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Evolution of ecological specialization and venom of a predatory marine gastropod

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Abstract

Understanding the evolution of ecological specialization is important for making inferences about the origins of biodiversity. Members of the predatory, marine gastropod genus *Conus* exhibit a variety of diets and the ability to capture prey is linked to a venom comprised of peptide neurotoxins, termed conotoxins. We identified conotoxin transcripts from *Conus leopardus*, a species of *Conus* that uniquely preys exclusively on hemichordates, and compared its venom duct transcriptome to that of four other *Conus* species to determine whether a shift to a specialized diet is associated with changes in the venom composition of this species. We also examined the secondary structure of predicted amino acid sequences of conotoxin transcripts of *C. leopardus* to identify substitutions that may be linked to specialization on hemichordates. We identified seven distinct conotoxin sequences from *C. leopardus* that appear to represent transcripts of seven distinct loci. Expression levels and the diversity of conotoxins expressed by *C. leopardus* are considerably less than those of other *Conus*. Moreover, gene products of two transcripts exhibited unique secondary structures that have not been previously observed from other *Conus*. These results suggest that transition to a specialist diet is associated with reduction in the number of components expressed in venoms of *Conus* and that diverse venoms of *Conus* are maintained in species with a broad dietary width.

Keywords: conotoxins, *Conus*, ecological specialization, gene expression

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Introduction

Much of evolution involves the origin of adaptations that enable organisms to utilize new resources or to use resources in new or more efficient ways. However, these adaptations and their contribution to the origins of biodiversity cannot be fully realized until their genetic foundation is identified. Members of the predatory, tropical marine gastropod genus *Conus* exhibit a number of feeding specializations and their ability to capture prey is linked to a venom comprised of peptide neurotoxins. Studies of the molecular evolution of genes that are directly associated with species' ecologies, such as those expressed in the venoms of *Conus*, will illuminate key issues in evolution, particularly the patterns of evolution of ecologically relevant genes and their association with ecological adaptations.

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Species of *Conus* prey on fishes, other marine snails, errant and sedentary polychaetes, and hemichordates (Kohn 1959). Based on phylogenetic reconstructions, errant polychaete-eating is the ancestral diet and the derived feeding habits evolved only a few times during the initial major diversification of *Conus* species (Duda *et al.* 2001; Duda & Palumbi 2004). The particular diets of vermivorous species also differ, with species-specific preferences for particular worm taxa (Kohn 1959, 1966, 1980; Marsh 1971; Kohn & Nybakken 1975; Leviten 1980; Reichelt & Kohn 1985; Kohn & Almasi 1993). *Conus* utilize an assortment of potent peptide neurotoxins, termed conotoxins, in their venom to capture prey. Conotoxins are small peptides consisting of 10–35 amino acids with highly conserved framework of cysteine (Cys) residues and are expressed in the venom ducts of *Conus*. These peptides target specific ion channels and receptors of prey (Olivera 1997; McIntosh *et al.* 1999; Terlau & Olivera 2004). The active components of cone snail venoms are remarkably diverse; venom of a single species may contain up to 200 different toxin peptides

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(Olivera *et al.* 1990, 1991). These toxins vary extensively within and between species, and across the snails' range of dietary preferences (e.g. Olivera *et al.* 1991; Walker *et al.* 1999; Conticello *et al.* 2001; Olivera 2002; Duda & Palumbi 2004; Corpuz *et al.* 2005).

Among cone snails, *Conus leopardus* is unique in that it is the only *Conus* species known to prey exclusively on the hemichordate *Ptychodera flava* (Kohn 1959, 1980; Pickens 1973; Kohn & Nybakken 1975; Röckel *et al.* 1995). While other vermivorous *Conus* include *P. flava* in their diets (e.g. *C. lividus*; Kohn 1959), no other *Conus* are known to feed solely on this species and *C. leopardus* is perhaps the only *Conus* to exhibit such a narrow dietary breadth. *Conus leopardus* is most closely related to other *Conus* that predominantly prey on sedentary polychaetes, errant and sedentary polychaetes, sedentary polychaetes and hemichordates, or fishes (Duda *et al.* 2001; see also Duda & Kohn 2005). Although reconstructed phylogenies are not well resolved with regards to the history of divergence of *C. leopardus* and its closest relatives, the diet of this species reflects the evolution of a unique feeding ecology and a shift to a narrow diet.

