Evolutionary Diversification of Multigene Families: Allelic Selection of Toxins in Predatory Cone Snails

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In order to investigate the evolution of conotoxin multigene families among two closely related vermivorous *Conus* species, we sequenced 104 four-loop conotoxin mRNAs from two individuals of *Conus ebraeus* and compared these with sequences already obtained from *Conus abbreviatus.* In contrast to the diversity of conotoxin sequences obtained from *C. abbreviatus,* only two common sequence variants were recovered from *C. ebraeus.* Segregation patterns of the variants in these two individuals and restriction digests of four-loop conotoxin amplification products from nine additional individuals suggest that the common variants are alleles from a single locus. These two putative alleles differ at nine positions that occur nonrandomly in the toxin-coding region of the sequences. Moreover, all substitutions are at nonsynonymous sites and are responsible for seven amino acid differences among the predicted amino acid sequences of the alleles. These results imply that conotoxin diversity is driven by strong diversifying selection and some form of frequency-dependent or overdominant selection at conotoxin loci, and they suggest that diverse conotoxin multigene families can originate from duplications at polymorphic loci. Furthermore, none of the sequences recovered from *C. ebraeus* appeared to be orthologs of loci from *C. abbreviatus,* and attempts to amplify orthologous sequences with locus-specific primers were unsuccessful among these species. These patterns suggest that venoms of closely related *Conus* species may differ due to the differential expression of conotoxin loci.

Introduction

Multigene families are ubiquitous in the genomes of many organisms and are associated with the evolution of complex adaptations (Ohno 1970, pp. 59–88; Ohta 1991, 1994; Hughes 1994; Li 1997, pp. 269–308). When functional diversity is advantageous, such as with major histocompatibility loci, it can be enhanced by the extreme divergence of gene family members through the operation of diversifying selection among loci (Ohta 1991, 1994; Hughes 1993, 1994; Hughes and Hughes 1993; Courty et al. 1996; Merritt et al. 1998; Zhang, Rosenberg, and Nei 1998) and, in some cases, overdominant selection among alleles (Hughes and Nei 1988, 1989). However, much of what is known about multigene family evolution is based on studies of these systems in single species, so it is unclear how gene families evolve during adaptive radiations of closely related taxa.

The gastropod genus *Conus* greatly diversified during an explosive adaptive radiation in the Pleistocene (Kohn 1990). Species are distinguished by a number of specialized diets and express a complex cocktail of peptide neurotoxins (''conotoxins'') used to capture prey. Previously, we reported the existence of a four-loop conotoxin multigene family in the vermivorous cone snail, *Conus abbreviatus,* and the operation of strong diversifying selection and high rates of nonsynonymous substitutions among these recently duplicated loci (Duda and Palumbi 1999*a*). Conotoxins are intricately related to a species' ability to paralyze its prey, yet it is unclear why they are diversifying so rapidly, how conotoxins are evolving among closely related species, and whether conotoxin diversity is generally maintained between loci, within loci, or both.

In order to further investigate the rapid diversification of conotoxin multigene families in *Conus,* we sequenced four-loop conotoxin mRNAs from another worm-eating species, *Conus ebraeus,* a close relative of *C. abbreviatus* (Duda and Palumbi 1999*b*). Based on calibrations from the fossil record and biogeographic history of *Conus,* these two species presumably diverged as recently as 1.7 MYA (Duda and Palumbi 1999*b*). Although the diets of these species are not identical, both primarily prey on eunicid and nereid polychaetes (Kohn 1959; Kohn and Nybakken 1975; Reichelt and Kohn 1985).

Because of their similar diets and close phylogenetic relationships, we expected to find loci in *C. ebraeus* that were similar to those found in *C. abbreviatus.* On the contrary, none of the sequences from the two species were alike, and at least 75% of the amino acids among the predicted amino acid sequences of conotoxins from these species were substituted, except the structure-defining cysteine residues. While nine distinct sequences that differed at an average of 53.5 and minimum of 16 nucleotides were identified from *C. abbreviatus* (Duda and Palumbi 1999*a*), only two distinct sequences, differing at only nine nucleotide positions, were recovered from *C. ebraeus.*

Materials and Methods

We collected specimens of *Conus ebraeus* from Kahe Point, Oahu, Hawaii, constructed cDNA libraries from 11 individuals (CebH1–CebH11), and sequenced a minimum of 50 four-loop conotoxin mRNA transcripts from each of two individuals with methods of Duda and Palumbi (1999*a*).

