated, suggesting that concerted evolution may play a critical role (Swanson and Vacquier 1998). Further work detailing sequence variation in lysin and its receptor at the population level (along with the functional consequences of these differences) may yield a clearer picture of the forces responsible for rapid change in this fascinating protein.

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