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Differentiation of Venoms of Predatory Marine Gastropods: Divergence of Orthologous Toxin Genes of Closely Related *Conus* Species with Different Dietary Specializations

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Abstract Venoms of *Conus* are remarkably diverse among species and the genes that encode conotoxins show high rates of evolution. Yet no prior studies have specifically explored how conotoxin gene evolution contributes to the differentiation of venoms of closely related *Conus* species. Previous investigations of four-loop conotoxin expression patterns of six closely related *Conus* species identified 12 sets of putative orthologous loci from these species, including eight pairs of loci that are coexpressed by two of these six species, *C. abbreviatus* and *C. miliaris*. Here I analyze the molecular evolution of orthologous conotoxin loci of these species and specifically examine the divergence of the eight orthologous counterparts of *C. abbreviatus* and *C. miliaris*. Tree and maximum likelihood-based analyses of these sequences reveal that positive selection promotes the divergence of orthologous genes among species and that the evolution of orthologues of *C. abbreviatus* and *C. miliaris* is asymmetric among species. The asymmetric evolution of conotoxin loci among species may result from lineage-specific dietary shifts or interspecific differences in the impact of selection from predator-prey interactions on conotoxin loci.

Keywords *Conus* · Gene family · Four-loop conotoxins · Adaptive evolution · Species interactions · Venom

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Introduction

The marine gastropod genus *Conus* is a hyperdiverse group of specialized predators that use venom to subdue prey. *Conus* venoms contain a plethora of peptides ('conopeptides') and venom composition differs immensely among species (Olivera et al. 1999). Although major shifts in diet (i.e., shifts from vermivory to piscivory) are relatively rare (Duda et al. 2001), vermivorous *Conus* species exhibit dramatic differences in feeding specializations such that even close relatives predominantly prey on different species (Kohn 1959, 1966, 1980, 2001; Kohn and Orians 1962; Marsh 1971; Kohn and Nybakken 1975; Leviten 1980; Reichelt and Kohn 1985; Kohn and Almasi 1993; Duda et al. 2001). Because venoms of *Conus* are presumably used primarily to capture prey, the correlation between interspecific differences in venom composition and feeding specialization suggests that venoms are under strong selection in response to dietary shifts or to coevolutionary interactions among predators and prey. Species with different feeding specializations may show differences in venom composition that reflect the evolution of venoms for use with particular prey. Venoms may also evolve in response to the development of resistance to venoms in prey via an arms race between the venoms of predators and the ion channels and cell receptors of prey that are blocked by venom components. Analyses of the molecular evolution of genes expressed in the venom among recently diverged *Conus* species can determine if selection drives the differentiation of venoms among species.

Conotoxins (i.e., disulfide bonded conopeptides) are encoded by large gene families (e.g., A-, I-, M-, O-, P-, and T-superfamilies). Strong positive selection drives the divergence of paralogous conotoxin loci within species

(Duda and Palumbi 1999a, 2000, 2004; Conticello et al. 2001) and differential expression of these loci promotes the differentiation of venoms among relatives (Duda and Palumbi 2000; Duda and Remigio 2008) and among species with different feeding modes (Duda and Palumbi 2004). As also reported for snake venom phospholipase A_2 genes (Lynch 2007; Gibbs and Rossiter 2008), the adaptive evolution of conotoxin genes creates distinct arsenals of conotoxins and the differential expression of these loci causes venom composition to differ among species. But does the adaptive evolution of conotoxin gene families contribute to the differentiation of venoms over timescales relevant to the evolution of feeding specializations of *Conus*? Although estimated rates of conotoxin gene evolution suggest that these genes are rapidly evolving [1.7–4.8% nonsynonymous substitutions per nonsynonymous site per million years (Duda and Palumbi 1999a)], positive selection may occur primarily among paralogous gene copies following gene duplication (Lynch and Conery 2003) and contribute little to the divergence of orthologues among species.