Because the venoms of *Conus* are used primarily to subdue prey, the evolution of the unique feeding mode exhibited by *C. leopardus* is likely to be associated with the evolution of its venom. Dietary shifts and venom evolution were examined previously in sea snakes (Li *et al.* 2005a, b). Phospholipase A_2 toxins of *Aipysurus eydouxii*, a sea snake that feeds on fish eggs and does not utilize venom to obtain food, are less diverse and exhibit slower rates of evolution than these toxins from sea snakes that use venom to capture prey (Li *et al.* 2005a). Moreover, a gene encoding the sole three-finger toxin expressed by *A. eydouxii* possesses a two-base deletion that significantly alters the function of this peptide (Li *et al.* 2005b). Clearly, the loss of a venomous feeding mode in this sea snake is associated with the reduced effectiveness of its toxins and decelerated evolution of genes that encode venom components (Li *et al.* 2005a, b). Shifts in diet from venomous to nonvenomous feeding modes may have predictable effects on the evolution of venoms, but the association of venom evolution and dietary shifts among venomous taxa remains largely unexplored despite the potential utility of these systems to examine the molecular basis of ecological adaptations.

The evolution of ecological specialization is of fundamental concern in biology because it is likely to foster adaptive radiations and the origins of biodiversity (Schluter 2000). The process of specialization typically includes the loss or reduction of characters that result in the origin of a narrow niche breadth (Futuyma & Moreno 1988). For predators, transitions from a generalist to specialist diet should be accompanied by the modification of characters that permit greater exploitation of a narrower breadth of prey species. These traits likely include chemosensory abilities, foraging behaviours and search strategies, physiological processes associated with digestion and waste removal, and other features involved with prey capture, with some traits being more labile than others. Among predatory taxa that use venom to subdue prey, the evolution of a specialized diet is likely to be strongly linked to the evolution of venoms, many of which are comprised of direct gene products such as the conotoxins of *Conus*. Thus, although the evolution of many of these characters may be difficult to trace for a species that has evolved a narrow diet, the evolution of venoms can be inferred from analyses of expression of genes encoding venom components. How has the venom of *C. leopardus* evolved with the origin of the specialized diet of this species? As noted above, *Conus* venoms are typically comprised of a plethora of conotoxins. Complex venoms may be associated with broad diets of species. Are transitions to more specialized feeding modes then accompanied by the evolution of a more streamlined venom such that fewer venom components are needed to capture a narrower range of prey? The answers to questions such as these can give broad insight into the evolution and maintenance of the diverse venom compositions of *Conus* and illuminate the genetic changes associated with shifts in feeding ecology and the origins of specialization.

To determine whether the dietary transition of *C. leopardus* is related to the evolution of its venom, we generated a cDNA library from mRNA recovered from the venom duct of a single individual of this species. We sequenced 200 transcripts obtained from this library to identify putative conotoxin transcripts. Because *C. leopardus* shows no close affinity to other *Conus* and venom duct transcriptomes have so far only been described from four other *Conus* species, we are limited in our ability to compare the venom composition of *C. leopardus* to that of these four species: *C. arenatus*, a species that preys on capitellid, eunicid, maldanid and neried polychaetes (Kohn 1968; Kohn & Nybakken 1975; Reichelt & Kohn 1985; Röckel *et al.* 1995); *C. striatus*, a piscivore (Röckel *et al.* 1995); and *C. pennaceus* and *C. textile*, two molluscivorous species (Röckel *et al.* 1995). Based on phylogenies derived from mitochondrial and nuclear sequence data, *C. arenatus* represents the closest relative of *C. leopardus* among the above four species (Duda *et al.* 2001; Duda & Kohn 2005) and the lineages that gave rise to *C. arenatus* and *C. leopardus* likely diverged about 21 million years ago (Duda & Kohn 2005). Because of the hindrance of not being able to make direct comparisons among a very close relative of *C. leopardus*, we were not able to specifically examine the evolution of conotoxin loci of *C. leopardus* or identify changes in expression of these genes. Nonetheless, we did compare the venom duct transcriptome of *C. leopardus* to that of the four other *Conus* listed above to determine whether the origin of a unique feeding mode in *C. leopardus* is associated with any major shifts in venom composition, such as reduced venom complexity,