Sequences were aligned by eye. Molecular phylograms were constructed with neighbor-joining from Kimura two-parameter distances among the sequences

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(MEGA; Kumar, Tamura, and Nei 1993) and included four-loop conotoxin sequences from *C. abbreviatus* and *Conus lividus* (GenBank accession numbers AF089901–AF090180; Duda and Palumbi 1999*a*). Identical sequences were represented only once, and bootstrap values were estimated from 500 replicates.

The corrected proportions of nonsynonymous substitutions per nonsynonymous site (D_n) and synonymous substitutions per synonymous site (D_s) were calculated among sequences recovered from *C. ebraeus* and among these sequences and the sequences previously recovered from *C. abbreviatus* and *C. lividus* (Duda and Palumbi 1999*a*). D_n and D_s were estimated from the beginning of the ''prepro'' region, a region that is translated with the mature conotoxin and is cleaved from the toxin during processing (Woodward et al. 1990; Colledge et al. 1992), to the last codon before the stop codon with method 1 of Ina (1995; program by T.F.D.). Significance of differences between D_n and D_s was estimated using a one-tailed *t*-test with infinite degrees of freedom (Kumar, Tamura, and Nei 1993). Significance levels were corrected for multiple tests with Hochberg's (1988) Bonferroni technique.

Preliminary sequence results from two individuals identified a polymorphism among the two common four-loop conotoxin mRNA sequences from *C. ebraeus* that contained an *Rsa*I restriction site. We amplified putative four-loop conotoxins from the remaining libraries ($n = 9$) and digested the amplification products as per manufacturer recommendations. Digested products were run on a 2.5% agarose/0.5 × TBE gel and visualized under ultraviolet light.

Because the general conotoxin primers we initially used might preferentially amplify nonorthologous conotoxin loci from *C. abbreviatus* and *C. ebraeus*, we designed locus-specific primers from sequences recovered from both species in order to test whether orthologous loci are expressed by these two closely related species. Two primers were designed from previously identified conotoxins from sequence groups A1–A8 from *C. abbreviatus* and L2 from *C. lividus* (Duda and Palumbi 1999*a*). The 5′ primer (ACTACAGCTGAGACTTC) was designed within the prepro region of these sequences and differed from sequences recovered from *C. ebraeus* at only one base at the 3′ end of the primer. The 3′ primer (GATCACGCTAGGGGAAT) was designed within the 3′ untranslated region (UTR) of these sequences and differed from sequences recovered from *C. ebraeus* at a number of bases distributed throughout the primer. This primer was designed from the consensus sequence from all sequences from groups A1–A8 and L2 and presumably should amplify all of these loci. We also designed primers from sequences from these same regions from recovered *C. ebraeus* four-loop conotoxins (5′ primer = ACTACAGCTGAGACTTA; 3′ primer = GATAGATCACGAAAGGGA). We used both sets of primers in amplifications with cDNA from two *C. abbreviatus* and two *C. ebraeus* individuals.

Results

We sequenced 51 and 53 mRNAs, respectively, from each of the two individuals analyzed (CebH1 and CebH2; GenBank accession numbers AF174268–AF174287). Forty of the sequences from CebH1 were identical. Twenty of the sequences from CebH2 were identical to the 40 obtained from CebH1, while 14 others were identical to each other and differed from the other set of sequences at nine positions. The remaining sequences had one or two positions that differed from the common sequences ($n = 23$), had an out-of-frame deletion ($n = 5$), or appeared to be PCR recombinants of the two common sequences ($n = 2$) (fig. 1). Such minor variants were considered PCR artifacts from the original amplification or during screening of clones (multiple screens of the same clones often contain random ''singleton'' errors; Villablanca, Roderick, and Palumbi 1998; France et al. 1999). Sequences with out-of-frame deletions or which appeared to be PCR-induced recombinants were excluded from further analyses.