Utilizing the same approach to identify nine four-loop conotoxin (O-superfamily) loci of *C. abbreviatus* [i.e., amplification of venom duct cDNA with primers designed to amplify a subset of four-loop conotoxin transcripts (Duda and Palumbi 1999a)], Duda and Palumbi (2000) obtained four-loop conotoxin transcript sequences of a close relative of *C. abbreviatus*, *C. ebraeus*. Four-loop conotoxins affect sodium (δ-conotoxins) and calcium (ω-conotoxins) ion channels (Olivera et al. 1999). Duda and Palumbi (2000) sought to determine if positive selection drives the differentiation of venoms among related *Conus* by examining the molecular evolution of orthologous loci of these two species that show differences in feeding specializations (Kohn 1959). The sequences obtained from *C. ebraeus* though were quite divergent from and apparently not orthologous to any of the nine loci identified previously from *C. abbreviatus*, and so analysis of the evolution of orthologous counterparts among species was not possible (Duda and Palumbi 2000). However, besides *C. ebraeus*, several other *Conus* species show close affinity to *C. abbreviatus*, including *C. aristophanes*, *C. chaldaeus*, *C. coronatus*, *C. dorreensis*, *C. fulgetrum*, *C. judaeus*, and *C. miliaris* (Duda and Palumbi 1999b; Duda et al. 2001; Espiritu et al. 2001; Duda and Kohn 2005; Duda and Rolán 2005). These species display unique dietary specializations and tend to prey on various species of eunicid and nereid polychaetes. For example, the major prey taxon of *C. abbreviatus* is *Eunice antennata* (Kohn 1959), while that of *C. miliaris* is *Lysidice collaris* (Kohn 1968, 1978, 2001).

To compare four-loop conotoxin expression patterns of a set of closely related *Conus* species, Duda and Remigio (2008) obtained four-loop conotoxin transcript sequences from five close relatives of *C. abbreviatus* including *C. aristophanes*, *C. coronatus*, *C. ebraeus*, *C. judaeus*, and *C. miliaris*. They identified transcript sequences from these species that appear to represent 12 sets of orthologous counterparts based on levels of sequence divergence and comparison of the reconstructed conotoxin gene tree to a hypothesized species tree of the six species examined. *C. miliaris* apparently coexpresses eight of the nine loci that were previously identified from *C. abbreviatus*. Using these data, I examined the molecular evolution of orthologous counterparts of *C. abbreviatus* and *C. miliaris* to determine if positive selection promotes the differentiation of venoms among *Conus* species through the divergence of orthologous conotoxin loci.

Materials and Methods

From analyses of 507 four-loop conotoxin sequences obtained from venom duct mRNA of at least two individuals of six closely related vermivorous *Conus* species, *C. abbreviatus*, *C. aristophanes*, *C. coronatus*, *C. ebraeus*, *C. judaeus*, and *C. miliairis* (GenBank accession numbers AF089983, AF089988, AF089995, AF089997, AF090006, AF090007, AF090035, AF090041, AF090055, AF174268, DQ644543–DQ644549, and EF108267–EF108288), Duda and Remigio (2008) identified 39 putative loci and 12 sets of presumed orthologous genes, including eight pairs of orthologues expressed by *C. abbreviatus* and *C. miliaris* (Fig. 1). Four of the eight orthologues expressed by *C. abbreviatus* and *C. miliaris* (locus pairs A3-M12, A4-M25, A6-M1, and A7-M26) are also apparently expressed by *C. coronatus* (Fig. 1). Because *C. abbreviatus* and *C. miliaris* are more closely related to each other than either is to *C. coronatus* (Duda and Remigio 2008), sequences from *C. coronatus* provide a foundation for examining nucleotide substitutions among the pairs of orthologues from *C. abbreviatus* and *C. miliaris* because the direction of change in the branches leading to sequences from these species can be rooted with sequences from *C. coronatus*. Two other pairs of orthologous counterparts from *C. abbreviatus* and *C. miliaris* (A8-M8 and A1-M23) occur in well-supported clades with other four-loop conotoxin sequences that presumably do not represent orthologsue of these sequences. As above, these sequences can be used to root the direction of change in these orthologues. The remaining two pairs of loci though (A2-M15 and A9-M19) do not show affinity to any of the other four-loop conotoxins in the gene tree.