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that can be inferred from these comparisons. We also examined the conotoxin transcripts of *C. leopardus* to identify substitutions or structural changes in encoded peptides that may be linked to the origin of a hemichordate-only diet.

Methods

Preparation of cDNA libraries

A specimen of *Conus leopardus* was collected from Kahe Point, Oahu, Hawaii, USA. The snail's venom duct was dissected and preserved in RNAlater (Ambion), kept on ice, and stored at –80 °C. Total mRNA was isolated from the venom duct using protocols described previously (Duda & Palumbi 1999). In brief, mRNA were extracted from lysed cells of venom duct tissue using magnetic beads attached to a 25-mer oligo dT and subsequently separated from the oligo dT with heat. We prepared a cDNA library using components of the CloneMiner cDNA Library Construction Kit and a TA cloning kit (Invitrogen) in an attempt to obtain full-length cDNA clones (Karnaoukhova *et al*. 2003). We slightly modified the manufacturer's protocol to permit cloning of cDNA from small quantities of mRNA as described here. Specifically, the cDNA-adapter complex formed by ligation reactions was size fractionated and ethanol precipitated. Then, the resulting pellet was resuspended in TE (pH 8.0), column-purified (QIAGEN) using two washes of guanidine hydrochloride (Amresco), and eluted with distilled water. Because initial mRNA abundance was low, we polymerase chain reaction (PCR) amplified the purified cDNA-adapter complex with a set of *att*B primers that are identical to regions of the adaptor sequences (GGGACAACTTTGTACAAAAAAGTTGG and CACAAC TTTGTACAAGAAAGTTGG-GT) using the following conditions: 40 cycles of 94 °C, 30 s; 52 °C, 30 s; and 72 °C, 30 s. We then ligated the PCR-amplified cDNA adapter with a PCR 2.1 vector and transformed the plasmid into chemically competent *Escherichia coli* with a TA Cloning Kit (Invitrogen).

Screening of cDNA libraries

White vs. blue colony screening was used to identify colonies with vector inserts. Several hundred white colonies were picked and used as templates for PCR utilizing M13 forward and reverse primers. Amplification products that were at least 300 bp long (i.e. below the expected minimum size of conotoxin transcripts plus partial vector sequences) were column-purified (QIAGEN) and sequenced in one or both directions (depending on the orientation of the polyA tail) using M13 forward and reverse primers. Purified sequences were submitted for sequencing at the University of Michigan DNA Sequencing Core.

Molecular analyses

We used nucleotide–nucleotide Basic Local Alignment Search Tool (http://ncbi.nlm.nih.gov/BLAST/) searches to identify putative conotoxin transcript sequences. For sequences that did not show affinity to published genes, we also examined translations of transcripts to identify Cys motifs that are characteristic of known conotoxin superfamilies. We aligned and edited sequences, including representatives of previously published conotoxin sequences, with GENEDOC (Nicholas & Nicholas 1997). Sequence analyses were performed in MEGA 2.1 (Kumar *et al*. 2001).