In the molecular phylogram, all sequences from CebH1 cluster together in a clade with 25 of the sequences from CebH2 (E1a; fig. 2), while the remaining sequences from CebH2 form a second clade (E1b; fig. 2). Bootstrap support for both of these clades is strong, with values of 96% and 88%, respectively (fig. 2). These two clades also cluster together among a group of loci from *C. abbreviatus* and *C. lividus* with strong bootstrap support (94%; fig. 2).

The mean pairwise Kimura two-parameter distance among sequences from clades E1a and E1b was 4.3% (SE = 0.6%), and the distance between the common sequence types (CebH1-a/CebH2-a and CebH2-i) was 3.6 (SE = 1.2%). The mean pairwise Kimura two-parameter distances among sequences from these clades and sequence groups A1–A8 and L2 ranged from 18.8% to 31.0% (table 1).

The conotoxin mRNA sequences we recovered consisted of 208 nt of sequence that code for a precursor peptide. The first 124-nt code for the prepro region, the next 84-nt code for the mature conotoxin, and the final 50 nt were untranslated (3′ UTR). All nine positions that differed between the two putative alleles of *C. ebraeus* occurred within the region that codes for the mature conotoxin; based on a goodness-of-fit test, the distribution of substitutions among these sequence regions was nonrandom ($P < 0.005$).

All nine substitutions between the putative alleles of *C. ebraeus* are nonsynonymous substitutions, although they are only responsible for seven amino acid differences between the predicted conotoxin peptides due to the co-occurrence of two substitutions in two codons (fig. 3). Based on a *t*-test (Kumar, Tamura, and Nei 1993), the proportion of nonsynonymous substitutions per nonsynonymous site across the translated region of these alleles (0.056; SE = 0.019) is significantly greater than the proportion of synonymous substitutions per synonymous site (0; SE = 0).

The D_n/D_s ratios among sequences from clades E1a and E1b and sequence groups A1–A8 and L2 were all >1 and range from 1.17 to 3.00 (table 1). D_n was significantly greater than D_s among clades E1a and E1b and clades A1, A3, A6, A8, and L2 (table 1). Three of 10 of these differences remained significant after cor-