Duda and Palumbi (1999a) calculated the proportion of synonymous substitutions per synonymous site (d_S) and proportion of nonsynonymous substitutions per

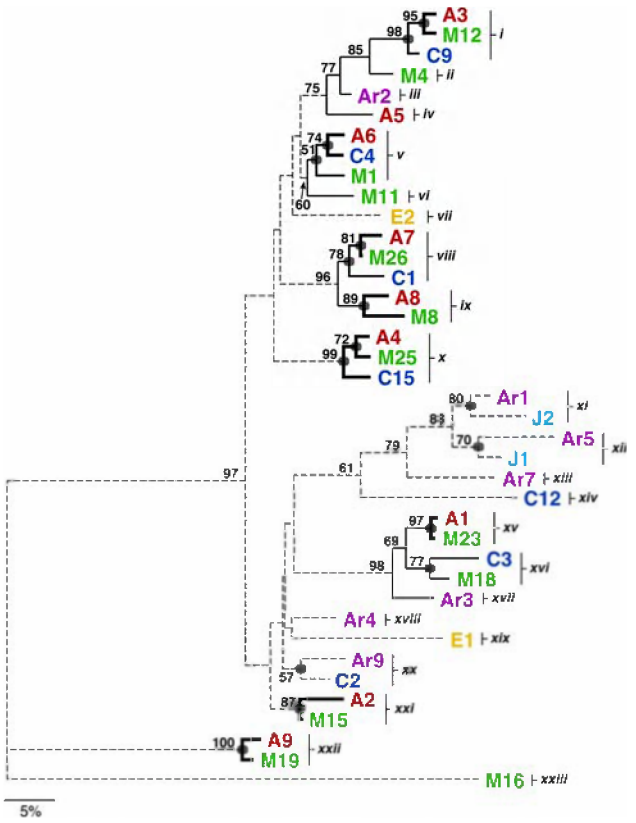

Fig. 1 Phylogram of 39 putative four-loop conotoxin loci of *C. abbreviatus*, *C. aristophanes*, *C. coronatus*, *C. ebraeus*, *C. judaeus*, and *C. miliaris* reconstructed with neighbor-joining based on maximum likelihood distances calculated with comparison of entire transcript sequences. The TrNef + G model was used to calculate maximum likelihood distances (rate matrix: [A–C] = 1.0000, [A–G] = 1.9398, [A–T] = 1.0000, [C–G] = 1.0000, [C–T] = 1.2029, [G–T] = 1.0000; gamma distribution shape parameter = 0.4634). Bootstrap scores for branches are shown at nodes. Loci are identified as by Duda and Remigio (2008): A—*C. abbreviatus*; Ar—*C. aristophanes*; C—*C. coronatus*; E—*C. ebraeus*; J—*C. judaeus*; and M—*C. miliaris*. Arbitrary numerical identifiers are given for each putative locus. Clades of sequences that represent sets of orthologous loci and single sequences that represent loci that are only expressed by single species are labeled with roman numerals (*i–xxiii*). Nodes at the bases of clades that contain putative orthologous loci are indicated by gray ovals. The eight pairs of putative orthologues of *C. abbreviatus* and *C. miliaris* are indicated by thick branches. Sets of sequences used to estimate an independent value of ω for each branch for particular pairs of orthologues of *C. abbreviatus* and *C. miliaris* are indicated by solid lines. This figure is modified from Fig. 2 of Duda and Remigio (2008)

nonsynonymous site (d_N) from pairwise comparisons, including sliding-window analyses, of eight paralogous four-loop conotoxin loci of *C. abbreviatus* with Ina's (1995) method. Their results revealed that positive selection had operated on these paralogous loci. To determine whether orthologous loci of recently diverged *Conus* species also show evidence of positive selection, I calculated d_S and d_N along branches of the gene tree of these loci using a maximum likelihood approach with PAML 3.15 (Yang 1997) and used likelihood ratio tests to determine whether ratios of d_N to d_S (ω) differ along branches. I first examined likelihood scores determined using a null model that set ω equal to one for all branches of the gene tree (model A; Table 1) and a model that estimated a single value of ω for all branches (model B; Table 1). I also examined two alternative models that estimated ω for branches connecting paralogous loci and constrained different values of ω to branches connecting all sets of putative orthologues: one model constrained a single value of ω that was estimated from the data to the branches connecting all putative orthologous loci (model C; Table 1), and the other constrained an ω value of 1 to branches connecting the orthologous counterparts (model D; Table 1). The likelihood scores determined from these models were compared to determine if ω ratios differ significantly along sets of branches associated with paralogous and orthologous loci and if these ratios are significantly different than a value of one. For these models, I used a data set containing all four-loop conotoxin transcripts of *C. abbreviatus*, *C. aristophanes*, *C. coronatus*, *C. ebraeus*, *C. judaeus*, and *C. miliaris* that were analyzed by Duda and Remigio (2008). All codons were examined except for those following the codon of the last cysteine residue because alignment of these codons required insertions/deletions that affected reading frame in some sequences and because stop codons occurred earlier in some sequences than others.