We directly compared the venom duct transcriptome of *C. leopardus* to that of *Conus arenatus*, *C. pennaceus*, *C. striatus* and *C. textile*. We first compared the proportion of conotoxin transcripts relative to nonconotoxin transcripts among transcriptomes of these five species. We examined the overall diversity of conotoxins expressed by these species by comparing the total number of unique conotoxin transcripts that were obtained. To determine whether species show differences in terms of the diversity of conotoxins in their transcriptomes, we also calculated and compared Shannon's diversity indices (Weaver & Shannon 1949) based on the frequencies of distinct conotoxin obtained from the cDNA libraries of these five species. Because the total number of transcripts ($n = 200$) that were sequenced from *C. leopardus* was smaller than that of *C. arenatus*, *C. pennaceus*, *C. striatus* and *C. textile*, we conducted permutation tests in which we randomly resampled 200 sequences 1000 times for each of these four species and calculated Shannon's diversity indices for each of the resampled data sets.

Conotoxin superfamilies are largely defined based on the arrangement of Cys residues in the mature peptides. For example, O-superfamily conotoxins display the following arrangement of cysteines: C-C-CC-C-C- (C = Cys and dashes refer to presence of one to several other amino acids). To determine if predicted amino acid sequences of *C. leopardus* show unique primary structures, we compared the predicted peptide sequences of the mature toxin region of the transcripts of this species to those known from other species. These latter sequences were obtained from GenBank and included 144 sequences of A-superfamily conotoxins from 33 *Conus* species, 146 O-superfamily conotoxins from 24 species and 48 T-superfamily conotoxins from 13 species. We specifically examined the numbers of amino acids that occur between the conserved Cys positions of published conotoxins of other *Conus* to the numbers that occur in translated conotoxin peptides of *C. leopardus*. We also examined transcripts of *C. leopardus* for the presence of premature termination codons, frameshift mutations and nonsynonymous substitutions within Cys codons of the transcripts of *C. leopardus*. Differences in functions of conotoxins are related to differences in the noncysteine

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Table 1 Characteristics of venom duct transcriptomes of *Conus*

Species	Total number of transcripts examined	Frequency of conotoxin transcripts	Number of distinct conotoxin transcripts	Frequency of most abundant conotoxin transcript	Shannon's diversity index	Reference
C. leopardus	200	0.200	7	0.650	1.24	This study
C. arenatus	370	0.511	39	0.190	3.01	Conticello *et al*. 2001
C. pennaceus	370	0.481	32	0.146	3.09	Conticello *et al*. 2001
C. striatus	429	0.515	19	0.421	1.91	Pi *et al*. 2006
C. textile	370	0.292	30	0.139	3.03	Conticello *et al*. 2001

Table 2 Primary structures of translated peptides encoded by the mature toxin coding region of transcripts of conotoxin superfamilies of *Conus leopardus* and other *Conus* species. The numbers of amino acids within the conserved Cys frameworks of the different conotoxin superfamilies are indicated in square brackets

	A-superfamily	O-superfamily	T-superfamily
C. leopardus	$C_1C_2[4]C_3[7]C_4$	$C_1[6]C_2[1\text{–}8]C_3C_4[2\text{–}4]C_5[3\text{–}5]C_6$	$C_1C_2[5]C_3C_4$
Other *Conus* species	$C_1C_2[3\text{–}4]C_3[3\text{–}9]C_4$	$C_1[6]C_2[4\text{–}9]C_3C_4[2\text{–}4]C_5[3\text{–}8]C_6$	$C_1C_2[4\text{–}7]C_3C_4$

components of these peptides (Lewis *et al*. 2000) and so drastic modifications of the peptides (i.e. incomplete translation or large changes in secondary structures) would presumably be linked to major changes in conotoxin function.

Results and discussion

From analyses of 200 sequences recovered from a cDNA library constructed from venom duct mRNA of *Conus leopardus*, we identified 40 transcript sequences that show identity to and can be aligned with known conotoxins of other *Conus* species. These sequences presumably represent transcripts of seven distinct conotoxin genes (GenBank Accession nos EF467314–EF467320). The remaining sequences appear to be transcripts of various 'housekeeping' genes or showed no obvious similarity to other conotoxins or sequences in GenBank.