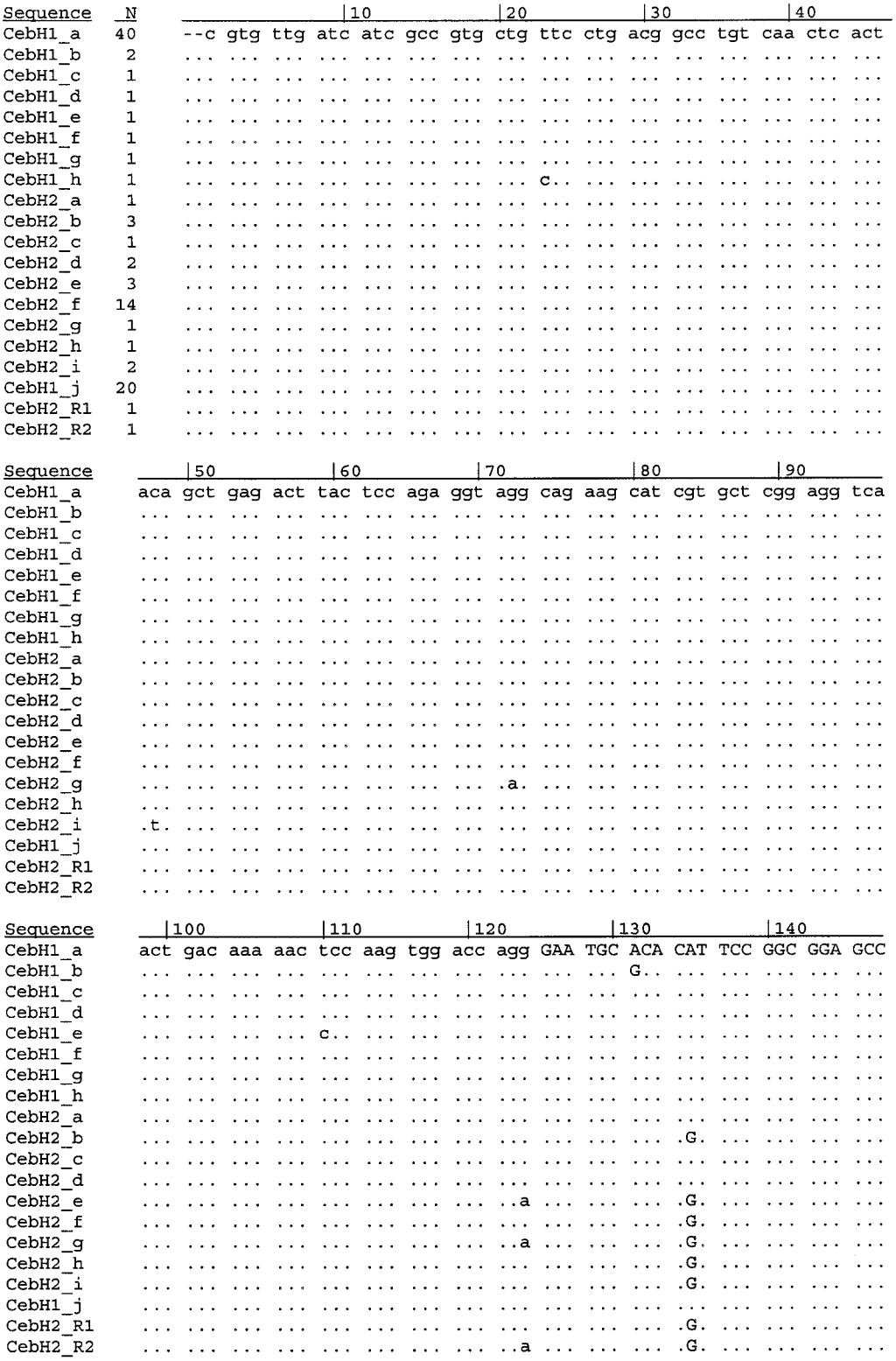

FIG. 1.—Variant four-loop conotoxin mRNA sequences recovered from two *Conus ebraeus* individuals. CebH2-R1 and CebH2-R2 appear to be PCR-induced recombinants. Sequences with out-of-frame deletions are not presented. The *Rsa*I restriction site is at positions 186–189 in sequences CebH2-d through CebH2-i. The prepro region sequence is shown in lowercase letters, the toxin region is shown in uppercase letters, and the 3′ untranslated region is shown in italics. *N* = number of sequences recovered. Proper nomenclature for conotoxins (Cruz et al. 1985; Gray, Olivera, and Cruz 1988): E1a = EbVIa; E1b = EbVIb.