To examine variation in ω ratios along branches of the conotoxin transcript phylogram and to specifically compare rates of evolution among the eight pairs of orthologous counterparts of *C. abbreviatus* and *C. miliaris*, I estimated ω for each branch in the tree with the free-ratio model in PAML. Pairwise estimates of d_N and d_S among orthologous sequences were also calculated using a maximum likelihood approach with PAML. Because orthologue pairs A2-M15 and A9-M19 did not show obvious identity to any other conotoxin transcripts, complete data sets containing all 39 sequences described and analyzed by Duda and Remigio (2008) were examined for calculations of pairwise and branch-specific estimate of these pairs. However, because this alignment did not include the codons that followed the sixth cysteine codon (see above) and because many of the branches in the conotoxin gene tree were not completely resolved, smaller data sets that only included sequences of well-resolved clades were utilized to calculate these statistics for the other putative orthologue pairs (see Fig. 1).

Results

All putative orthologous pairs exhibit at least 1 and as many as 15 nonsynonymous substitutions within the 25–

Table 1 Models used to test hypotheses concerning the molecular evolution of orthologous four-loop conotoxin loci

Model	–lnL	ω_{para}	ω_{ortho}
A. $\omega = 1$ for all branches	1807.8	[1]	[1]
B. Single value of ω estimated for all branches: $\omega_{para} = \omega_{ortho}$	1777.3	5.31	5.31
C. Two values of ω estimated for branches: ω_{para} and ω_{ortho}	1776.9	4.60	8.00
D. Two values of ω for branches: ω_{para} estimated, $\omega_{ortho} = 1$	1788.8	4.23	[1]
E. Independent values of ω estimated for each branch	1752.0	–	–

Note: ratios of the proportion of nonsynonymous and synonymous substitutions per respective site (ω) are estimated or constrained for all branches or sets of branches leading to orthologous (ω_{ortho}) and paralogous loci (ω_{para}). Likelihood scores (–lnL) and ω ratios are presented for each model when applicable

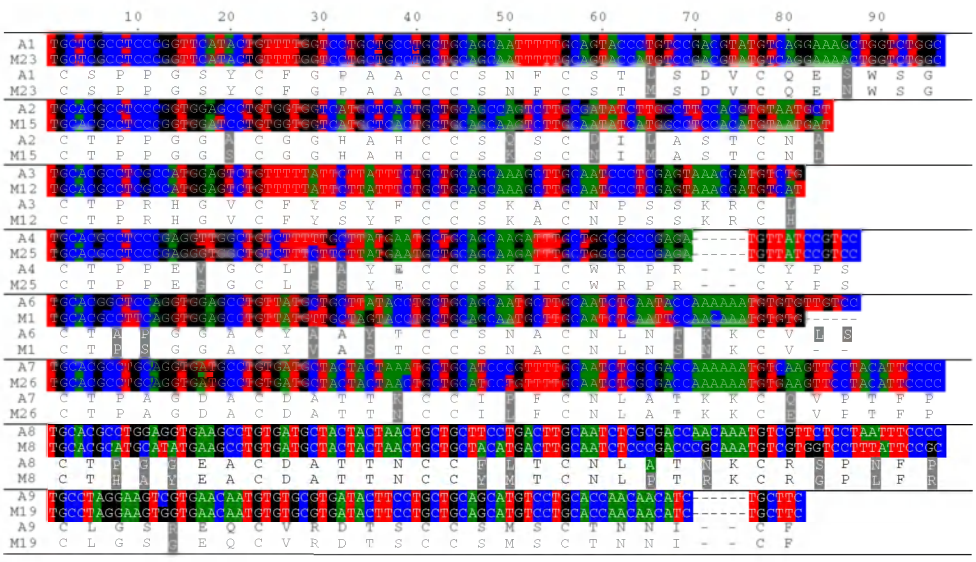

Fig. 2 Alignment of nucleotide and predicted amino acid sequences of sets of putative orthologous loci of *C. abbreviatus* and *C. miliaris*. Shaded amino acids indicate amino acid substitutions among pairs of orthologues. Horizontal lines delimit pairs of putative orthologous loci