Conticello *et al*. (2001) and Pi *et al*. (2006) used a similar approach as ours to describe the diversity of conotoxins expressed by four other *Conus*. Approximately 51%, 48% and 29% of the sequences obtained by Conticello *et al*. (2001) from *C. arenatus*, *C. pennaceus* and *C. textile*, respectively, and 52% of those obtained by Pi *et al*. (2006) from *C. striatus* were conotoxin transcripts. Thus, the proportion of conotoxins expressed by *C. leopardus* relative to other genes (i.e. 20%) is less than that observed for other *Conus* (Table 1). However, while our data were obtained from cDNA constructed from venom duct mRNA of a single individual of *C. leopardus*, Conticello *et al*. (2001) constructed cDNA libraries of *C. arenatus*, *C. pennaceus*, and *C. textile* from pooled mRNA of 20–30 individuals of these species and Pi *et al*. (2006) used multiple specimens to construct the cDNA library of *C. striatus* (the exact number of specimens used was not given). Although the pooling of mRNA could increase the diversity of conotoxins recovered if conotoxin expression patterns differ among individuals of a species or if allelic diversity at conotoxin loci is large (see below), it should not affect the proportions of recovered conotoxin transcripts.

Based on predicted amino acid sequences and identity to members of various conotoxin superfamilies (Fig. 1), the majority (i.e. 35 of 40) of the conotoxin transcripts we obtained belong to the O-superfamily of conotoxins (Fig. 2). These transcripts apparently represent four distinct genetic loci that differ at a minimum of 50 nucleotide sites within their coding regions. Four of the recovered sequences are members of the T-superfamily (Fig. 2) that presumably represent two distinct genetic loci that differ at a minimum of 21 sites. One of the recovered transcripts is a member of the A-superfamily (Fig. 2).

The number of distinct conotoxin transcripts that were recovered from *C. leopardus* ($n = 7$) is considerably less than the numbers of distinct transcripts obtained from *C. arenatus*, *C. pennaceus*, *C. striatus* and *C. textile* (Table 1), but this could reflect differences in the total numbers of conotoxin transcripts obtained from these species. Nonetheless, the majority of the 40 conotoxin transcripts obtained from *C. leopardus* consists of a single distinct sequence that was obtained 26 times and represents 65% of the conotoxins transcripts that were recovered from this species (Fig. 2; Table 1). Venom duct transcriptomes of the four other *Conus* instead exhibit a more even blend of conotoxins and only *C. striatus* possessed a distinct conotoxin with an observed frequency greater than 0.2 (Table 1). As expected from

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A-superfamily
Leo-A1   ...LTLDRASDDTDVAAEIMSGLIALAIDSCCSDSDCNANHPDMCS

O-superfamily
Leo-O1   MKLTCMMLVAVLFLTAWTFVTANVSRNGLENLFPEERHEMMNPEAAKLNNRDCVKAGTACGFPKPEPACCSSWCIFVCT
Leo-O2   MKLTCVLIIAVLFLTACQLVTADYSGDEQQYRAMRLIDAMRNFGDTRSCGRRGKPCPCCRGFRCTGSFCRKWQ
Leo-O3   MKLTCVVIVAVLFLTACQLATADISGGMRKHRALRSTTKLSRSPFDCSSPGAFCGLVPCCDSCNVLGRCGSGLHV
Leo-O4   MKLTCVVIVAVLFLTACQLTTADISRGTRKHRALRSAARLTPFPVECRPYRGICGSFGLCC

T-superfamily
Leo-T1   MRCLPVFIILLLLIPSAPSVDAQPKTEDDVPLASLHDNAKLTLQGLWDKRCCPNLFYCCPDRRK
Leo-T2   MRCLPVFIILPLLIPSAPSVDAQPMTEDDVPLASFHEQTLQELWNKRPCCPLIPGCCR
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Fig. 1 Predicted amino acid sequences of the transcripts recovered from *Conus leopardus*. Presumed cleavage sites of prepro and toxin regions are underlined; Leo-A1 was a partial sequence and no obvious cleavage site (i.e. an arginine residue) near the toxin region was apparent.