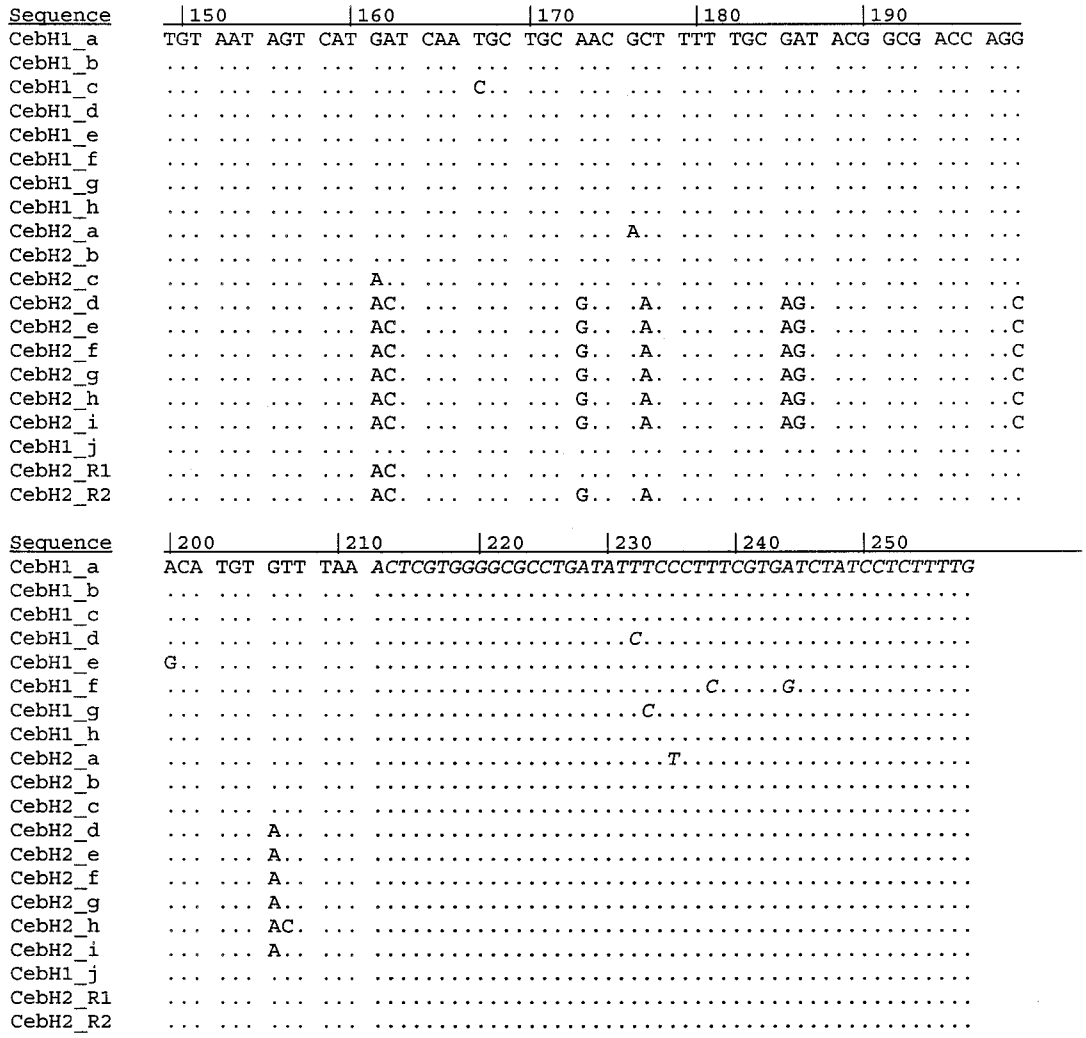

FIG. 1 *(Continued)*

recting for multiple tests (Hochberg 1988), as indicated in table 1.

One position that differed between the two common sequences obtained from *C. ebraeus* occurred within an *Rsa*I restriction site (fig. 1). The digestion patterns of four-loop conotoxin amplifications from the nine other specimens showed that individuals possess either one of the two common sequences or both (fig. 4). Moreover, the segregation of sequences among individuals was in Hardy-Weinberg equilibrium ($P > 0.75$).

Amplifications with the primers designed from sequence groups A1–A8 from *C. abbreviatus* and L2 from *C. lividus* were successful only with cDNA from *C. abbreviatus*, and not with cDNA from *C. ebraeus* (amplifications with cDNA from *C. lividus* were not attempted). Similarly, amplifications with the primers designed from *C. ebraeus* sequences were successful only with cDNA from *C. ebraeus*, and not with cDNA from *C. abbreviatus.*

Discussion

We interpret the sequence differences among four-loop conotoxin mRNAs from *C. ebraeus* to indicate that they are alleles of a single locus diverging under strong diversifying selection. An alternative explanation is that there is more than one locus and the minor sequence variants obtained represent alleles from many loci, and not artifacts due to PCR or other induced errors. However, the segregation of the common sequence types in Hardy-Weinberg equilibrium, as demonstrated by the restriction digests (fig. 4), supports the view that the two common mRNA types are from a single locus. If these mRNAs are from more than one locus, there must have been either amplification biases or variable amounts of concerted evolution occurring among these loci at the individual level. The latter theory is unlikely, while the former seems unjustified in light of the fact that both common mRNAs were apparently recovered from several individuals (fig. 4).