32 codons of the toxin coding regions of these transcripts. These substitutions are responsible for between 1 and 10 amino acid substitutions within the predicted amino acid sequences of the mature conotoxin peptides (Fig. 2). One pair of putative orthologous that differ at three nonsynonymous sites, A6-M1, also exhibit differences in stop codon position; although polarity of change is not obvious, presumably a substitution within the second position of a leucine codon (TTG) gave rise to a stop codon in sequence M1 (Fig. 2). Differences at synonymous sites were only observed for two pairs of putative orthologues: at positions 69 and 75 for orthologue pair A2-M15 and at position 81 for pair A3-M12 (Fig. 2). The latter substitution occurs in one of the two possible pathways of substitution to account for two substitutions within a single codon (i.e., CTG [L] → CTT [L] → CAT [H]); the second pathway involves two nonsynonymous substitutions (i.e., CTG [L] → CAG [Q] → CAT [H]). Thus, of 41 substitutions among pairs of putative orthologues, only 2 or 3 synonymous substitutions (depending on the actual pathway of substitution for the codon mentioned above) were observed.

As expected based on previous analyses of paralogous conotoxin transcripts of *C. abbreviatus* (Duda and Palumbi 1999a), examination of the evolution of the paralogous loci analyzed here revealed strong evidence of positive selection. The ratio of d_N to d_S (ω) is 5.30 for all branches of the gene tree for the model in which ω was held constant (model B) and this model is significantly better than the model (model A) that constrained ω to be 1 (Table 2). An alternative model that constrained a different value of ω for sets of putative orthologues from *C. abbreviatus* and *C. miliaris* (model C) was not significantly better at explaining the data than the model that used one estimate of ω for all branches of the tree (model B) (Tables 1, 2). Furthermore,

Table 2 Hypothesis testing and model comparison with likelihood ratio tests (see Table 1)

Hypothesis tested	Model comparison	2ΔlnL	p
$\omega_{para} = \omega_{ortho} = 1$	A & B	61.0	$<10^{-6}$
$\omega_{para} = \omega_{ortho}$	B & C	0.8	0.670
$\omega_{para} \neq \omega_{ortho} = 1$	C & D	23.8	7×10^{-6}

the model that constrained ω to be 1 for branches connecting orthologous counterparts (model D) had a significantly worse likelihood score than the model that estimated ω for these branches (model C) (Table 2). The significance of these results is further emphasized because the alignments analyzed did not include the one to six codons that immediately followed the sixth and final cysteine codon and orthologous counterparts from *C. abbreviatus* and *C. miliaris* exhibit a total of 10.5 nonsynonymous substitutions and only 1.5 synonymous substitutions in this region (see Fig. 2).

Pairwise estimates of d_N and d_S among the eight putative orthologue pairs of *C. abbreviatus* and *C. miliaris* show that six pairs have d_N to d_S ratios >1; only pairs A2-M15 and A3-M12 show ratios <1 (Table 3). Lineage-specific (i.e., free-ratio) estimates of d_N and d_S along branches connecting these pairs reveal that both counterparts of four locus pairs have diverged from the predicted ancestral sequence (locus pairs A2-M15, A4-M25, A7-M26, nd A8-M8; Table 3). However, four sequences show no evidence of divergence, including M23, A3, A6 and M19. Overall, nonsynonymous divergence is greater for sequences from *C. abbreviatus* for five locus pairs (A1-M23, A2-M15, A4-M25, A7-M26, and A9-M19) and for sequences from *C. miliaris* for three locus pairs (A3-M12, A6-M1, A8-M8) (Table 3). The average rates of divergence of orthologous counterparts of *C. abbreviatus* and *C. miliaris* are 0.026 and 0.048, respectively, and sequences of *C. miliaris* show greater variation in rates ($s = 0.071$) than sequences of *C. abbreviatus* ($s = 0.021$).