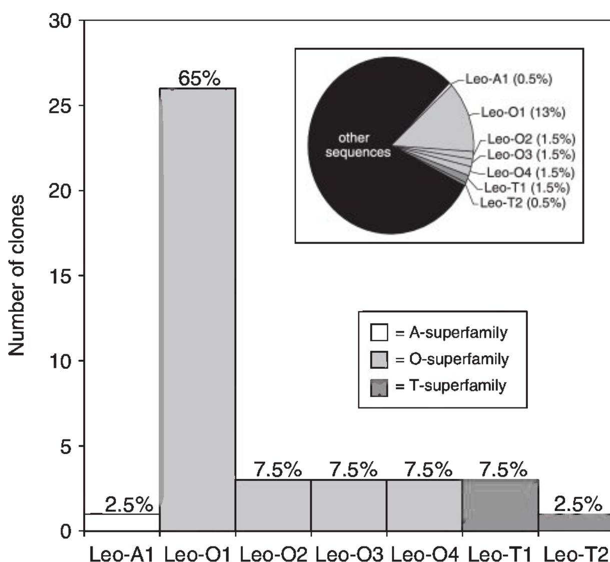

Fig. 2 Numbers of conotoxin transcripts recovered from a cDNA library of *Conus leopardus*. Pie diagram: per cent recovery of conotoxin transcripts relative to the total number of transcripts sequenced from the cDNA library. Graph: frequency of conotoxin transcripts relative to the total number of conotoxin transcripts recovered (per cent recovery relative to the total number of conotoxin transcript sequences indicated above bars).

these results, Shannon's diversity indices show a similar trend with *C. leopardus* exhibiting the lowest diversity of expressed conotoxin transcripts among these five species (Table 1). Permutation tests show that the low diversity index of *C. leopardus* was not biased by the fact that fewer numbers of transcripts were examined from this species: diversity indices of resampled data sets of *C. arenatus* (values ranged from 2.33 to 3.78), *C. pennaceus* (2.56–3.19), *C. striatus* (1.55–2.16) and *C. textile* (2.32–3.07) were all greater than the estimated diversity index of *C. leopardus* (1.24, Table 1).

As mentioned above, both Conticello *et al*. (2001) and Pi *et al*. (2006) used pooled mRNA from up to 30 specimens to construct the cDNA libraries they screened and so the greater diversity of venom components that they detected may not accurately reflect the actual diversity or expression levels of conotoxins expressed by single individuals if individuals of these species show variation in expression. Consequently, although our results imply that venoms of *C. leopardus* are unique in that they are largely comprised of a single conotoxin and are less diverse in composition than venoms of congeners, the low diversity and skewed expression could simply be an artefact of having examined the venom duct transcriptome of a single individual. Studies of levels of intraspecific diversity of expressed conotoxins of these and other species though could strengthen our claims. We also need to consider that differences in diversity of venom composition of *C. leopardus* relative to that of the other four *Conus* is a result of differences in the methods used to generate these results. For example, PCR amplification of cDNA could bias the recovery of particular transcripts. However, we recently utilized this technique with other *Conus* species and preliminary examination of the sequences recovered shows that the diversity of conotoxin sequences obtained from these species is comparable to the diversity obtained from *C. arenatus*, *C. pennaceus*, *C. striatus* and *C. textile*.

We inspected transcript sequences of *C. leopardus* to identify premature stop codons that would result in incomplete translation of conotoxin peptides. One stop codon (TAA) occurred immediately after the pair of adjacent third and fourth Cys codons in the mature toxin region of one of the distinct O-superfamily conotoxin transcripts (Leo-O4, Fig. 1). While the presence of this stop codon would presumably result in a peptide with four Cys residues in a unique arrangement for conotoxins (i.e. C-C-CC) and the reverse arrangement characterizes A-superfamily conotoxins (i.e. CC-C-C), no other described conotoxins show this particular structure. It is unclear whether the translated peptide would possess a novel function or be nonfunctional. Moreover, based on frequencies of recovery of the distinct sequences (Fig. 2), this transcript comprises just 7.5% of the conotoxin transcripts we detected and could represent an expressed pseudogene. Because this transcript was observed three times and is quite divergent from the

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other O-superfamily transcripts we recovered, we assume that the premature stop codon is not a result of a polymerase error that occurred during the amplification process (see Duda & Palumbi 2000). No premature stop codons were observed in the other conotoxin transcripts.