Alleles at this locus show evidence of strong diversifying selection within the toxin-coding regions of these sequences (fig. 1). Indeed, five of the seven predicted amino acid differences are radical changes. The pattern of diversifying selection and radical substitutions is similar to the pattern observed among eight putative loci in *C. abbreviatus*, in which the signal of diversi-

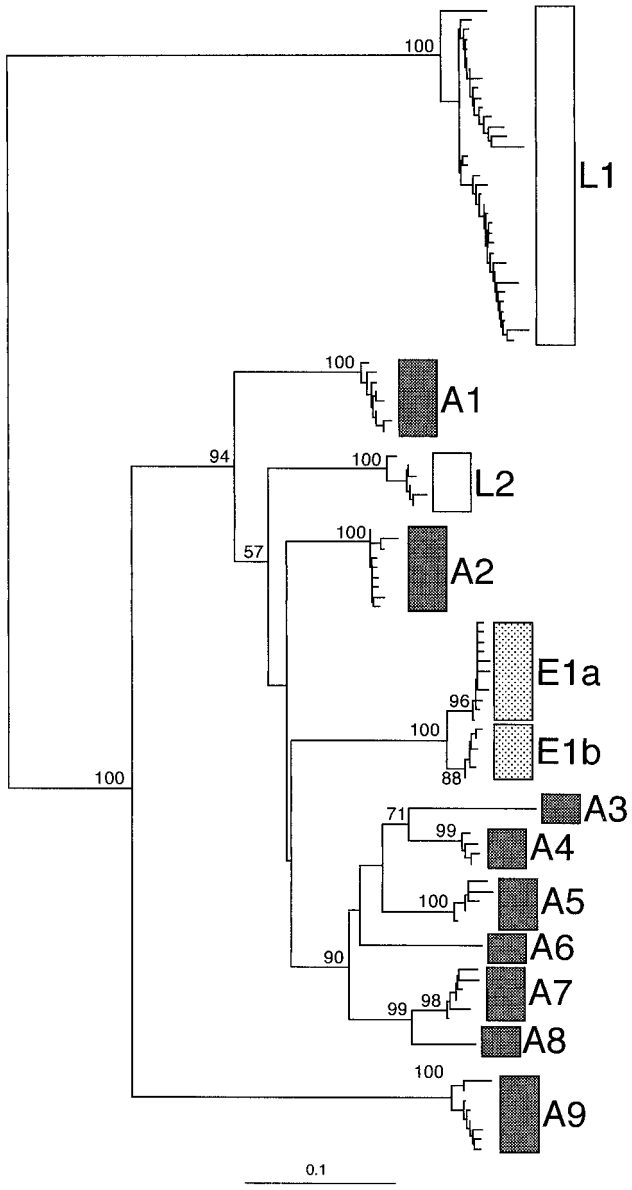

FIG. 2.—Molecular phylogram of *Conus abbreviatus*, *Conus ebraeus*, and *Conus lividus* four-loop conotoxin mRNAs. The phylogram was reconstructed from Kimura two-parameter distances among sequences. Bootstrap values are presented on branches. E1a = sequences CebH1-a to CebH1-h, CebH2-a to CebH2-c, and CebH2j; E1b = sequences CebH2-d to CebH2-i (see fig. 1).

Table 1
Mean Kimura Two-Parameter Pairwise Distances and D_n/D_s Ratios Among Sequences from *Conus ebraeus* (E1a and E1b), and *Conus abbreviatus* (A1–A8), and *Conus lividus* (L2) Sequence Groups

Sequence Group	Mean Distance (%)		D_n/D_s[a]	
	E1a	E1b	E1a	E1b
A1	26.6	25.0	**2.92** ***	**3.00** ***
A2	19.4	18.9	1.83	2.22
A3	30.5	31.0	1.81 *	2.56 ***
A4	25.5	26.5	1.17	1.66
A5	25.5	25.8	1.36	1.80
A6	24.7	24.6	2.43 **	**2.98** ***
A7	23.7	21.3	1.36	1.67
A8	25.9	24.0	2.33 **	2.38 **
L2	23.3	23.3	2.31 **	2.13 **