Table 3 Lineage-specific and pairwise maximum likelihood estimates of nonsynonymous substitutions per nonsynonymous site (d_N) and synonymous substitutions per synonymous site (d_S) among putative orthologous conotoxin loci of *C. abbreviatus* (A) and *C. miliaris* (M)

Locus	Lineage-specific		Pairwise	
	d_N (*N*)	d_S (*S*)	d_N (*N*)	d_S (*S*)
A1	0.016 (63.0)	0.000 (18.0)	0.031 (67.0)	0.000 (29.0)
M23	0.000 (63.0)	0.000 (18.0)		
A2	0.054 (67.5)	0.116 (19.5)	0.084 (65.2)	0.112 (18.8)
M15	0.016 (67.5)	0.000 (19.5)		
A3	0.000 (64.8)	0.000 (16.2)	0.017 (68.2)	0.068 (12.8)
M12	0.019 (64.8)	0.050 (16.2)		
A4	0.032 (70.2)	0.000 (16.8)	0.051 (62.0)	0.001 (19.0)
M25	0.016 (70.2)	0.000 (16.8)		
A6	0.000 (61.9)	0.000 (19.1)	0.134 (60.4)	0.001 (20.6)
M1	0.129 (61.9)	0.000 (19.1)		
A7	0.028 (74.0)	0.000 (22.0)	0.043 (72.2)	0.000 (23.8)
M26	0.014 (74.0)	0.000 (22.0)		
A8	0.056 (74.0)	0.000 (22.0)	0.227 (80.0)	0.002 (16.0)
M8	0.191 (74.0)	0.000 (22.0)		
A9	0.019 (67.5)	0.000 (19.5)	0.015 (66.0)	0.000 (9.0)
M19	0.000 (67.5)	0.000 (19.5)		

Note: numbers of nonsynonymous (*N*) and synonymous (*S*) sites compared are indicated in parentheses. Pairs of putative orthologues are shown together

Discussion

Duda and Remigio (2008) identified eight four-loop conotoxin transcript sequences from *C. miliaris* that appear to be orthologues of eight of nine loci reported previously from *C. abbreviatus* (Fig. 1). Although orthology is difficult to assess and may be contentious for those pairs of sequences that differ at many sites (e.g., putative orthologue pairs A2-M15 and A6-M1 differ at seven nucleotide positions and pair A8-M8 differs at 15 positions; see Fig. 1), alleles of a conotoxin locus of *C. ebraeus*, as determined based on segregation patterns of variant sequences among individuals, differ at nine nonsynonymous sites (Duda and Palumbi 2000). Also, *C. abbreviatus* and *C. miliaris* show ~2% synonymous divergence at a nuclear calmodulin locus (Duda and Kohn 2005), and except for two cases (i.e., pairs A2-M15 and A3-M12), pairwise estimates of d_S among putative orthologous conotoxin loci of these taxa are <2% (Table 3).

Analyses of duplicate genes from whole genomes have revealed that only a small percentage of paralogous genes experiences bouts of positive selection and that these episodes usually occur soon after the duplication event that gave rise to these genes (Lynch and Conery 2003). On the contrary, putative orthologous conotoxin genes of two closely related *Conus* species, *C. abbreviatus* and *C. miliaris*, have diverged largely due to the accumulation of nonsynonymous substitutions (Table 3, Fig. 2). Moreover, analyses of models that assume different ratios of ω for branches connecting putative orthologous and paralogous loci show that these ratios are not significantly different and that they are not equal to 1 (Tables 1 and 2). Based on levels of divergence of loci and the topology of the gene tree (see Fig. 1), gene duplication events occurred much earlier than the separation of these species. Although functions of gene products of orthologous counterparts of *C. abbreviatus* and *C. miliaris* have not been examined, conotoxin peptides that differ at few amino acid sites show differences in specificity for particular ion channel subtypes (Lewis et al. 2000) and so the evolution of these genes is likely to be related to changes in conotoxin function.