We also examined the primary structure of the transcripts of *C. leopardus* to determine if these sequences possess any unique substitutions or insertions/deletions (indels) that suggest a change in function of their gene products. Alignment of the transcript sequences to other conotoxin sequences obtained from GenBank revealed several indels for many of the transcripts of *C. leopardus*. However, these indels did not affect reading frame as they occurred in multiples of three. Conotoxin peptides are characterized by a highly conserved and unique arrangement of Cys residues that comprise the backbone of these molecules. The codons that encode Cys residues in the mature toxin region of the seven transcripts of *C. leopardus* are intact. No other amino acids show the same levels of conservation as the Cys residues, but although the numbers of other amino acids in 'loops' of conotoxin molecules that are bordered by the Cys residues are variable, these numbers appear to have bounds based on examination of sequences from other *Conus*. Numbers of amino acids within the Cys framework of all but one of the conotoxin transcripts of *C. leopardus* are comparable to those found in other published conotoxin sequences (Table 2). The exception is one of the O-conotoxin transcripts (Leo-O2) that contained only one amino acid in the loop immediately following the second Cys residue; all other described O-superfamily members possess a minimum of four amino acids in this region (Table 2, see also Fig. 1). This transcript comprises just 7.5% of the expressed conotoxin transcripts, but the altered secondary structure of its gene product may be functionally significant.

In summation, our results imply that the venom composition of *C. leopardus* is considerably less diverse than that of other *Conus*. The evolution of a specialized diet in this species appears to be associated with changes in conotoxin expression patterns and the evolution of a more streamlined venom that is dominated by few components. Changes in expression were also linked to the origins of piscivory in *Conus* (Duda & Palumbi 2004) and so conotoxin gene regulation appears to be strongly coupled with the evolution of dietary changes in this genus. Because the narrow diet of *C. leopardus* appears to be correlated with a fairly homogeneous venom, the incredibly diverse venom compositions that typify most *Conus* are likely maintained in part because of the relatively broad diets that these species exhibit. In addition, predicted amino acid sequences of two expressed conotoxin genes of *C. leopardus* show unique secondary structures that have not been observed previously from *Conus*. We have not functionally characterized the encoded peptides of these genes nor examined their effect in hemichordates and so do not know if the changes are functionally significant, but these novel peptides may target specific neuronal targets in hemichordates and may have evolved in response to the origin of the hemichordate-only diet of this species.

Finally, observations on the foraging behaviour of *C. leopardus* in aquaria suggest that this species consumes prey without first immobilizing them with venom (Kohn 1959). Other features also imply that *C. leopardus* may have undergone a shift to a nonvenomous feeding mode, including possession of relatively small radular teeth (~0.7 mm; Bergh 1895), and inability of the venom of this species to elicit any response in typical prey of *Conus* (e.g. fishes, marine snails and polychaetes) unless relatively large volumes of venom are injected (Endean & Rudkin 1965; assays with hemichordates though have not been conducted). Nonetheless, venom ducts of *C. leopardus* show no apparent signs of atrophy and, similar to other vermivorous *Conus*, the lengths of these ducts of *C. leopardus* are about three times the lengths of shells of individuals of this species (T.F.D., personal observations; Endean & Rudkin 1965). Our results too show that *C. leopardus* indeed expresses conotoxins in its venom. Thus, the earlier observations of this species in aquaria may simply reflect the fact that different feeding behaviours are exhibited in captivity than in nature and relatively small radular teeth may be entirely appropriate for injecting venom into hemichordates.

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