[a] The significance of the difference between D_n and D_s is noted below D_n/D_s (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P < 0.001$; D_n/D_s ratios in bold type denote differences that remain significant at the 0.05 level after correcting for multiple tests).

fying selection was most prominent within the region that codes for the mature conotoxin (Duda and Palumbi 1999*a*). As with those results, we do not know whether conotoxins produced by the two alleles recovered from *C. ebraeus* are functionally distinct. However, results from other studies show that four-loop conotoxins that differ in amino acid sequence have different specificities for particular cell channels (Cruz et al. 1985; Olivera et al. 1985, 1990, 1991; Monje et al. 1993; Fainzilber et al. 1994, 1995; Kristipati et al. 1994; Hasson et al. 1995; Kits et al. 1996; Gandia et al. 1997).

These results for *C. ebraeus* contrast with results from other *Conus* species in that diversifying selection in *C. ebraeus* was detected among alleles at a single locus, whereas diversifying selection in *C. abbreviatus* was apparent among loci expressed in single individuals (Duda and Palumbi 1999*a*). An explanation for both observations is that there is strong overall selection for the expression of a diverse set of toxins in *Conus* regardless of the particular genetic architecture that produces toxins in a species. Among some species, there are many conotoxin genes, and selection drives their divergence. Among others, like *C. ebraeus*, there are few loci, and selection drives the divergence of alleles at these loci. This selection pressure may be due to a coevolutionary

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                    |20                  |40
vliiavlfltacqlttaetysrgrqkhrarrstdknskwtrECTHSGGACNSHDQCCNA
.....................................................R.........T...DD
...................s...k.....l.......l...G..PP....GGHAH..SQ