The results presented here demonstrate that the adaptive evolution of conotoxin loci clearly contributes to the differentiation of venoms among species and that positive

selection is not exclusively linked to the early divergence of conotoxin paralogs. These results also further cement the notion that conotoxin gene evolution is driven by strong positive selection and that it is not necessary to invoke hypermutation to explain the tremendous diversity of venom components found in *Conus*. The divergence of members of conotoxin gene families is obviously a dynamic and progressive process that acts to create new conopeptides. This pattern likely arises from strong and continual selection pressures that drive the adaptive evolution of conotoxin genes. Lynch (2007) observed a similar pattern in the evolution of snake venom phospholipase A_2 genes in which positive selection is not only confined to gene duplication events but also appears to be associated with speciation events as well. Lynch (2007) suggests that the evolution of venoms of snakes is driven primarily by dietary shifts after speciation: while some genes are lost because their products are not effective against the new prey, others are retained and refined through positive selection. A similar process may occur in *Conus*, especially if dietary shifts are linked to speciation in this group.

Divergence of orthologous counterparts of *C. abbreviatus* and *C. miliaris* also appears to be asymmetric (Table 3); that is, sequences of one species show greater rates of nonsynonymous substitution than their counterparts in the other species. First, five of eight sequences of *C. abbreviatus* show greater divergence from predicted ancestral sequences than orthologous counterparts of *C. miliaris*, including two sequences of *C. miliaris* (sequences M23 and M19) that have not diverged from predicted ancestral sequences (Table 3). Differences between rates of divergence for these orthologue pairs range from 0.014 to 0.038 (average, 0.021). Second, the remaining three of eight sequences of *C. miliaris* show greater divergence than counterparts from *C. abbreviatus*, including two sequences from *C. abbreviatus* (sequences A3 and A6) that show no divergence from predicted ancestral sequences (Table 3). Differences in rates of divergence between these pairs range from 0.019 to 0.135 (average, 0.094). This pattern suggests that since the separation of these species, the evolution of the orthologous conotoxin loci examined is largely asymmetric among species, especially for locus pairs A1-M23, A3-M12, A6-M1, and A9-M19, in which one member of these pairs shows no divergence from the predicted ancestral sequence, and pairs A6-M1 and A8-M8, in which the counterpart from *C. miliaris* shows pronounced divergence from predicted ancestral sequences (Table 3).

Steinke et al. (2006) identified a number of genes of fish that were subject to positive selection and that show asymmetric rates of evolution among species. The authors suggest that these genes likely contributed to the unique evolution of these species, but the exact roles of the genes on the diversification of these fish are unknown. In *Drosophila*, the divergence of five protein coding genes associated with the male-specific lethal complex, a gene complex associated with dosage compensation in males, is asymmetric, with signals of positive selection for four loci in *D. melanogaster* and for one locus in *D. simulans* (Rodriguez et al. 2007). Rodriguez et al. (2007) propose that selection must have been strong enough to have affected these four loci in *D. melanogaster* or that changes in one member of the complex triggered the evolution of the other members. The gene that shows evidence of adaptive evolution solely in *D. simulans* is the only one of the five genes that is expressed by both males and females (all others are solely expressed by males), and so perhaps it has evolved independently from the other loci (Rodriguez et al. 2007). Rodriguez et al. (2007) hypothesize that selection pressures may be related to genetic conflict in association with male-killing bacteria or retroelements, but it is not clear why these pressures were lineage-specific.

Because *Conus* venoms are presumably used primarily to subdue prey and conotoxin gene evolution is hence likely to be strongly linked to predator-prey interactions, observation of asymmetric rates of evolution of conotoxin genes suggests that the strength of selection driven by these interactions is locus and species-specific. As suggested to explain the evolution of snake venoms (Lynch 2007) and the rapid evolution of conotoxin gene family members in *Conus* (Duda and Palumbi 1999a), venom evolution may be driven by the modification of venoms for use on new prey following a dietary shift. It is difficult to reconstruct diets of ancestral lineages and infer whether dietary shifts have occurred in *Conus*. Nonetheless, while other *Conus* feed on the preferred prey of *C. abbreviatus*, *Eunice antennata* [e.g., *C. rattus* (Kohn 1959)], close relatives of *C. abbreviatus*, including *C. miliaris*, do not show preferences for this species (Kohn 1968, 1978, 2001) and so the diet of *C. abbreviatus* may be derived. Broader analyses of venoms and diets of additional closely related species as well as populations of widespread species would further bolster our understanding of the evolution of venoms and feeding specializations of *Conus*. Also, functional studies that examine the effects of gene products of conotoxin genes that have substantially diverged from predicted ancestral sequences and gene products of ancestral sequences in preferred prey of these species could be instrumental in demonstrating the basis for the adaptive evolution of these genes.

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