|60
FCDTATRTCV-
..S...S..I.
S.NILAS..NA
```

FIG. 3.—Predicted amino acid sequences (single-letter code) of the two common conotoxin mRNAs obtained from *Conus ebraeus* and a sequence from *Conus abbreviatus*. Sequence 1 = CebH1-a/CebH2-j; sequence 2 = CebH2-f (see table 1); sequence 3 = locus A2 from *C. abbreviatus*. For the prepro region, the amino acid code is in lowercase letters; for the toxin region, the amino acid code is in uppercase letters.

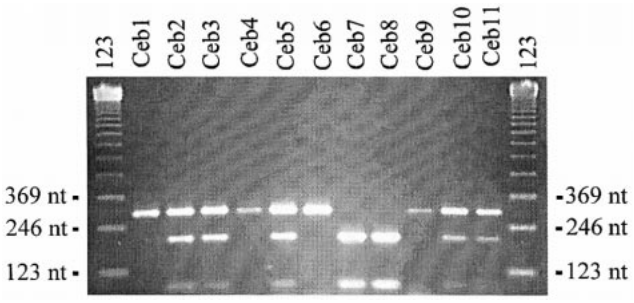

FIG. 4.—*Rsa*I restriction digests of four-loop conotoxin amplifications from 11 *Conus ebraeus* individuals. Individuals 1, 4, 6, and 9 are homozygous for the uncut allele; individuals 2, 3, 5, 10, and 11 are heterozygous for the cut and uncut alleles; individuals 7 and 8 are homozygous for the cut allele. There are 123 nucleotide standards in the outer lanes; the sizes of the three smallest fragments of the standard are noted next to these lanes.

''arms race'' between the weaponry of the predator and the targets of the prey or other mechanisms involving predator-prey relationships (Duda and Palumbi 1999*a*). We do not know if *C. ebraeus* has fewer genomic copies of conotoxin loci than *C. abbreviatus* or if differences between species are due to transcriptional silencing of loci that remain in the genome of *C. ebraeus,* but this can be tested through Southern blot analyses of the genomes of these species.

The operation of diversifying selection among alleles at a single locus has been observed among alleles at human and mouse class I and II major histocompatibility complex (MHC) loci (Hughes and Nei 1988, 1989) and alleles of the gamete recognition protein bindin in sea urchins (Palumbi 1999). Hughes and Nei (1988) suggest that high rates of nonsynonymous substitutions compared with rates of synonymous substitutions in the antigen recognition site of class I MHC genes are evidence that MHC loci are subject to overdominant selection or a form of frequency-dependent selection. This type of selection leads to an excess of heterozygotes (Hughes and Nei 1988) and ancient allele clades within species (Takahata 1993). Similarly, the expression of multiple conotoxin alleles may provide heterozygous individuals of *C. ebraeus* with a greater ability to successfully paralyze potential prey items, and this could generate overdominant selection. However, we did not observe a strong excess of heterozygotes in the small number of individuals tested, and the four-loop conotoxin alleles appear to be recently derived because there are no silent substitutions among them. Thus, it remains unclear whether frequency-dependent or overdominant selection is operating on conotoxin loci.

In other *Conus* species, conotoxin diversity was found to be associated with an ongoing process of gene duplication and divergence (Duda and Palumbi 1999*a*). The striking diversification of alleles at a single locus in *C. ebraeus* shows that conotoxin diversity is also driven by diversifying selection within loci and suggests that conotoxin gene family diversity may at least originate from duplications at already-polymorphic loci. Ohno (1970, pp. 65–67) suggested that gene duplications could serve as a mechanism to fix allelic diversity, particularly in cases of heterozygote advantage, and cited Koehn and Rasmussen's (1967) work on a single polymorphic esterase locus in one species of catostomid fish and two monomorphic esterase loci in a related species as possible evidence for this scenario. As demonstrated by the extreme divergence of four-loop conotoxin loci in *C. abbreviatus* and alleles in *C. ebraeus,* selection for conotoxin diversity is particularly intense. Gene duplications may serve as a mechanism by which this diversity is maintained in *Conus.* Moreover, in circumstances where the production of diverse gene products is advantageous, such as with MHC and odorant receptors (Buck and Axel 1991; Buck 1992; Hughes and Hughes 1993; Ngai et al. 1993; Sullivan et al. 1996), gene family diversity may be preceded by periods of allelic diversity.

As revealed from the molecular phylogram and levels of divergence of four-loop conotoxins from *C. ebraeus, C. abbreviatus,* and *C. lividus* (fig. 2 and table 1), the sequences recovered from *C. ebraeus* do not appear to be orthologs of any previously identified putative loci from *C. abbreviatus* or *C. lividus.* In fact, the sequences recovered from *C. ebraeus* are as divergent from sequence groups A1–A8 of *C. abbreviatus* as they are from the sequences from sequence group L2 of *C. lividus* (fig. 2), even though *C. abbreviatus* and *C. ebraeus* are more closely related to each other than either species is to *C. lividus* (Duda and Palumbi 1999*b*).

Negative results from amplifications with locus-specific primers imply that the *C. ebraeus* individuals examined are not expressing orthologs of the *C. abbreviatus* loci from sequence groups A1–A8, nor are *C. abbreviatus* individuals expressing orthologs of the *C. ebraeus* locus. These results suggest that conotoxins are differentially expressed among related *Conus* species. Conotoxins might therefore represent not only a rapidly diverging gene family, but also one in which the active or expressed members are constantly changing. These hypotheses can be tested through comparisons of expressed and genomic conotoxin sequences from these two species.

The pattern of diversifying selection observed among the *C. ebraeus* sequences and the loci from *C. abbreviatus* and *C. lividus* (table 1) is similar to the pattern observed among nonorthologous loci from *C. abbreviatus* (Duda and Palumbi 1999*a*). This suggests that conotoxins are diversifying between as well as within species.

In conclusion, venom diversity among *Conus* species is facilitated by four factors: (1) diversifying selection among loci, as revealed by the rapid evolution of conotoxin loci in *C. abbreviatus* (Duda and Palumbi 1999*a*); (2) diversifying selection within loci, as shown by the concentration of substitutions within the toxin-coding region among two alleles from *C. ebraeus*; (3) gene duplications, possibly of already polymorphic loci, that create a genetic architecture that can maintain a diverse set of loci; and (4) differential expression of conotoxins, as implied from the absence of identifiable expressed orthologous sequences from two closely related *Conus* species, *C. abbreviatus* and *C. ebraeus.*

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